Fluorescence Imaging

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Correlations between Molecular Numbers and Molecular Masses in an Evanescent Field and Their Applications in Probing Molecular Interactions**

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Single-molecule detection (SMD) technology has made remarkable progress within this decade.[1] It has been demonstrated that it is a unique technique for exploiting the fundamental physical and chemical principles of molecules, down to the single-molecule level, and that it could lead to significant biological and medical applications in the near future. A small probe volume to minimize the background effect is a critical factor for detecting individual molecules in solution. Confocal microscopy[2] or evanescent-wave excitation[3] are two valid techniques to optically confine the excitation volume. An evanescent wave is generated by total internal reflection (TIR) of the light at the interface between two media of different refractive indices, when a parallel beam of light propagates to the interface from a dense medium with a refractive index n_2 to a less dense medium with a refractive index n_1, and the incident angle θ exceeds the critical angle $\theta_c = \arcsin(n_2/n_1)$. The penetration distance z_p away from the interface, located at $z = 0$, is calculated by Equation (1):[4]

$$z_p = \frac{\lambda_0}{4\pi\sqrt{n_2^2 \sin^2\theta - n_1^2}} \quad (1)$$

in which λ_0 is the wavelength of the light. In our experiments, a laser at $\lambda_0 = 488$ nm was focused at an angle of incidence of 40–45° onto a fused silica prism ($n_2 = 1.46$). The laser beam was refracted through the prism at an angle of incidence $\theta =$ 71–74° at the fused silica/water ($n_1 = 1.33$, $\theta_c = 66°$) interface. The penetration distance z_p was 107–89 nm. The scheme for exciting a single fluorophore in an evanescent field has been employed by several research groups. Funatsu et al.[5] used refined TIR fluorescence microscopy to visualize a single fluorophore in a solution and observed adenosine triphosphate turnover reactions. Evanescent-wave excitation[6] was also adopted at the boundary of a cover slip and a polyacrylamide gel for the detection of fluorophores diffusing in and out of the gel. Yeung and co-workers[3] measured the diffusion and photodecomposition of single molecules in solution, and studied the electrostatic trapping and absorption/desorption of protein molecules at a solid/liquid interface. Fang and Tan[7] demonstrated a new fluorescence method for SMD and imaging by using an optical-fiber probe. The fluorophores were excited by the evanescent-wave field that was produced on the core surface of an optical fiber. Gai et al.[8] simultaneously determined the velocities of single molecules flowing near the channel wall and at the center of the channel by using the evanescent-field excitation mode combined with the traditional wide-field microscope.

Heparin consists of repeating disaccharide units of hexuronic acids linked to either N-sulfated or N-acetylated glucosamine units by a (1→4) bond. Both units can be sulfated to a different extent, the hexuronic acid at the C2 carbon atom and the glucosamine at C6.[9] Heparin is known for its interaction with many biologically important proteins such as proinflammatory chemokines, growth factors, extracellular matrix proteins, leukocyte proteinases, and cytokines.[10] The biological activity of heparin is strongly affected by binding to target proteins. Strong binding of heparin to the granulocyte–macrophage colony-stimulating factor (GM-CSF) and fibroblast growth factor (FGF) has been reported in the literature.[11] The granulocyte colony-stimulating factor (G-CSF) is a glycoprotein that can stimulate the proliferation and differentiation of hematopoietic progenitor cells of the neutrophil lineage and also increase the functional activity of fully differentiated neutrophils. The interaction between standard heparin, low-molecular-weight heparin (LMWH), and G-CSF has been studied by Liang et al.[12] with capillary zone electrophoresis.

We designed an experiment to detect fluorescein isothiocyanate (FITC, $M_w = 398$), FITC-deoxythymidine monophosphate (FITC-T, $M_w = 701$), FITC-12-mer oligonucleotides ($M_w = 4358$), FITC-18-mer oligonucleotides ($M_w = 6338$), and FITC-12 bp DNA ($M_w = 8318$) independently in an evanescent field at the single-molecule level and counted their molecular numbers. The numbers of molecules decreased with increasing molecular weight. We then tested a series of single fluorophores of 2-aminoacridone (2-AMAC), oligochitosan labeled with 2-AMAC, and heparin labeled with 2-AMAC, and similar results were obtained. We therefore established, on the basis of these experimental results, a new method for detecting the molecular interactions of heparin and G-CSF as model compounds in a free solution by using a single-molecule fluorescence imaging system.

The imaging system was similar to that used in previous studies,[3] except that an electron-multiplier charge coupled

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device (EMCCD) was used instead of an intensified CCD. The detection of single Rhodamine 6G molecules was used to calibrate the system and the results were the same as those in previous reports.[3,7] A series of oligonucleotides of different lengths and labeled with FITC at the 5′ end were used as a model system. A small detection volume and low fluorophore concentration were employed in SMD,[3,7] which was mainly a statistical argument for high confidence in the results. Our experimental data demonstrated that there were excellent linear relationships between the molecular concentrations and the numbers of molecules observed in the images. As shown in Figure 1, the numbers of bright spots were proportional to the concentrations of FITC, FITC-T, FITC-12-mer oligonucleotides, FITC-18-mer oligonucleotides, and FITC-12 bp DNA. Based on these five linear fits, we quantitatively calculated the molecular numbers in the evanescent field for each individual solution at a concentration of 1000 nM (Figure 2). The expected numbers of molecules of the test compounds detected in the evanescent field should be undifferentiated, with the same concentration of fluorophores under the same fluorescence imaging conditions. The experimental results, however, indicated that the numbers of molecules that were detected in the evanescent field decreased with increasing molecular weight. We also counted the molecule numbers of real samples (at 1000 nM) and compared them with the calculated values (Figure 2); the two groups of molecule numbers are almost the same. This unexpected result indicates that, as a consequence of steric hindrance, larger molecules labeled with a single fluorophore cannot be completely immersed in the 100-nm-deep evanescent field near the surface. Although the number of molecules detected in the evanescent field was constant for each specified molecule at a given concentration, the numbers of molecules detected under the same conditions can be different for different molecules because of the variations in molecular masses and/or structures.

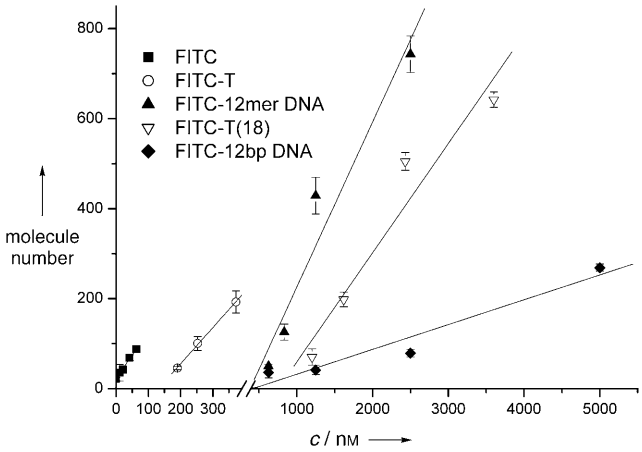

Figure 1. The linear relationships between the numbers of molecules of FITC, FITC-T, FITC-12-mer oligonucleotides, FITC-18-mer oligonucleotides, and FITC-12 bp DNA and different concentrations of each type of molecule. Samples (3 μL) were placed on the surface of the prism. The results were obtained from 128×128 pixel subframe images that were acquired by excitation with a 488-nm Ar^+ laser (100× NA 1.3 objective). The laser power was measured at about 25 mW before reaching the prism. Each pixel represents a square with 0.16-μm edges, as calibrated by the microscope stage micrometer. The excitation depth was about 100 nm (see text) and the volume of each pixel was 2.56×10^{-18} L. The EMCCD was maintained at −60 °C; the gain and exposure time were set at 255 and 100 ms, respectively. A mechanical shutter with 3-s closure was used to minimize photobleaching by blocking the laser beam when data were not being collected.

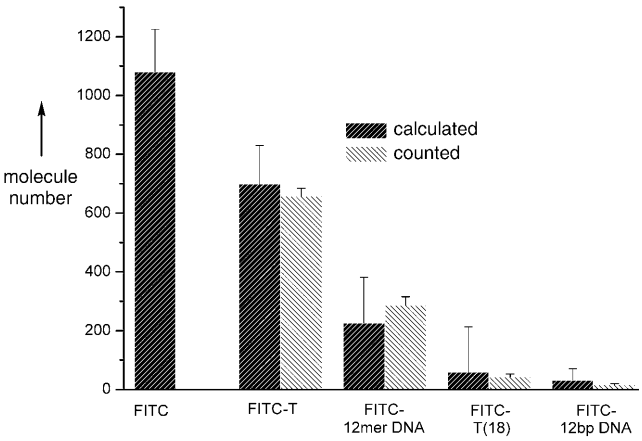

Figure 2. Comparison of the experimental and calculated numbers of individual molecules of FITC, FITC-T, FITC-12-mer oligonucleotides, FITC-18-mer oligonucleotides, and FITC-12 bp DNA at the same fluorophore concentration (1000 nM). All other experimental conditions were the same as those in Figure 1.

Oligochitosan and heparin molecules were derivatized with 2-AMAC according to the literature.[13] The 2-AMAC-glycan derivatives were produced by a "one-pot" Schiff reaction at the reducing end (the *N*-acetylglucosamine residues) followed by reduction, and one carbohydrate molecule was labeled with one fluorophore. After four extraction steps with tetrahydrofuran, free 2-AMAC dye was totally removed from the 2-AMAC-labeled oligochitosan and heparin solution. The fluorescence concentration and purity of the derivatives were determined by fluorescence spectrometry and high-performance thin-layer chromatography.

As shown in Figure 3a, the numbers of molecules were proportional to the concentrations of 2-AMAC, 2-AMAC-oligochitosan, and 2-AMAC-heparin. Similarly, the calculated molecular numbers for each individual solution (Figure 3b) decreased with an increase in molecular weight.

Oligochitosan is a mixture of chitosan oligomers with a degree of polymerization of 3–10,[14] obtained by the enzymatic hydrolysis of chitosan. Its average molecular weight was about 1800 according to mass spectrometry (data not shown). Heparin is a glycosaminoglycan with an average molecular mass of about 14000. Oligochitosan and heparin molecules were both derivatized with 2-AMAC. All of the bright spots in the fluorescence images corresponded to the 2-AMAC-derivatized molecules.

The two series of correlated experimental data allow us to hypothesize that the interaction of molecules can be probed in a mixture without separation, based on the decreased numbers of product molecules in the probed area after

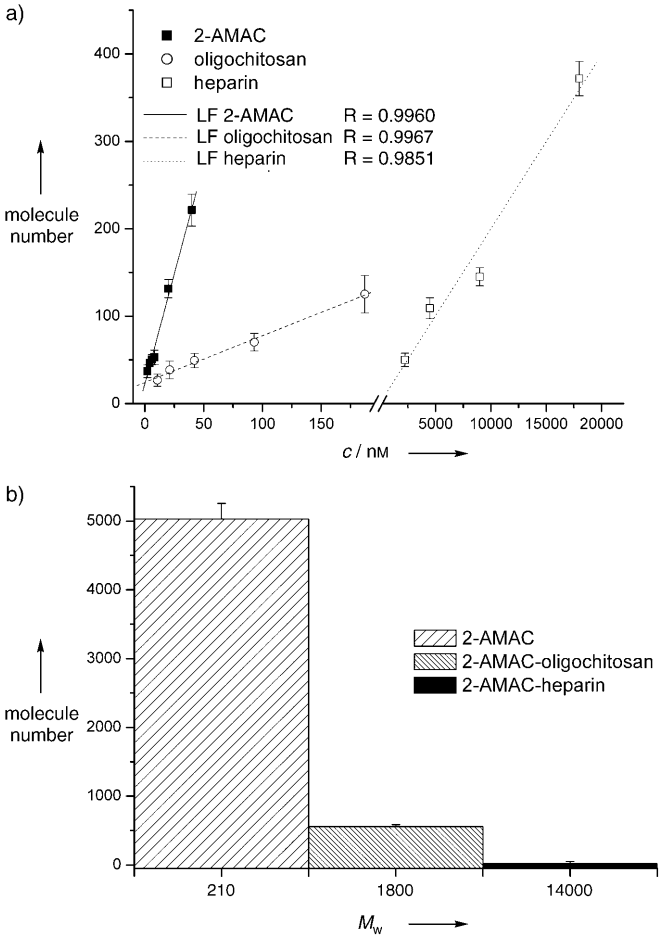

Figure 3. a) The linear relationships between the numbers of molecules of 2-AMAC (■), 2-AMAC-oligochitosan (○), and 2-AMAC-heparin (□) and different concentrations of each type of molecule. LF = linear fit. b) Comparison of the numbers of individual molecules of 2-AMAC, 2-AMAC-oligochitosan, and 2-AMAC-heparin at the same fluorophore concentration (1000 nM). All other experimental conditions were the same as those in Figure 1.

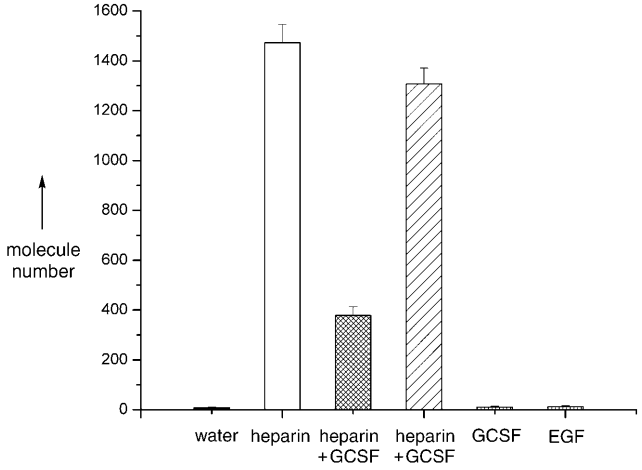

Figure 4. The numbers of molecules counted in purified water, heparin (73 μM), a mixture of heparin (73 μM) with G-CSF (6.5 μM), a mixture of heparin (73 μM) with EGF (34.72 μM), G-CSF (6.5 μM), and EGF (34.72 μM). The experimental conditions were the same as those indicated in Figure 1.

reaction relative to the numbers of reactant molecules. To test this hypothesis, the complex of heparin with G-CSF was studied as a model.

The binding of heparin with G-CSF was demonstrated by Liang et al.[12] with capillary zone electrophoresis. The heparin-G-CSF complex has a higher molecular mass (M_w = 14000 + 18987) than heparin itself (M_w = 14000), and therefore the number of heparin-G-CSF complex molecules observed in the evanescent field should be less than that of the heparin molecules. The imaging results fully supported our hypothesis (see Figure 4). Furthermore, we imaged a mixture of heparin and epidermal growth factor (EGF) to confirm that the decrease in the number of heparin-G-CSF complex molecules was not a result of the co-existing protein molecules. EGF is an ideal negative control for the formation of heparin-G-CSF complex because EGF molecules do not bind to heparin at all.[15] At EGF concentrations up to 34.72 μM, the number of heparin molecules counted in the evanescent field was about the same as the number counted for the solution that did not contain EGF. The G-CSF and EGF molecules were also counted separately to determine whether their fluorescence backgrounds were comparable with that of water, as both of these proteins had no fluorescence. This step was to ensure that the numbers of extra G-CSF molecules in solution did not contribute to the count of the heparin-G-CSF complexes when using this technique. The results in Figure 4 confirm that the existence of G-CSF and EGF molecules at this concentration level does not significantly increase the background signal. The findings support our hypothesis that the formation of a macromolecular complex changes the number of molecules detected in the evanescent field, which means that the interaction of macromolecules can be probed with this technique. Conversely, the data prove that the number of molecules decreases with an increase of molecular weight in the evanescent field.

In summary, with our single-molecule fluorescence imaging technique we discovered that the numbers of molecules probed in the evanescent field decreased with an increase of molecular weight as a consequence of steric hindrance. Our results suggest that this technique and phenomenon could be potentially useful for screening undefined molecular interactions at extremely low concentrations without any separation, for which other methods may not be applicable. This finding could be especially useful for studying the interactions of biological molecules. Future investigations will focus on quantitative measurements of the relationship between molecule numbers and molecular mass, quantitative determination of binding constants, and exploration of the effects of binding constants on the numbers of molecules detected in a probed area.

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