A Multichannel Time-Tagged Time-Resolved (TTTR) Model for Quantification of Oligomer Concentrations Based on Antibunching Effect

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ABSTRACT: Molecule/protein aggregation causes many devastating and incurable diseases in human bodies. For example, studies have revealed that protein oligomers formed at the early stage are toxic and may be mostly responsible for some diseases. In the fundamental research, differentiation of different protein oligomers and quantification of the concentrations are important and challenging. Here, we have developed a multichannel time-tagged time-resolved (TTTR) confocal fluorescence model based on antibunching effect to solve the problem. The key point of the model is that n-oligomers labeled with n-dyes cannot emit more than n photons at one time. By assuming that all labeling dyes behave perfectly as noninteractive individual dyes, the analytic relationship between photon-emission probability and oligomer concentrations has been derived. Simulations have been carried out to verify the model, in which differentiation and concentration quantification of up to tetraoligomers can be realized with a relative error <10% in an eight-channel TTTR confocal setup with eight single-photon detectors.

1. INTRODUCTION

Molecule aggregation is a usually observed phenomenon in solutions, which is important in life sciences. For example, aggregation of proteins is related to a wide variety of human diseases.1–4 Understanding the aggregation process is of fundamental importance. However, there is a challenge in precise differentiation of different oligomer aggregates and accurate quantification of the concentrations. Many offline methods have been used to differentiate different oligomers, such as polyacrylamide gel electrophoresis,5,6 capillary electrophoresis,7 and thioflavin T fluorescent method.8–11 Fluorescence correlation spectroscopy (FCS) can also gain information about the size of species in solution.12,13 However, FCS has a limited size resolution and usually yields average hydrodynamic size for a population of aggregates. Photon count histogram14 method and burst analysis15 are oligomer quantification methods based on fluorescence brightness analysis. Dual-focus FCS can also be used to analyze the absolute diffusion size of molecules/particles16 Fluorescence-detection-based flow cytometry also can quantify particle size with high throughput.17 Here, we propose an alternative model to quantify concentrations of different oligomers on the basis of fluorescence antibunching effect18–21 by assuming no interaction (such as energy/charge transfer, singlet-to-triplet-conversion, photobleaching, and so on) among the labeling dyes within the same oligomers. The model employs multichannel time-tagged and time-resolved (TTTR) confocal fluorescence measurement similar to that in the previous report.22 Simulations have been carried out for concentration quantification for up to tetraoligomers using an eight-channel TTTR setup with eight single-photon detectors.
excitation. That is, in our model, all labeling dyes are assumed to behave as individual unperturbed single-photon emitters. Besides, photobleaching is also not considered. Note that there are many exceptions. Experimentally, antibunching effect has also been observed for closely packed oligomers/aggregate systems because of complicated excited-state interactions among dyes within the same oligomers/aggregate, including energy transfer, electron transfer, singlet-to-triplet-conversion, singlet–singlet annihilation, singlet–triplet annihilation, and so on. Therefore, the model presented in this report has its limitations.

By assuming Gaussian point spread function for the confocal system, during one excitation period, the probability for the dye tagged on a monomer to emit one photon, \( p_i \), is given by:

\[
p_i = I_{0,i} \exp \left( -\frac{2(x_i^2 + y_i^2)}{w_{xy}^2} - \frac{2z_i^2}{w_z^2} \right)
\]

where \((x_i, y_i, z_i)\) is the location of the monomer \(i\), and \(w_{xy}\) and \(w_z\) represent the radii in the \(x-y\) plane and the light sheet, respectively.

\( V_0 = (\pi/2)^3 w_{xy}^2 w_z \) is the effective confocal volume. \( I_{0,i} \) represents the fluorescence intensity of the molecule in the center of the confocal volume. \( I_{0,i} \) is related to laser power, molecular absorption cross section, quantum yield, and collection efficiency of the instrument (see the Supporting Information (SI) for details).

For a solution with only \( N_1 \) number of monomers, the probability of emitting one photon in one excitation period, \( P_1 \), can be written as

\[
P_1 = \sum_{i=1}^{N_1} (p_i) \prod_{j=1, j \neq i}^{N_1} (1 - p_j)
\]

where \(i\) and \(j\) denote different monomer molecules.

As the time interval among neighboring pulses (for example, \( \sim 100 \) ns for an excitation repetition rate of \( 10 \) MHz) is much shorter than the diffusion time of molecules (\( \sim \)ms), the positions of oligomers in a few consecutive pulses are approximately the same. Then, photon-emission probability in consecutive pulses can be written out. For example, the probability of emitting one photon in the first excitation and no photon emission in the second pulse, \( P_{1,0} \), can be written as

\[
P_{1,0} = \sum_{i=1}^{N_1} (p_i)(1 - p_i) \prod_{j=1, j \neq i}^{N_1} (1 - p_j)
\]

Since all molecules have the same probability to appear at any location in the solution, the time-averaged single-photon-emission probabilities \( \bar{P}_{1} \) and \( \bar{P}_{1,0} \) are equal to the space-averaged emission probability, i.e.,

\[
\bar{P}_1 = \sum_{i=1}^{N_1} \left( \frac{\iint (1 - p_i) dx_i dy_i dz_i}{V} \right)
\]

\[
\bar{P}_{1,0} = \sum_{i=1}^{N_1} \left( \frac{\iint (1 - p_i) dx_i dy_i dz_i}{V} \right) \prod_{j=1, j \neq i}^{N_1} \frac{\iint (1 - p_j) dx_j dy_j dz_j}{V}
\]

where \( V \) is the volume of the sample cell. Since the volume of the sample cell \( V \) is usually much larger than the effective confocal volume \( V_0 \), the number of monomers in the sample volume is much larger than in the confocal volume. Then,

\[
\bar{P}_1 = \sum_{i=1}^{N_1} \left( \frac{\iint (1 - p_i) dx_i dy_i dz_i}{V} \right) = c_i N_A I_0 \bar{P}_0
\]

\[
\bar{P}_{1,0} = \sum_{i=1}^{N_1} \left( \frac{\iint (1 - p_i) dx_i dy_i dz_i}{V} \right) \prod_{j=1, j \neq i}^{N_1} \frac{\iint (1 - p_j) dx_j dy_j dz_j}{V}
\]

\[
= c_i N_A \left( I_0 - \frac{1}{V} \int_0^1 \frac{dI}{I} \right) V \bar{P}_0
\]

(5)

\( N_A \) is the Avogadro constant, \( c_1 \) is the concentration of the monomer, and \( c_i = \frac{N_i}{N_0} \). \( P_0 \) and \( P_{1,0} \) represent the probabilities of no photon emission in one pulse and in two consecutive pulses, respectively. \( P_0 \) and \( P_{1,0} \) are written as

\[
P_0 = \prod_{i=1}^{N_1} \frac{\iint (1 - p_i) dx_i dy_i dz_i}{V}
\]

\[
P_{1,0} = \prod_{i=1}^{N_1} \frac{\iint (1 - p_i) dx_i dy_i dz_i}{V} \prod_{k=1}^{N_2} \frac{\iint (1 - p_k) dx_k dy_k dz_k}{V}
\]

(6)

For a mixture of monomers and dimers with molecule numbers of \( N_1 \) and \( N_2 \), respectively, \( P_1 \), \( P_{1,0} \), and \( P_2 \) (average probabilities for two-photon emission in one excitation) can be written as

\[
P_1 = \sum_{i=1}^{N_1} \frac{\iint p_i dx_i dy_i dz_i}{V} + \sum_{i=1}^{N_2} \frac{\iint 2p_i(1 - p_i) dx_i dy_i dz_i}{V}
\]

\[= c_1 N_A I_0 V_0 + 2c_2 N_A \left( I_0 - \frac{1}{V} \int_0^1 \frac{dI}{I} \right) V \bar{P}_0
\]

\[
\bar{P}_1 = \frac{1}{2} (\bar{P}_0)^2 + \sum_{i=1}^{N_1} \left( \frac{\iint p_i dx_i dy_i dz_i}{V} \right) = \frac{1}{2} (\bar{P}_0)^2 + c_2 N_A \left( I_0 - \frac{1}{V} \int_0^1 \frac{dI}{I} \right) I_0 \bar{P}_0
\]

(7)

\[
P_{1,0} = \sum_{i=1}^{N_1} \left( \frac{\iint (1 - p_i) dx_i dy_i dz_i}{V} \right) \prod_{j=1, j \neq i}^{N_1} \frac{\iint (1 - p_j) dx_j dy_j dz_j}{V}
\]

\[
+ \sum_{j=1}^{N_2} \left( \frac{\iint 2p_j(1 - p_j) dx_j dy_j dz_j}{V} \right) \prod_{k=1, k \neq j}^{N_2} \frac{\iint (1 - p_k) dx_k dy_k dz_k}{V}
\]

\[= c_1 N_A \left( I_0 - \frac{1}{V} \int_0^1 \frac{dI}{I} \right) V_0 + 2c_2 N_A \left( I_0 - \frac{1}{V} \int_0^1 \frac{dI}{I} \right)^2 + 3 \frac{1}{V} \int_0^1 \frac{dI}{I} \left( I_0 - \frac{1}{V} \int_0^1 \frac{dI}{I} \right)
\]

\[\cdot \left( \frac{1}{8} I_0 \right)^2 \bar{P}_0 \]

In the same manner, for a mixture of monomers, dimers, trimers, and tetramers with molecule numbers of \( N_1 \), \( N_2 \), \( N_3 \), and \( N_4 \), respectively, the photon-emission probabilities can be derived.
Theoretically, photon-emission probabilities \( \bar{P} \) and \( \bar{P}_0 \) can be derived. However, it is more complex. Therefore, here we limit aggregations up to tetraoligomers for simplicity.

Photon-emission probabilities \( \bar{P}_i \) can all be measured from multichannel TTTR confocal fluorescence experiments via the below equations

\[
\bar{P}_i = \sum_{n=1}^{N_i} \frac{\int p^i(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 2p^i(1-p)^2(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 4p^i(1-p)^3(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 4p^i(1-p)^3(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 6p^i(1-p)^4(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 6p^i(1-p)^4(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 8p^i(1-p)^5(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 8p^i(1-p)^5(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 10p^i(1-p)^6(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 10p^i(1-p)^6(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 12p^i(1-p)^7(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 12p^i(1-p)^7(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 14p^i(1-p)^8(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 14p^i(1-p)^8(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 16p^i(1-p)^9(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 16p^i(1-p)^9(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 18p^i(1-p)^10(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 18p^i(1-p)^10(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 20p^i(1-p)^11(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 20p^i(1-p)^11(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 22p^i(1-p)^12(x, y, z) \, dx \, dy \, dz}{V} + \sum_{n=1}^{N_i} \frac{\int 22p^i(1-p)^12(x, y, z) \, dx \, dy \, dz}{V}
\]

where \( c_1 \) and \( c_4 \) are the concentrations of trimers and tetramers, respectively, and \( c_3 = \frac{N_t}{N_{TTTR}} \) and \( c_4 = \frac{N_t}{N_{TTTR}} \). Theoretically, photon-emission probabilities for oligomers larger than tetramers can also be derived. However, it is more complex. Therefore, here we limit aggregations up to tetraoligomers for simplicity.

Photon-emission probabilities \( \bar{P}_i \) and \( \bar{P}_0 \) can all be measured from multichannel TTTR confocal fluorescence experiments via the below equations

\[
\bar{P}_i = \frac{M_i}{t \cdot f} \quad \text{and} \quad \bar{P}_0 = \frac{M_0}{t \cdot f}
\]

where \( M_i \) is the number of pulses with the photon-emission number of \( n, M_{1,0} \) is the number of two consecutive pulses with one photon emission in the first pulse and no photon emission in the second pulse, \( t \) is the measurement time, and \( f \) is the laser excitation frequency. Then, by solving eq 8, oligomer concentrations \( c_1, c_2, c_3, \) and \( c_4 \) can be obtained.

For the channel number of TTTR single-photon recording, practically it is possible to use eight channels. Then, in simulations, we have adopted an eight-channel TTTR confocal configuration, in which each single-photon detectors are

Figure 1. Illustration of a multichannel TTTR confocal setup and photon emission from a monomer and a dimer excited by laser pulses.

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required. For each fluorescence photon, each channel will have 1/8 probability to receive the photon. However, in the case of multiphoton emission in one pulse, we need to consider wrapping of multiphotons in one detector. That is, in the case of two or more photon emissions, there is a probability for more than one photon to arrive in one channel but the channel can only detect one photon because of a long dead time of the single-photon detector. So, from the histogram of photon numbers detected by detectors ($M_{\text{det}}$), deconvolution is needed to give a real histogram of the photon number distribution ($M_n$) (see the SI for details).

2.2. Simulation Results. Simulations have been carried out to verify the model. A periodic cubic box (side length of 6000 nm), much larger than the confocal volume ($w_{xy} = 300$ nm, $w_z = 1500$ nm), was used to simulate the sample cell. The concentrations of oligomers in the sample cell were set in the range of several nanomolars to ensure that there were only several oligomers in the confocal volume at the same time. The laser excitation wavelength $\lambda$ was 646 nm, the laser excitation frequency $f = 1 \times 10^7$ Hz, $w_{xy}$ was 300 nm, and $w_z$ was 1500 nm. The laser power varied from 0.1 to 20 $\mu$W. The numerical aperture of the objective lens NA was 1.2, and the objective collection efficiency $\eta_1$ was 28.44% when the medium between the sample and the objective was water. Optical components' efficiency $\eta_2$ and detector efficiency $\eta_3$ were supposed to be 0.85 and 0.85, respectively. Properties of all fluorophores tagged on monomers and dimers were assumed to be identical. Molar absorption coefficient $\varepsilon$ was $1.30 \times 10^5$ L mol$^{-1}$ cm$^{-1}$ at the excitation wavelength, and quantum yield was $Q = 0.8$. All of the simulations above were implemented in MATLAB with home-written scripts (see the SI for simulation details).

2.2.1. Monomer and Monomer–Dimer Systems. First, a solution of monomers (labeled with one dye) and a mixture of monomers and dimers (labeled with one and two dyes, respectively) were simulated. The excitation power, $P_{\text{laser}}$, was set at 15 $\mu$W (corresponding $I_0$ of 0.1421).

Table 1. Concentrations and Fluorescence Brightness Derived from the Model for the Monomer-Only and Dimer-Only Systems

| parameters | monomer-only solution | dimer-only solution |
|------------|-----------------------|---------------------|
| $P_{\text{laser}}$ ($\mu$W) | 15 | 15 |
| $I_0$ | 0.1421 | 0.1419 | 0.1422 |
| $c_1$ (nM) | 1.0000 | 1.0042 | 0.4989 |
| $c_2$ (nM) | 0.0008 | 0.5000 | 0.0054 |

Figure 3. Simulation results of two monomer-to-tetramer mixture solutions. (a) Probability of $n$-photon emission in a pulse, $P_{\text{deconv}}$ (b) deconvoluted probability of $n$-photon emission by one oligomer in a pulse, $P_{\text{deconv}}$. For the mixture solution 1, input concentrations were $c_1 = 0.1$ nM, $c_2 = 0.1$ nM, $c_3 = 0$ nM, and $c_4 = 0.1$ nM with simulation time $t = 490$ s and total photon number $\sim 8.50 \times 10^7$. For the mixture solution 2, input concentrations were $c_1 = 0.1$ nM, $c_2 = 0.05$ nM, $c_3 = 0.1$ nM, and $c_4 = 0.05$ nM with simulation time $t = 510$ s and total photon number $\sim 8.50 \times 10^7$. Laser power was set at 16 $\mu$W (corresponding $I_0$ of 0.1516).
monomer, dimers, trimers, and tetramers were simulated. The concentrations are mentioned in the caption of Figure 3. Simulation parameters for the mixture of concentrations of monomers, dimers, trimers, and tetramers were $D_1 = 1.00 \times 10^{-6}$ cm$^2$ s$^{-1}$ and $D_2 = 7.94 \times 10^{-7}$ cm$^2$ s$^{-1}$, respectively.

Figure 2 shows the simulation results for monomer-only (concentration $c_1 = 1$ nM) and dimer-only (concentration $c_2 = 0.5$ nM) solutions. Figure 2a shows the photon-emission probability of only one monomer or one dimer, $P_{\text{deconv}}$. For example, the probability of two-photon emission in one excitation is zero for a monomer and nonzero for a dimer. Table 1 lists the probability of two-photon emission by a single dimer $P_{2, \text{deconv}}$ in eq 11 was obtained by removing the contribution of two monomers

$$\frac{1}{2} \bar{P}_1^2.$$ 

$$\bar{P}_{1, \text{deconv}} = \bar{P}_1$$ 

$$\bar{P}_{2, \text{deconv}} = \bar{P}_2 - \frac{1}{2} \bar{P}_1^2$$

(11)

It can be clearly seen from Figure 2b that the deconvoluted probability of two-photon emission in one excitation is zero for a monomer and nonzero for a dimer. Table 1 lists the concentrations and fluorescence brightness derived from the model. For the two-sample system, all simulated $c_1$ and $c_2$ values are within 1% relative error from the input values.

### 2.2.2. Mixture of Monomers, Dimers, Trimers, and Tetramers

Two mixture solutions containing different concentrations of monomers, dimers, trimers, and tetramers were simulated. The concentrations are mentioned in the caption of Figure 3. Simulation parameters for the mixture of monomer, dimers, trimers, and tetramers were $D_1 = 1.00 \times 10^{-6}$ cm$^2$ s$^{-1}$, $D_2 = 7.94 \times 10^{-7}$ cm$^2$ s$^{-1}$, $D_3 = 6.93 \times 10^{-7}$ cm$^2$ s$^{-1}$, and $D_4 = 6.30 \times 10^{-7}$ cm$^2$ s$^{-1}$, respectively, and laser power $P_{\text{laser}} = 16$ μW. Figure 3a shows the probability of n-photon emission $P_n$. Figure 3b shows the deconvoluted probability of a single oligomer $P_{n, \text{deconv}}$, in which

$$\bar{P}_{1, \text{deconv}} = \bar{P}_1$$ 

$$\bar{P}_{2, \text{deconv}} = \bar{P}_2 - \frac{1}{2} \bar{P}_1^2$$ 

$$\bar{P}_{3, \text{deconv}} = \bar{P}_3 - \bar{P}_2 \bar{P}_1 + \frac{1}{3} \bar{P}_1^3$$ 

$$\bar{P}_{4, \text{deconv}} = \bar{P}_4 - \bar{P}_3 \bar{P}_1 - \frac{1}{2} \bar{P}_2^2 + \bar{P}_1 \bar{P}_2 \bar{P}_3 - \frac{1}{4} \bar{P}_1^4$$

(12)

Because of the same total dye concentrations and similar oligomer concentrations, there is almost no difference in $\bar{P}_n$ and $P_{n, \text{deconv}}$ for $n = 0, 1, 2$ between the two systems. In addition, even for $n = 3$ and $4$, the difference in $\bar{P}_n$ and $P_{n, \text{deconv}}$ is also small. However, we can still extract the oligomer concentration from the simulation data (Table 2). All $c_1$, $c_2$, $c_3$, and $c_4$ values derived from the model are within 6% relative error from the input values.

#### 2.2.3. Fluorescence Brightness Effect on the Simulation Results

Fluorescence brightness is directly related to the signal-to-noise ratio of data and hence has a very important role in the accuracy of concentration quantification. Simulations of mixture solution of monomers and dimers with different excitation powers were conducted, which are equivalent to simulations with different fluorescence quantum yields. Figure 4 shows that with increasing excitation power,
However, a monomer-to-tetramer mixture is more sensitive to noise. The relative error is around 10−50% for the concentrations (Figure 5c,d). To obtain a relative error less than 20%, the background noise should be lower than 200 cps and the detector noise should be lower than 100 cps (Figure 5d). In practical conditions, a background noise of less than 200 cps can be achieved and a detector noise can be low to 25 cps.

3. CONCLUSIONS

On the basis of fluorescence antibunching property of individual dye molecules, we have derived a new statistic model to quantify oligomer concentrations in solution. In the model, an analytical relationship between photon-emission probability under pulsed excitation and the concentrations of oligomers has been derived. Simulation results have showed that the model can accurately obtain concentrations of oligomers with a relative error within 10% for mixtures of monomers and dimers, and monomers, dimers, trimers, and tetramers at low-noise conditions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01387.

Simulation details and procedures for deconvolution photon-wrapping effect in the multiphoton counting system (PDF)

Figure 5. Relative error of obtained concentrations and fluorescence brightness for (a, b) monomer−dimer mixture and (c, d) monomer-to-tetramer mixture solutions at different noise levels. In (a) and (c), detector noise was fixed at 100 cps. In (b) and (d), background noise was fixed at 200 cps. For the monomer−dimer mixture solutions, input concentrations were $c_1 = 0.5$ nM and $c_2 = 0.25$ nM, and laser power was set at 15 μW (corresponding $I_0$ of 0.1421) with simulation time $t = 260$ s. For the monomer-to-tetramer solutions, input concentrations were $c_1 = 0.2$ nM, $c_2 = 0.1$ nM, $c_3 = 0.1$ nM, and $c_4 = 0.05$ nM, and laser power was set at 15 μW (corresponding $I_0$ of 0.1421) with simulation time $t = 350$ s.

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Notes

The authors declare no competing financial interest.

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