Fluorogenic organocatalytic reactions

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Chapter 6
Towards Single Molecule detection of organocatalytic reactions

6.1. Introduction
Although the theoretical description of chemical reactions is typically based on single molecules, practical experimental data are almost always obtained from large ensembles of molecules.\textsuperscript{1–3} Any property thus obtained is an average over the whole ensemble. Developments in optical techniques over the past decades have provided new powerful tools to explore reactions at the single molecular level. The pioneering work in this area was limited to studies at low temperature for example at liquid helium temperature, but nowadays single molecule spectroscopy is applied at room temperature and even in living cells.\textsuperscript{1,2,4} Optical single molecule spectroscopy allows observing the dynamical state changes in one molecule in a condensed phase sample.\textsuperscript{1,5–9}

In the context of the present work our ultimate goal is to monitor reactions of individual molecules in the field of organocatalysis. In chapter 3 we described our studies of the Michael addition reactions of fluorogenic acceptors (compounds 15 and 17) with benzyl mercaptan and with dimethyl malonate in the presence of organocatalysts.

The binding interactions between the catalysts and the Michael acceptors were shown to give a small fluorescence response, but a strong enhancement of the fluorescence was observed as result of the addition of the nucleophile
to the Michael acceptor. We also demonstrated in Chapter 3 that two immobilized catalysts are effective for this reaction, and that the reaction in solution could be catalyzed by a catalyst bearing a perylene imide fluorescent label. These results set the stage for the single molecule studies described in this chapter. We decided to use total internal reflection fluorescence microscopy to image the change in fluorescence by adding the solution of compound 15 to the immobilized catalysts 30 and 31. The catalysts are not fluorescent themselves. We expect, however, that due to binding of compound 15 to the catalysts fluorescent spots will appear.

![Scheme 2. Immobilized catalysts 30 and 31 on the glass surface.](image)

The single molecules of interest must be detected in the presence of solvent molecules and other molecules which are present in the photoexcited volume. The fluorescent photons emitted by the molecule of interest provide the signal. The main challenge in these measurements is to increase the signal-to-background and signal-to-noise ratios. The noise arises from fluctuation effects and the background is produced by photons from any source other than the target single molecule.\(^1\) Use of a small emission volume, high absorption cross section at the laser wavelength, high fluorescence quantum yield, high photostability, and operation below saturation of molecular absorption all contribute to improving the signal to noise and background ratios.\(^2\)

Total internal reflection fluorescence microscopy (TIRFM) is a technique which is ideal for imaging events that occur at a surface.\(^3\) This technique can be applied in two forms, which are called through-objective TIRFM and prism-based TIRFM (Scheme 3).\(^4,5\) In the case of prism-based TIRF, the excitation light path and the emission channel are separated. In prism-based TIRF, a prism is attached to the coverslip's surface which directs a focused light beam or laser towards the coverslip/medium interface. With the help of the prism the angle of the penetrating light is adjusted to the critical angle. In the case of objective-type TIRF, the excitation light path and the emission channel share the objective and other optical elements of the microscope. The light, usually laser light, is directed to the specimen through the objective, which also collects the emitted fluorescence light.\(^6,7\) Modern TIRF microscopy systems are usually objective-based.

![Scheme 3. Schematic representation of (a) prism-based TIRFM, (b) through-objective TIRFM.](image)

6.2. Imaging with TIRFM

In general, total internal reflection illumination can be applied in imaging of small particles or single molecules on the surface of samples such as a cell membrane on top of a coverslip.\(^8\) Using this method, selective visualization and spectroscopy of contact regions of cell and substrate is possible. Also the kinetic rates of binding of extracellular and intracellular proteins to cell surfaces can be measured.\(^9\) In these measurements Raman scattering from water molecules, incident light breaking through filters, luminescence arising from the objective lens, oil and dust are all sources of background that should be suppressed.\(^10\) Beside the application of TIRFM in biology, this method has also been applied to track various catalytic processes.\(^11,12\) In 2010 Blum and co-workers used TIRFM to study the spatial distribution of chemical reactivity of a triethoxysilane-modified surface at the solution/surface interface. Binding of a BODIPY bearing platinum complex to thiourea containing coverslips was investigated (Scheme 4). They found that the chemical reaction of one platinum complex does not have an effect on the location of the other chemical reactions.\(^13\)
to the Michael acceptor. We also demonstrated in Chapter 3 that two immobilized catalysts are effective for this reaction, and that the reaction in solution could be catalyzed by a catalyst bearing a perylene imide fluorescent label.

These results set the stage for the single molecule studies described in this chapter. We decided to use total internal reflection fluorescence microscopy to image the change in fluorescence by adding the solution of compound 15 to the immobilized catalysts 30 and 31. The catalysts are not fluorescent themselves. We expect, however, that due to binding of compound 15 to the catalysts fluorescent spots will appear.

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Towards Single Molecule detection of organocatalytic reactions

Scheme 4. The reaction of platinum complex-BODIPY with surface thiourea groups rapidly immobilizes the complex through platinum-sulfur covalent bond formation.15

Herten and co-workers used TIRFM with simultaneous laser excitation at 532 nm and 488 nm to study oxidation reaction pathways of an oxallyl styryl-BODIPY with meta-chloroperoxybenzoic acid (Scheme 5). Dual-color detection in the wavelength ranges of 570 – 615 nm for the yellow fluorescence (reactant) and 500 – 525 nm for the green emission (product) was realized by using an emCCD camera. According to their observations most of the individual transformations occur in a direct manner but a few of them include a dark intermediate state.16

Scheme 5. Epoxidation reaction of an immobilized substrate on a cover slip.16

The fluorogenic reduction reaction between nonfluorescent resazurin and hydroxylamine catalyzed by Au nanocatalysts has been studied by different research groups.17,21,22 Chen and co-workers could determine the location of catalytic events in the reductive N-deoxygenation of resazurin to resorufin by NH₂OH. Gold nanorods were used as the catalyst and super-resolution fluorescence microscopy and scanning electron microscopy (SEM) were applied for imaging of the single catalyst and to quantify its activity.17

6.3. Experiment design

A characteristic of enantioselective organocatalytic bond forming reactions such as the Michael addition, aldol condensation, Henry reaction and many others, is that the catalyst brings two reagents together in a favorable orientation, and activates at least one of the reagents for the reaction to occur. Direct observation of the transition state of the reaction is currently beyond the power of experiment. Computational methods have made great progress in the description of transition states, but the quantitative accuracy that is required to make a decisive statement about mechanisms is not easily achieved. Another approach to get insight into a reaction mechanism is the study of reaction kinetics: by monitoring the rate of the reaction as a function of concentration of the catalyst and the reagents, much can be learned. In many cases, the overall organocatalytic reaction is first-order in all three
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Towards Single Molecule detection of organocatalytic reactions

23 This is compatible with a simple reaction mechanism as shown in Scheme 7.

Scheme 7. Bond formation between two reactants using a bifunctional catalyst.

One of the reagents (reversibly) forms a complex with the catalyst, activating the reagent by deprotonation (turning it into a nucleophile) or by covalent activation e.g. as an enamine or iminium ion (we use the term “complex” here for generality). The other reagent reacts with this complex, possible via an intermediate ternary complex. In many cases the concentration of the complexes in solution is fairly low. Single molecule fluorescence may be able to make them detectable by adding a constraint on the experiment, namely the spatial position of one of the reaction partners. We immobilize this on a glass cover slip, and use fluorescence microscopy to detect binding events in which a transient local fluorescence is observed. There are several possible implementations. In the present work, we immobilize the catalysts (Takemoto catalyst 30 and cinchona alkaloid derivative 31, see Chapter 3), and add the maleimide-BODIPY dye 15 as one of the reagents. We detect the dye during its binding to the catalyst due to the fluorescent spots on the cover slips. When benzyl mercaptan is added, it may react with the complex of catalyst and 15, leading to release of the product and a shorter duration of the binding. In a complementary experiment in this project, Dongdong Zheng in our laboratory immobilized a maleimide unit on the glass surface, and studied the binding of the fluorescent catalyst 32 of which we demonstrated in chapter 3 that it is an active catalyst for the Michael addition (Scheme 8).

Scheme 8. Observation of the interaction between catalyst 32 and reactants. In this case, one of the reagents (the Michael acceptor) is immobilized on the cover slip, the catalyst is fluorescent. When the catalyst binds to the immobilized reagent, a fluorescent spot is detected in the TIRF image. When the catalyst is free in solution it diffuses fast on the timescale of the image acquisition, and it just gives a broad background.

6.4. TIRFM of organocatalytic Michael addition of benzyl mercaptan to compound 15

We used immobilized catalyst 30 on the surface of cover slips for TIRF measurements. The coverslip with immobilized catalyst was attached to a glass standard Joint Socket using 5 min epoxy glue from Devcon (Figure 1).

Figure 1. Photograph of the reaction chamber used in TIRF measurements.

In the experiments, the argon ion laser (see Chapter 2) was set to 496 nm as the excitation wavelength, and the power was 85 mW. The emission was selected using a 496 nm long pass filter combined with a notch filter 488 ± 10 nm to block the unwanted scattered light from the excitation beam. The
Towards Single Molecule detection of organocatalytic reactions components.\textsuperscript{23} This is compatible with a simple reaction mechanism as shown in Scheme 7.

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6.4. TIRFM of organocatalytic Michael addition of benzyl mercaptan to compound 15

We used immobilized catalyst \textsuperscript{30} on the surface of cover slips for TIRF measurements. The coverslip with immobilized catalyst was attached to a glass standard Joint Socket using 5 min epoxy glue from Devcon (Figure 1).

In the experiments, the argon ion laser (see Chapter 2) was set to 496 nm as the excitation wavelength, and the power was 85 mW. The emission was selected using a 496 nm long pass filter combined with a notch filter 488 ± 10 nm to block the unwanted scattered light from the excitation beam. The
exposure time for each image and the interval time were chosen to be 20 ms and 1 ms, respectively. First, the images of the cover slip (500 images in series) were taken by the use of the TIRF microscope with the sCMOS imaging camera. The catalyst is not fluorescent. So, in this movie we do not expect any fluorescent spot on the surface of the catalyst. In some cases, however, because of contamination from the environment some fluorescent spots appeared on the surface of the cover slip. These spots can be distinguished from the fluorescent species that resulted from binding to the surface because these contaminants give stable bright spots, while the spots that result from the interaction between catalyst and the substrate appear and disappear during the recording process. After recording the movie of the cover slip with immobilized catalyst, a solution of compound 15 in acetonitrile (10^4 M) was prepared. After adding 100 µL of the solution to the cuvette attached to the cover slip, a second series of images (movie) was recorded. It is expected that by adding compound 15 fluorescent single molecules appear as a result of attaching the compound to one of the catalyst molecules on the surface. Because this attachment is a reversible process, the compound can bind and dissociate again from the catalyst. This process is seen as blinking points which appear and disappear in different positions in the recorded movie. In the next step, 100 µL of 10^7 M benzyl mercaptan solution in acetonitrile was added to the solution and the movie was recorded again under otherwise identical conditions. Benzyl mercaptan is known to react with compound 15 to produce the fluorescent compound 38. So, compound 15 will be detached from the catalyst. While the background fluorescence is expected to increases due to producing fluorescent product in solution, the fluorescent single molecules on the surface of the coverslip disappear. A schematic representation of the expected events is shown in Scheme 9.

We analyzed the recorded movies by the use of a custom-made MATLAB program to obtain information about the lifetimes of the fluorescent spots that resulted from interaction between compound 15 and catalyst 30. In the program the background fluorescence is removed and noise is suppressed by applying a Gaussian blur filter and a boxcar filter to each image, and taking the difference of the two filtered images. Next, the program identifies the positions of fluorescent spots (called “particles”, because this type of analysis is typically used for tracking of fluorescent particles in series of images). In the next step time traces are constructed by assuming that particles in the same position in subsequent images are the same species. A certain tolerance on the difference of the positions is applied here (2 pixels). In this way for each particle a certain residence time is determined, given by the number of
Towards Single Molecule detection of organocatalytic reactions

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subsequent frames in which it appears times the time per frame. For acceptance of a spot as a particle, two threshold parameters are defined. One of them is the maximum size of a particle in pixels. Each pixel equals to 63 nm. Another one is the minimum fluorescence intensity of the particle. For the experiments summarized in Table 1 the total numbers of fluorescent particles range from 12000 to 72000 particles depending on the selected size and intensity thresholds for the particles.

After fitting the resulting fluorescence decay curve as a sum of three exponential components, three time constants, \( \tau_1 \), \( \tau_2 \), \( \tau_3 \), were obtained (entry 2, Table 1). We also analyzed the data choosing smaller size limits of the particles, and found two time constants, \( \tau_1 \approx 6.5 \text{ ms} \) and \( \tau_2 \approx 23 \text{ ms} \) (entry 1, Table 1). The rare cases of long binding times give rise to the third component but can be ignored when the number of binding events is smaller. The time constants, however, do not change significantly (Figure 2).

<table>
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<tr>
<th>Entry</th>
<th>Catalyst</th>
<th>Solvent</th>
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<th>( \tau_2 ) (ms)</th>
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<th>( \tau_3 ) (ms)</th>
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Note: Entries 1-6 are the results of three different coverslips with catalyst 30. In every case analysis was done by choosing two different sizes for the particles. ACN: Acetonitrile; BZ: Benzene;

After fitting the resulting fluorescence decay curve as a sum of three exponential components, three time constants, \( \tau_1 \approx 10 \text{ ms} \), \( \tau_2 = 30 \text{ ms} \) and \( \tau_3 = 110 \text{ ms} \) were obtained (entry 2, Table 1). We also analyzed the data choosing smaller size limits of the particles, and found two time constants, \( \tau_1 \approx 6.5 \text{ ms} \) and \( \tau_2 \approx 23 \text{ ms} \) (entry 1, Table 1). The rare cases of long binding times give rise to the third component but can be ignored when the number of binding events is smaller. The time constants, however, do not change significantly (Figure 2).

Figure 2. Histogram of binding times of the fluorescent particles resulting from addition of compound 15 in acetonitrile to the cover slip with catalyst 30. The different curves resulted by choosing 6 pixels or 8 pixels for the size of the particle.

Then we repeated the measurement with two other cover slips with immobilized catalyst 30 under very similar conditions. The results are in fair agreement with each other (entries 3-6, Table 1), (Figure 3).

Figure 3. Histogram of binding times of the fluorescent particles resulting from addition of compound 15 in acetonitrile to three different cover slips with catalyst 30 (entries 2-7 in Table 1).

Under the present experimental conditions, the time constant of the fast process is less than the exposure time of one frame (20 ms). The particles that
subsequent frames in which it appears times the time per frame. For acceptance of a spot as a particle, two threshold parameters are defined. One of them is the maximum size of a particle in pixels. Each pixel equals to 63 nm. Another one is the maximum fluorescence intensity of the particle. For the experiments summarized in Table 1 the total numbers of fluorescent particles range from 12000 to 72000 particles depending on the selected size and intensity thresholds for the particles.

Table 1. Time constants resulted from analyzing the collected data from TIRFM.

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<th>Entry</th>
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<th>Catalyst</th>
<th>Size, (pixels)</th>
<th>( t_1 ) (ms)</th>
<th>( A_1 ) (%)</th>
<th>( t_2 ) (ms)</th>
<th>( A_2 ) (%)</th>
<th>( t_3 ) (ms)</th>
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<td>14</td>
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<td>-</td>
<td>-</td>
<td>14</td>
<td>50</td>
<td>2</td>
</tr>
</tbody>
</table>

*CAT*: Catalyst; *SOL*: Solvent; *Size*: average binding time \( t_{ave} = (\sum A_i \tau_i)/ (\sum A_i) \); \( N \) : average number of particles per frame in the first 200 frames; \( \sum A_i \) entries 1-6 are the results of three different coverslips with catalyst 30. In every case analysis was done by choosing two different sizes for the particles. *Acetonitrile*; *Benzene*;

After fitting the resulting fluorescence decay curve as a sum of three exponential components, three time constants, \( t_1 = 10 \) ms, \( t_2 = 30 \) ms and \( t_3 = 110 \) ms were obtained (entry 2, Table 1). We also analyzed the data choosing smaller size limits of the particles, and found two time constants, \( t_1 = 6.5 \) ms and \( t_2 = 23 \) ms (entry 1, Table 1). The rare cases of long binding times give rise to the third component but can be ignored when the number of binding events is smaller. The time constants, however, do not change significantly (Figure 2).

Figure 2. Histogram of binding times of the fluorescent particles resulting from addition of compound 15 in acetonitrile to the cover slip with catalyst 30. The different curves resulted by choosing 6 pixels or 8 pixels for the size of the particle. Then we repeated the measurement with two other cover slips with immobilized catalyst 30 under very similar conditions. The results are in fair agreement with each other (entries 3-6, Table 1), (Figure 3).

Figure 3. Histogram of binding times of the fluorescent particles resulting from addition of compound 15 in acetonitrile to three different cover slips with catalyst 30 (entries 2-7 in Table 1).

Under the present experimental conditions, the time constant of the fast process is less than the exposure time of one frame (20 ms). The particles that...
Chapter 6

Table 2. Time constants resulting from analyzing the collected data from TIRFM for different concentrations of compound 15 in acetonitrile after adding to immobilized catalyst 30. The selected size and the intensity for threshold were 6 and 8 respectively.

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>τ1 (ms)</th>
<th>A1 (%)</th>
<th>τ2 (ms)</th>
<th>A2 (%)</th>
<th>τ3 (ms)</th>
<th>A3 (%)</th>
<th>n</th>
<th>η</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0.79 \times 10^{-5}$</td>
<td>8.5</td>
<td>97</td>
<td>27</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>141</td>
</tr>
<tr>
<td>$1.9 \times 10^{-5}$</td>
<td>9</td>
<td>85</td>
<td>23</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>11</td>
<td>148</td>
</tr>
<tr>
<td>$4 \times 10^{-5}$</td>
<td>11</td>
<td>98.5</td>
<td>30</td>
<td>1.4</td>
<td>120</td>
<td>0.1</td>
<td>13</td>
<td>160</td>
</tr>
</tbody>
</table>

*average binding time $t_{\text{av}} = \sum A_i t_i / \sum A_i$, $^\ddagger$ average number of particles per frame in the first 200 frames.

We used different laser powers to record the movies to distinguish the hydrogen bonding dissociation from photobleaching. 100 μL of the solution of compound 15 ($2.38 \times 10^{-3}$ M) was added to the glass cell attached to the coverslip with immobilized catalyst 30.

Table 3. Time constants from analyzing the collected data from TIRFM with different power of laser using $2.38 \times 10^{-3}$ M of compound 15 in acetonitrile after addition to immobilized catalyst 30. The selected size and the intensity for threshold were 6 and 8, respectively.

<table>
<thead>
<tr>
<th>Power of laser (mW)</th>
<th>τ1 (ms)</th>
<th>A1 (%)</th>
<th>τ2 (ms)</th>
<th>A2 (%)</th>
<th>τ3 (ms)</th>
<th>A3 (%)</th>
<th>n</th>
<th>η</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>7</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>107</td>
</tr>
<tr>
<td>31</td>
<td>9</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>130</td>
</tr>
<tr>
<td>120</td>
<td>97.2</td>
<td>10</td>
<td>2.5</td>
<td>110</td>
<td>0.3</td>
<td>14</td>
<td>270</td>
<td></td>
</tr>
</tbody>
</table>

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The movie of the event was recorded under the same condition as previous experiments. The results are summarized in Table 3. Applying low power of the laser, one time constant was obtained while increasing the power of laser resulted in three time constants probably because the number of long-lasting particles is not noticeable at lower laser power, where fewer particles are detected.

We used immobilized catalyst 31 on the surface of glass and recorded the interaction between this catalyst and compound 15 with the same excitation wavelength and emission filter (entries 10-15, Table 1). Smaller size and lower intensity than in the case of catalyst 30 should be chosen to pick up the desired particles.

Towards Single Molecule detection of organocatalytic reactions. In each experiment we recorded the number of particles observed in each frame. As can be seen in Figure 4 the number of particles decreases quite clearly during the experiment in the case of catalyst 30, which suggests that photobleaching occurs, or the catalyst is deactivated. This issue should be addressed in future research.

![Graph showing number of fluorescent particles per frame with catalyst 30 and 31](attachment:graph.png)

Figure 4. Number of fluorescent particles per frame; (a) with catalyst 30, (b) with catalyst 31.

We used different concentrations of compound 15 in acetonitrile, expecting a smaller number of events at lower concentrations. The result is summarized in Table 2. The solution of compound 15 with concentration about $\sim 10^{-6}$ M was too dilute to observe the fluorescent particles and the solution with concentration $\sim 10^{-7}$ M was too concentrated to allow the molecules to be detected individually.

The solution with $\sim 10^{-6}$ M was found to be the most suitable for the single molecule detection. By increasing the concentration of the solution of compound 15 the binding time does not change a lot. The lifetime decay curve is fitted as bi-exponential at low concentration. By increasing the concentration the fitting pattern had to be changed to tri-exponential to fit the data properly.

occur in one frame only may be due to non-specific binding, or result from noise. Therefore, the first frame was excluded from the fit. The presence of three components in the distribution of binding times probably reflects a heterogeneity in the strengths of the binding at different sites. For comparison we also calculated the average lifetime $\tau_{av}$.

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Towards Single Molecule detection of organocatalytic reactions

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Table 2. Time constants resulting from analyzing the collected data from TIRFM with different concentrations of compound 15 in acetonitrile after addition to immobilized catalyst 30.

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>( t_1 ) (ms)</th>
<th>( A_1 ) (%)</th>
<th>( t_2 ) (ms)</th>
<th>( A_2 ) (%)</th>
<th>( t_3 ) (ms)</th>
<th>( A_3 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 0.79 \times 10^2 )</td>
<td>8.5</td>
<td>97</td>
<td>27</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( 1.5 \times 10^2 )</td>
<td>9</td>
<td>85</td>
<td>23</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( 4 \times 10^2 )</td>
<td>11</td>
<td>98.5</td>
<td>80</td>
<td>1.4</td>
<td>120</td>
<td>0.1</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Power of laser (mW)</th>
<th>( t_1 ) (ms)</th>
<th>( A_1 ) (%)</th>
<th>( t_2 ) (ms)</th>
<th>( A_2 ) (%)</th>
<th>( t_3 ) (ms)</th>
<th>( A_3 ) (%)</th>
<th>( \tau_{av} ) (ms)</th>
<th>( N_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>7</td>
<td>100</td>
<td>-</td>
<td>-</td>
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<tr>
<td>31</td>
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<td>9</td>
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<tr>
<td>120</td>
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<td>97.2</td>
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<td>2.5</td>
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<td>270</td>
</tr>
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![Fluorescence decay curve](image)

Figure 5. Fluorescence decay curve of the particles resulted from addition of compound 15 solution to the cover slips with (a) catalyst 30 (b) catalyst 31.

The final step in the experiment is the addition of the thiol. After adding 100 µl of 2.38 × 10^−7 M benzyl mercaptan we collected the movie under the standard conditions. Unfortunately, a highly fluorescent background quickly came up due to formation of the fluorescent adduct of compound 15 and benzyl mercaptan. This made it impossible to detect the single molecule binding events.

Currently, Dongdong Zheng is working on interaction between fluorescent catalyst 32 and an immobilized maleimide derivative on the surface of the cover slip. In his preliminary experiments, two time constants 35 ms (90%), 120 ms (10%) were found by fitting the binding times with a bi-exponential pattern. The average binding time is longer in that case than in the present experiment. It is conceivable that the maleimide in the BODIPY 15 is sterically hindered when binding to the catalyst, which is not the case in the system that Zheng is investigating (Scheme 6).

### 6.5. Conclusion

In this chapter we presented the results of preliminary measurements on the interaction between compound 15 and immobilized catalysts 30 and 31 by use of TIRFM. More work is required to achieve the desired results. Some instrumental improvement is required to increase the quality of the movies, removing the fluctuating background light which arises from the sources other than the fluorescent particles from the reaction. The time resolution of the present experiment is insufficient to accurately determine the short residence times. Further reduction of the exposure time is necessary. In principle, the integrated intensity of a spot due to a single molecule in a single frame should be proportional to the time it is actually bound. Thus, the intensity in the first and last frames of a binding trace should be lower than in the intermediate ones in which the binding lasts during the whole integration period. Such a refined analysis is beyond the scope of the present preliminary work.

The program to analyze the data needs to be improved to choose the desired particles with more certainty. Beside these, the process of preparing the sample must be improved to avoid fluorescence contaminants from glassware, solvent and the environment. Up to this point, the results show that TIRFM is a promising method to achieve more information about the catalytic processes and figuring out the dynamics of the interactions that play a role during the process. This work is part of the current research program in our group. We hope that the combination of our preliminary experiments with the complementary studies which are ongoing can help to achieve a quantitative insight and better understanding of the mechanisms of organocatalytic reactions.

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Chapter 6

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