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Citation for published version (APA):

DOI:
10.1021/acs.langmuir.9b02199

Document status and date:
Published: 13/10/2019

Document Version:
Publisher’s PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

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Single-Dimer Formation Rate Reveals Heterogeneous Particle Surface Reactivity

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Supporting Information

ABSTRACT: Biofunctionalized micro- and nanoparticles are important for a wide range of applications, but methodologies to measure, modulate, and model interactions between individual particles are scarce. Here, we describe a technique to measure the aggregation rate of two particles to a single dimer, by recording the trajectory that a particle follows on the surface of another particle as a function of time. The trajectory and the interparticle potential are controlled by a magnetic field. Particles were studied with and without conjugated antibodies in a wide range of pH conditions. The data show that the aggregation process strongly depends on the particle surface charge density and hardly on the antibody surface coverage. Furthermore, microscopy videos of single particle dimers reveal the presence of reactive patches and thus heterogeneity in the particle surface reactivity. The aggregation rates measured with the single-dimer experiment are compared to data from an ensemble aggregation experiment. Quantitative agreement is obtained using a model that includes the influence of surface heterogeneity on particle aggregation. This single-dimer experiment clarifies how heterogeneities in particle reactivity play a role in colloidal stability.

INTRODUCTION

Micro- and nanoparticles are widely used for biomedical applications such as drug delivery,1−8 magnetic resonance imaging,1 biosensing,9−11 and cancer therapy.12 The particles are made of various materials, e.g., magnetic iron oxides,1−3,9,10 silica,11 polymers,12 gold,6,13 silver,14 and combinations thereof.15 Furthermore, the particles are coated and bio-functionalized to give them the desired biomedical properties.

A major challenge in developing biomedical applications is to control colloidal stability and minimize particle aggregation. The aggregation is typically irreversible and can cause large variabilities in the measurements. For example, particle aggregation is an important factor determining the efficiency of drug delivery processes,16−19 and aggregation can strongly affect the coefficient of variation and the limit of detection of particle-based assays.20

The stability of colloidal suspensions can be measured by optical methods such as dynamic light scattering (DLS) and turbidity.16−20 In previous work, we developed an ensemble method to quantify particle aggregation rates in solution, named the optomagnetic cluster (OMC) experiment.21 In the OMC experiment, clusters of particles are formed and the average rate of dimer formation of an ensemble of particles is quantified by the analysis of the optical Mie scattering signal. Smaller amounts of material can be analyzed using flow cytometry22 or microscopic imaging.23 However, these methods do not reveal heterogeneities of surface reactivity of individual particles.

Single particles can be studied with techniques such as atomic force microscopy (AFM), total internal reflection microscopy (TRIM), and particle tweezers, e.g., optical,24 acoustic,25 or magnetic tweezers.26 In colloidal AFM,27 a single particle is attached to the apex of a cantilever and is pushed onto another surface to probe the interaction potential. AFM can be used to probe particle−particle interactions,28,29 but most literature has studied particle−substrate interactions.30 In TIRM, the height of a particle above a surface is monitored, while the particle is attracted using gravitational, optical,31 or magnetic forces.32 In particle tweezers, particles can be trapped and manipulated using applied fields. With all of these methods, one can measure the repulsive parts of particle−substrate and particle−particle potentials. However, these methods were not developed to quantify the kinetics of an interparticle aggregation process, which requires repeated probing of the stochastic association process and extraction of the rate of aggregation from time-dependent statistical data.

Here, we describe a measurement technique wherein repeated association and dissociation events are observed on single dimers of particles so that their individual aggregation...
rate can be quantified. The particles are magnetic and brought into each other’s proximity by magnetic dipole–dipole forces. The attractive magnetic force brings the surfaces of the particles very close to each other, to a distance of several nanometers. This close proximity gives a high effective attempt frequency so that aggregation kinetics can be studied even when particles have strong repulsive interactions and a high energy barrier for association.

The single-dimer aggregation (SDA) experiment is sketched in Figure 1a. A first particle is immobilized on a substrate, and a second particle is attracted onto the first one by magnetic dipole–dipole forces. The dipole forces result from an applied magnetic field that magnetizes the particles. To be able to determine if the dimer is aggregated, a precessing magnetic field is used; see Figure 1a. When the dimer is not aggregated, the secondary particle can follow the precessing motion of the magnetic field, making a circular motion path on top of the primary particle. Upon particle aggregation, the secondary particle becomes immobilized and stops following the rotating magnetic field. (b) Microscopy image of a quarter of a full field of view of primary particles. (c) Microscope images showing how a single secondary particle is trapped onto a primary particle (upper row) and how a circulating secondary particle stops circulating upon aggregation (lower row). The full recording is given in Supporting Information Video S1. (d) Cumulative number of rotations for a single dimer. In the free state, the dimer follows the (upper row) and how a circulating secondary particle stops circulating upon aggregation (lower row). The full recording is given in Supporting Information Video S1. (d) Cumulative number of rotations for a single dimer. In the free state, the dimer follows the trajectory of the second particle. When the dimer is aggregated, the second particle is bound to the first particle and does not perform a circular motion. Transient events between bound and unbound states are determined by analyzing the time series of microscopy images, revealing the kinetics of the particle aggregation process. In the experiment, multiple particle dimers are simultaneously imaged over time (Figure 1b,c); transitions are determined between aggregated and nonaggregated states (Figure 1d,e), and from the statistics, the aggregation rate is determined (Figure 1f).

In this study, we investigate particles with diameters of 0.5 and 1.0 μm, and the dependence is measured of the aggregation rate on charge conditions (ζ-potential) and biomolecular coating conditions (antibody surface density). The data shows a dominant role of the particle surface charge on the aggregation rate and indicates that the immobilized antibodies only weakly influence the aggregation rate. Using a model that includes heterogeneity in the particle surface reactivity, we will demonstrate a quantitative agreement between the aggregation rate obtained with the single-dimer experiment and with an ensemble-based method.21

### MATERIALS AND METHODS

#### Materials

Carboxylated superparamagnetic Masterbeads (nominal size 0.5 μm, hydrodynamic diameter from DLS is 258 nm with a coefficient of variation 25%) were purchased from Ademtech, and carboxylated MyOne C1 Dynabeads (nominal size 1.0 μm, hydrodynamic diameter from DLS is 1050 nm with a coefficient of variation 2%) were purchased from ThermoFischer. Monoclonal mouse IgG against cardiac troponin I (cTnI) was supplied by Hytest. Buffer components: phosphate-buffered saline (PBS) tablets, citric acid anhydrous, sodium citrate dihydrate, potassium chloride, and Pluronic F-127, 2-(N-morpholino)ethanesulfonic acid (MES) were obtained against from ThermoFischer. Monoclonal mouse IgG against cardiac troponin I (cTnI) was supplied by Hytest. Buffer components: phosphate-buffered saline (PBS) tablets, citric acid anhydrous, sodium citrate dihydrate, potassium chloride, and Pluronic F-127, 2-(N-morpholino)ethanesulfonic acid (MES) were obtained from Sigma-Aldrich. Also, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (sulfo-NHS), bovine serum albumin (BSA, >98% pure), and protein LoBind Eppendorf tubes were obtained from Sigma-Aldrich. Amine-terminated poly(ethylene glycol) (PEG) with a molecular weight of 5 kDa (Blockmaster CES10) was purchased from JSR Microsciences.
Glass substrates of size 26 × 22 mm² and thickness 0.16−0.19 mm were obtained from Menzel Gläser.

**Particle Functionalization.** Magnetic particles (Ademtech Masterbeads, 528 nm) were functionalized through an EDC-NHS reaction with different surface coverages of monoclonal mouse IgG against cardiac troponin I (cTnl) and blocked with the amine-terminated PEG (5 kDa). All steps were performed at room temperature.

The stock particles (50 mg/mL) were first magnetically washed four times with a 50 mM MES solution of pH 6.2 containing 60 mg/mL Pluronic F-127 to wash away the storage buffer. Between each washing step, the particles were shortly vortexed to redisperse them. The final concentration after the washing procedure was 20 mg/mL. The particle solution was then sonicated two times 10 s to undo the possible particle aggregation that occurred during storage or washing steps.

Subsequently, the carboxyl groups on the particles were activated by incubating the particles in a solution of 10 mM EDC and 10 mg/mL NHS for 30 min on a roller bench. These solutions were prepared within 5 min before using them, to minimize the hydrolysis of the compounds prior to the activation step. After the activation step, the particle solutions were magnetically washed twice with MES buffer, redispersed by vortexing, and sonicated two times during 10 s.

Monoclonal mouse antibodies against cTnl were incubated with the particles during 2 h on the roller bench, to covalently attach the antibodies via their primary amines. Hereafter, a solution of amineterminated 5 kDa PEG was added to the particle solution at an end concentration of 0.8 µM to saturate the remaining active carboxyl groups on the surface of the particles. The mixture was incubated overnight on the roller bench.

Finally, the particle solution was magnetically washed three times and sonicated, after which the solution was stored at an end concentration of 10 mg/mL at 6 °C.

**Surface Functionalization.** To immobilize the primary particles on a glass substrate, the glass was first rinsed consecutively with acetone, isopropanol, and methanol in a sonic bath for 10 min each. After each rinsing step, the substrate was dried with a nitrogen gun. During the first incubation step, goat-anti-mouse IgG was physisorbed onto the glass substrate for 60 min (200 nM in PBS). In the second incubation step, the remaining uncovered surface area was blocked with a 10 mg/mL BSA in PBS solution for 15 min. Then, in the third step, the primary particles were incubated at a 500 fM particle concentration to bind to the functionalized substrate for 60 min. The polyclonal goat-anti-mouse IgG on the substrate binds to the monoclonal mouse-anti-cTnl antibodies on the particles. During the last incubation step, a 0.8 µM solution of mouse IgG solution was incubated for 60 min to block the remaining goat-anti-mouse IgG on the surface. This prevents secondary particles, which may also contain mouse IgG, to bind to the substrate. For experimental details on the surface functionalization, see Section S1 of the Supporting Information.

**Quantification of Antibody Coverage on the Particles.** The coverage of antibodies on the particles after functionalization was quantified by a supernatant assay with a commercial Easy-Titer Mouse IgG assay kit (Thermo Scientific catalogue number 23 300). In these experiments, protein LoBind tubes were used. From the antibody concentration in the supernatant, the antibody coverage was calculated. This calculation gave an average number of immobilized antibodies per particle, without information about the orientation or functionality of the antibodies. The error in the antibody coverage is determined from the standard deviation of three measurements.

**ζ-Potential Measurements.** The average surface charge of the particles was quantified by measuring the ζ-potential of the particles with a Malvern Zetasizer Nano ZS. Particles were diluted to 0.1 mg/mL, and triplicate measurements were performed either in PBS buffer (10 mM phosphate buffer, pH 7.4, ionic strength 150 mM) or in citric acid buffer of different pH values (10 mM citric acid buffer, ionic strength 150 mM). The error in the determination of the pH of the buffer solutions is about 0.1. At these high salt concentrations, the operating voltage was limited to max. 10 V to prevent electrolysis at the electrodes, which decreases the signal-to-noise ratio in the measurements. The uncertainty in the ζ-potential measurement is relatively large due to the low absolute value of the ζ-potential of the measured particles (Δζ ≈ 2 mV).

**Experimental Setup with Magnetic Field and Microscopic Imaging.** The single-dimer aggregation experiment is conceptually depicted in Figure 1. The experimental setup is schematically shown in Figure S2a, and a photographic image is shown in Figure S2b. To create out-of-plane rotating magnetic fields, five electromagnets are located around the sample. Four electromagnets are placed around the sample, creating a magnetic field in the plane of the sample, and one electromagnet below the sample creates an out-of-plane field component. The current flowing through the coils of the electromagnets is generated by a voltage source that is driven with Matlab. The sample is placed in a polystyrene ketone sample holder, which is located in the middle of the electromagnets. The sample is illuminated by a Leica fiber optic light source coming from the side, which is directed onto the sample by a silver right-angle prism mirror.

The sample is imaged with bright-field microscopy by a Leica DM6000B microscope with a 63× water immersion objective and a 2X internal magnification. Recordings are made with an Andor Neo sCMOS camera; standard recording settings are a 30 ms exposure time, a 5 Hz frame rate, and 3000 frames (10 min). For these experiments, a homemade flow cell is used in which all of the incubation steps are performed; see Figure S2c. The flow cell consists of a cleaned glass slide with a sticker made of optical-grade plastic attached to it, containing an open channel for the liquid flow. The inlet and outlet are made of flexible silicone tubing sealed with a UV-curable gel. A Harvard apparatus 11 plus syringe pump is used to pull the liquid through the flow cell.

**Analysis Software.** Recordings of the single-dimer aggregation experiment were analyzed with a homemade Matlab script. The script consists of three steps: (i) detecting and tracking primary particles, (ii) detecting when a secondary particle gets magnetically trapped on a primary particle, and (iii) detecting the binding and unbinding of the rotating dimer.

Primary particles in solution appear as high-intensity spots on a lower-intensity background (Figure 1b). The locations of individual particles were determined by calculating the center of intensity of the high-intensity spots. The locations of particles in subsequent frames were correlated to obtain the trajectory of an individual particle. A drift correction was performed based on the average motion of the primary particles during the recording.

At some point during the recording, a freely diffusing secondary particle can become trapped in one of the primary particles. Because these particles are only 528 nm in diameter, when two of them get trapped, they appear as a single elongated diffraction-limited spot (Figure 1c). The center position of the diffraction-limited spot changes upon a trapping event, making it possible to detect trapping by thresholding the change in the position of a primary particle (Figure S3a). If a third particle is trapped on the dimer, this system was not tracked any further.

As soon as a secondary particle is trapped on the primary particle, the experiment starts for this dimer. The secondary particle makes a circular motion path on the primary particle. This is observed as a rotation of the elongated diffraction-limited spot. The orientation of the long axis of the diffraction-limited spot was tracked over time, and by thresholding on the rotation speed, the binding and unbinding were detected as a decrease or increase in the rotation speed, respectively (Figure S3b).

**SINGLE-DIMER AGGREGATION (SDA) EXPERIMENT**

We have developed an experimental technique, to study the kinetics of particle aggregation on single dimers. The principle of the experiment is shown in Figure 1. Single superparamagnetic particles are immobilized on a glass substrate in a multivalent fashion, i.e., these particles are not able to rotate freely in any direction. These immobilized particles will...
be referred to as the primary particles. Subsequently, due to an applied magnetic field \( B = 6 \text{ mT} \), individual particles, called secondary particles, are magnetically trapped on the primary particles and form a dimer (Figure 1a). The particles are now held together by a magnetic dipole–dipole force in the direction of the magnetic field. The magnetic force is high enough to keep the secondary particles magnetically trapped throughout the whole experiment (∼10 min). On the other hand, the magnetic force is much weaker than the forces that underlie a chemically aggregated state of the dimer. The orientation of the magnetic field is chosen to be tilted with respect to the horizontal plane of the substrate. This ensures that the secondary particles do not touch the substrate (∼100 nm distance between the secondary particles and the substrate) and allows for the application of a rotating field for detection purposes.

To be able to detect if a dimer is in a nonaggregated (free) or in an aggregated (bound) state, the orientation of the applied magnetic field is continuously rotated around the vertical axis so that the field performs a precession motion trajectory; see Figure 1a. In a free state, the secondary particle follows the magnetic field orientation and therefore makes a circular motion path on top of the primary particle. In a bound state, the secondary particle is immobilized and cannot follow the rotation of the magnetic field. By determining the state of the dimer as a function of time, association events, as well as dissociation events, can be identified. The time-to-aggregation is defined as the time that the dimer spends in the free state. From statics of the time-to-aggregation, the aggregation rate can be calculated.

In the experiment, multiple single dimers are simultaneously imaged with bright-field microscopy. Figure 1b shows a quarter of a full field of view with individual primary particles. Several microscope images of a single-dimer aggregation experiment are shown in Figure 1c. The first row of images shows the trapping of a secondary particle onto the primary particle. The two 500 nm particles in the dimer cannot individually be optically resolved; thus, the dimer appears as a single elongated diffraction-limited spot in the microscope. The second row shows how a freely rotating dimer switches to a bound state. Supporting Information Video S1 shows the full recording of this dimer. The microscopy recordings are analyzed with a homemade Matlab script (described in Materials and Methods Section).

Figure 1d shows the cumulative number of rotations of a single dimer over time. In the free state, the secondary particle rotates along with the magnetic field \( \omega/2\pi = 0.5 \text{ Hz} \) and makes complete rotations. In the bound state, the secondary particle cannot make a complete field rotation; it shows a weak wiggling motion indicating that it is bound but not fully immobilized. Figure 1e shows a complete time trace of a single dimer, distinguishing the bound state and the free state based on the rotated angle between two consecutive frames. The orange line shows the state of the dimer as detected by the analysis software. Multiple aggregation events are observed for the same dimer with different times-to-aggregation. The wiggling motion in the bound state is also observed in this plot. The upward spikes in the signal are due to the transient passage of particles in solution through the microscopic field of view, which perturbs the image analysis of the dimer. The range of measurable times-to-aggregation is limited on the low side by the field rotation frequency and the angular resolution. On the high side, the times-to-aggregation are limited by the total duration of the experiment (for more detail, see Section S4 of the Supporting Information).

Times-to-aggregation of all dimers in the field of view can be presented in a survival plot; see Figure 1f. When plotted on linear-logarithmic \( x\)-\( y \) scales, then the observation of a straight line implies that the process can be described by a single aggregation rate \( k_{agg} \). The data is fitted to obtain the average \( k_{agg} \) and the uncertainty in \( k_{agg} \).

The single-dimer aggregation experiment allows studies of the aggregation behavior for many types of magnetic particles, surface chemistries, surface charge, buffer conditions, and magnetic field conditions. In the following paragraph, we first describe the influence of the particle surface charge density on the aggregation rate by varying the pH of the solution, and second, we investigate the influence of the surface coating of the particles by varying the antibody coverage on the secondary particle. Thereafter, we will discuss the heterogeneity observed in the aggregation process.

### AGGREGATION RATE DEPENDS ON PARTICLE SURFACE CHARGE

The most important factor for stabilizing colloids in buffer solutions is the particle surface charge density, which is often expressed in terms of the \( \zeta \)-potential. Generally, by increasing the surface charge, the absolute value of the \( \zeta \)-potential increases and the aggregation rate decreases.\(^{33,34} \) Using the single-dimer aggregation experiment, we investigated and quantified the aggregation rate as a function of \( \zeta \)-potential by varying the pH in several citric acid buffers.

Two different types of particles were used: 0.5 \( \mu \text{m} \) Ademtech particles coated with monoclonal antibodies (∼10% antibody surface coverage) against cardiac troponin I (cTnI) and blocked with a 5 kDa PEG (for details about the particle coating and immobilization, see the Materials and Methods Section), or uncoated MyOne carboxylic acid particles with a diameter of 1.0 \( \mu \text{m} \). The pH dependence of the \( \zeta \)-potential of both types of particles was measured and is shown in Figure 2a. The carboxylic particles show a three times larger absolute value of the \( \zeta \)-potential than the antibody-coated particles. Using these two types of particles, two dimer systems are compared on their aggregation properties: an equal-particle system with dimers consisting of two antibody-coated 0.5 \( \mu \text{m} \) particles and dimers consisting of two different particles (Figure 2b). The 0.5–0.5 \( \mu \text{m} \) dimers were studied at a 6 mT magnetic field and the 0.5–1.0 \( \mu \text{m} \) dimers at a 4 mT magnetic field, to keep the magnetic dipole–dipole forces the same in the two dimer systems.

Figure 2c shows the aggregation data of the two dimer systems. The equal-particle dimer system aggregated immediately (i.e., within about a second) for pH ≤ 5.1 and shows a finite aggregation rate of about 0.2 s\(^{-1} \) for pH ≥ 6.1. Already at pH 6.1, a fraction of the dimers shows immediate aggregation upon dimer formation, indicating that the conditions are at the edge of the measurable rate window. The aggregation rate shown in Figure 2c is determined from the dimer subpopulation showing nonzero times-to-aggregation. Between pH 5.1 and 6.1, a transition takes place where all or a few particles show immediate aggregation upon dimer formation. This pH range where the dimer aggregation behavior strongly changes is indicated in Figure 2c by the green area.

For the different-particle dimer system, a binary behavior is observed: for pH ≤ 4.6, aggregation occurs immediately upon dimer formation, and for pH ≥ 4.8, no aggregation at all occurs.
during the time of an experiment (Supporting Information Videos S2 and S3 show an example of the immediate aggregation and no aggregation, respectively). Apparently, the aggregation rate strongly depends on the surface charge of the particles. When changing the pH of the buffer from 4.6 to 4.8, the ζ-potential of the 0.5 μm particles does not change significantly, but the ζ-potential of the 1.0 μm particles changes by about 6 mV. This leads to a very large change in the aggregation rate of at least 4 orders of magnitude, i.e., the rate traverses the complete range of measurable rate constants (Figure 2c). The pH range where the dimer aggregation behavior strongly changes is indicated in blue. This data clearly demonstrates that particle aggregation in buffer solutions is strongly dependent on electrostatic interactions.

### AGGREGATION RATE FOR ANTIBODY-COATED PARTICLES

The antibody coverage on the 0.5 μm particles was varied to study the influence of the surface coating on the aggregation rate. The particles were functionalized via EDC-NHS with different concentrations of monoclonal antibodies against cTnI and subsequently blocked with 5 kDa PEG. Figure 3a shows the measured antibody coverage as determined with the supernatant assay described in Materials and Methods section. The graph shows that functionalizing the particles with a higher antibody concentration leads to a higher antibody coverage, until saturation occurs at a coverage of about 10^4.
antibodies per particle, which we define as a 100% antibody coverage. This corresponds to an average surface area of 100 nm² per antibody, assuming a smooth spherical surface of the particle. The green circles indicate the antibody concentrations that were used for the subsequent experiments: no antibodies, ~10% antibody coverage, and ~100% antibody coverage.

The ζ-potential of the particles, measured in PBS at pH 7.4, decreases due to the functionalization process and shows no significant difference for the three antibody coverages given the uncertainty intervals; see Figure 3b. This is an important observation, because it allows us to study the influence of the antibody coverage on the aggregation rate, independent of the surface charge density on the particles.

The experiment as a function of antibody coating was performed with particles of equal size (0.5 μm). The primary particles were coated with a ~10% coverage of antibodies and blocked with PEG in all experiments. The secondary particles had either no antibodies, ~10% antibody coverage, or ~100% antibody coverage. Figure 3c shows the measured aggregation rate for each experiment (survival plots of time-to-aggregation are shown in Section S5 of the Supporting Information). The measured values for no and 10% antibody coverages are equal within the error bars, and the aggregation rate for a 100% antibody coverage is slightly higher. It should be noted that Figure 3c has a linear y-scale, whereas Figure 2c has a logarithmic y-scale. Therefore, the differences in the aggregation rate for different Ab coverages (cf. Figure 3c) are extremely small compared to the differences in the aggregation rate as a function of pH (cf. Figure 2c). Clearly, the aggregation rate depends only very weakly on the antibody coverage. The very weak dependence on surface coating has also been observed for other molecular systems (details are added to Section S6 of the Supporting Information).

It is interesting to discuss the results of the single-dimer aggregation (SDA) experiments with respect to earlier protein aggregation studies. The latter studies have shown that monoclonal antibodies at high concentrations ([mAb] > 60 mg/mL) suffer from significant protein aggregation. In the SDA experiment, the high coverage of mAbs on the particles leads to a high local mAb concentration at the interface between two magnetically confined particles. The local antibody concentration in the interaction volume between two particles in a dimer can be calculated using the antibody surface coverage and by estimating the interaction volume as a cylinder centered around the contact point of the particles having a length of 10 nm. For particles with a mAb coverage of ~10%, the local mAb concentration is already about 100 mg/mL. Therefore, the observed particle aggregation might be caused by the aggregation of mAbs.

In the described SDA experiments, the primary particle has a 10% antibody surface coverage, which implies that there are always antibodies present at the contact point of the primary and secondary particles. This might explain why we observe only small differences in the aggregation rate when varying the antibody density on the secondary particle. In the SDA experiment of this paper, the primary particles were multi-valently immobilized via antibodies on the primary particle; therefore, the antibody coverage on the primary particles could not be reduced. In the follow-up work, it will be interesting to develop novel primary particle immobilization strategies that will allow scaling of the antibody surface density on the primary particle.

### Interdimer and Intradimer Heterogeneities

An experimental method that resolves single particles and single dimers allows one to investigate interdimer and intradimer variations in the aggregation rate. We have studied to what extent such differences can be observed in our single-dimer aggregation experiment. Since the experiments have limited aggregation event statistics per individual dimer, only large interdimer differences in the aggregation rates can be resolved. Large differences are seen only in certain conditions, such as in the pH 5.1 equal-particle experiment of Figure 2c, where some dimers are immediately bound and other dimers show repeated aggregation and disaggregation.

Intradimer heterogeneities have also been observed. Figure 4 shows an example of a time trace of a dimer (10% Ab coverage, pH = 7.4, ionic strength 150 mM), where nine aggregation events have been related to their corresponding dimer angle, as indicated in the colored squares on the right. The data shows that aggregation events occur at preferential dimer angles: the secondary particle binds at well-defined positions on the primary particle. In this case, the large majority of aggregation events occurs at the dimer angle indicated in purple and the other angles occur rarely. This is a direct observation of preferential binding locations on the primary particle and heterogeneity of particle reactivity, resulting from the single-dimer resolution of the experiment.

This feature of the single-dimer experiment can be used to study the presence of reactive patches on a particle surface by its influence on particle aggregation. Reactive patches can arise, for example, by the unfolding of proteins on the particle surface or incomplete particle functionalization, causing...
Figure 5. Simulation of aggregation in the case of heterogeneous surface reactivity. (a) Heterogeneity in surface reactivity is simulated as N reactive patches on a nonreactive particle. An interaction volume is defined by two spherical caps centered around the contact point between particles. Aggregation can only occur when the interaction area on both particles contains at least one reactive patch. (b) Total probed interaction area on both particles depends on the experiment type and the motion of the secondary particle. For the SDA experiment, a rolling secondary particle probes more area on the secondary particle compared to the shoving case. In the OMC experiment, only the two initial spherical interaction areas have interaction. (c) Simulated aggregation rate as a function of the coverage of reactive patches on the particles, with $R_{\text{patch}} = 2.5$ nm, $R_{\text{particle}} = 250$ nm, and $k_{\text{patch}} = 1$ s$^{-1}$. Experimental results for the system of particles with a 10% Ab coverage are indicated by the horizontal bars.

In the SDA simulation, one of the particles is fixed in a certain random orientation, mimicking the immobilized primary particle. A second particle approaches the primary particle in random orientation at an angle of 45 degrees with respect to the vertical axis, mimicking the trapped secondary particle. The secondary particle is now moved in a circular fashion over the particle. The rotation frequency is chosen equal to the experimentally used field rotation frequency $f = 0.5$ Hz. The particles interact with each other only at the surface area close to the point of contact between the particles. An interaction volume is defined as shown in Figure 5a. This interaction volume creates an interaction area on both particles of a spherical cap centered around the contact point. The interaction distance, the width of the interaction volume, is chosen to be equal to 10 nm, as it is unlikely that bond formation occurs at longer distances.

In each simulation step ($\Delta t = 10^{-3}$ s), the program checks on both particles if there is overlap between reactive patches on the particle and its interaction area. When both particles have at least one reactive patch in their interaction area, then there is a possibility for aggregation; see Figure 5a. The probability for aggregation during a single time step is given by eq 2

$$P_{\text{agg,SDA}} = \Delta t \cdot k_{\text{patch}}$$

Using random numbers, the program checks if aggregation occurs. If so, the time-to-aggregation is determined, and otherwise, the secondary particle is moved further for a new time step. The effective aggregation rate is obtained from the simulation by a survival plot of multiple times-to-aggregation originating from multiple single dimers.

Figure 5b shows the contact configurations of the two particles. In the SDA experiment, the secondary particle moves over the surface of the primary particle. Here, we distinguish two limiting cases: rolling and shoving. A shoving secondary particle slides over the primary particle and exposes only a single contact area. A rolling secondary particle rolls over the primary particle and thereby exposes its equatorial area, indicated in orange in Figure 5b. In the OMC experiment,

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**SIMULATIONS OF HETEROGENEOUS PARTICLE SURFACE REACTIVITY**

To interpret the measured aggregation rates, a model and simulation code have been developed to study the effect of heterogeneity in the particle surface reactivity on measured aggregation rates. The simulation has been developed for both the single-dimer aggregation (SDA) experiment and the previously described ensemble optomagnetic cluster (OMC) experiment. This allows us to quantitatively compare the effect of different molecular systems, hypotheses on the patchiness of particle reactivity can be tested.

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**Quantitative Analysis**

In the SDA experiment, one of the particles is fixed in a certain random orientation, mimicking the immobilized primary particle. A second particle approaches the primary particle in random orientation at an angle of 45 degrees with respect to the vertical axis, mimicking the trapped secondary particle. The secondary particle is now moved in a circular fashion over the particle. The rotation frequency is chosen equal to the experimentally used field rotation frequency $f = 0.5$ Hz. The particles interact with each other only at the surface area close to the point of contact between the particles. An interaction volume is defined as shown in Figure 5a. This interaction volume creates an interaction area on both particles of a spherical cap centered around the contact point. The interaction distance, the width of the interaction volume, is chosen to be equal to 10 nm, as it is unlikely that bond formation occurs at longer distances.

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$$P_{\text{agg,SDA}} = \Delta t \cdot k_{\text{patch}}$$

Using random numbers, the program checks if aggregation occurs. If so, the time-to-aggregation is determined, and otherwise, the secondary particle is moved further for a new time step. The effective aggregation rate is obtained from the simulation by a survival plot of multiple times-to-aggregation originating from multiple single dimers.

Figure 5b shows the contact configurations of the two particles. In the SDA experiment, the secondary particle moves over the surface of the primary particle. Here, we distinguish two limiting cases: rolling and shoving. A shoving secondary particle slides over the primary particle and exposes only a single contact area. A rolling secondary particle rolls over the primary particle and thereby exposes its equatorial area, indicated in orange in Figure 5b. In the OMC experiment,
dimers rotate as a whole; so, only single contact areas of the particles are exposed.

The dimer formation rate in the OMC experiment was modeled as follows. During the magnetic actuation pulse, a magnetic dimer i contains two particles each with N patches, brought together in a random orientation. Throughout the remaining time of the actuation pulse, $t_{act,i}$ the particles interact in the same orientation. In case there is overlap on both particles between a reactive patch and its interaction area, aggregation occurs with a probability given by eq 3

$$P_{agg,OMC} = k_{patch}t_{int,i}$$

(3)

The aggregation rate $k_{agg}^{OMC}$ in the simulation is determined in the same way as experimentally (eq 2 from ref 21).

Figure 5c shows the effective aggregation rates obtained from the simulations as a function of the reactive surface coverage $\eta_{fl}$ for $k_{patch} = 1$ s$^{-1}$. As expected, high reactive surface coverages lead to high aggregation rates. For the SDA simulation, coverages over a few percent give an aggregation rate equal to the patch aggregation rate. For the OMC simulation, the aggregation rate levels off at $10^{-1}$ s$^{-1}$ because the aggregation rate is limited by the inverse of the mean interaction time ($\langle t_{int} \rangle^{-1} = 0.1$ s$^{-1}$).

The simulations of the OMC experiment show that the aggregation rates depend on the orientation. At low reactive surface coverages, the shoving particles give a higher aggregation rate than rolling particles. This difference is caused by a reactivity bias. Dimers that do not show aggregation events during the experiment, because one of the particles has no reactive patch in the exposed surface area, do not contribute to measurement statistics in the survival plot. This means that the deduced aggregation rates are biased toward reactive dimers, which is more pronounced for shoving particles because they have a higher chance to lack a reactive patch in the exposed contact area.

We can now compare experimental results with results from the simulations. Aggregation rates have been measured on the 0.5 μm particles with a 10% Ab coverage, in both the SDA and OMC experiments. The found aggregation rates, including their uncertainty intervals, are presented in Figure 5c by the green and orange horizontal bars. The experimental and simulated rates are in agreement for $k_{patch} = 1$ s$^{-1}$, a reactive surface coverage of 0.04–0.07% (corresponding to 40–70 patches per particle) and when the secondary particle makes a shoving motion in the SDA experiment. Simulations performed with different patch aggregation rates show that agreement is achieved in a narrow range of patch aggregation rates: $k_{patch} = 1.0 \pm 0.3$ s$^{-1}$. A shoving motion of the secondary particle indicates that the mechanical torque due to its anisotropy is larger than the torque exerted due to shoving-induced friction; this is an interesting mechanistic result that merits further study.

In conclusion, the simulations described in this section allow a comparison between aggregation rates measured with the SDA and OMC experiments. Due to the presence of reactive patches on the particles (see Figure 4), the motion configuration and exposed surface areas of the particles (see Figure 5b) appear to have a large influence on the measured aggregation rates. Further studies should focus on unraveling the precise nature of the patches and their reactivity.

## CONCLUSIONS

In this paper, we described a new experimental method to investigate particle aggregation on single particle dimers. The nonspecific aggregation has been studied between particles with and without conjugated antibodies, in a wide range of pH conditions. The data shows that the aggregation rate strongly depends on the particle surface charge density, with variations over more than 4 orders of magnitude when changing the pH of the solution. Varying antibody type and surface coverage resulted in only a factor 1.5 change in the aggregation rate.

Video microscopy of aggregation and disaggregation events of individual dimers revealed discrete areas with high reactivity, i.e., strong heterogeneity in surface reactivity of the particles. Apparently, reactive patches are present on the surface of the particles. Simulations on the aggregation of heterogeneously reactive particles resulted in a quantitative agreement between the experimental data of the single-dimer aggregation experiment and an ensemble-based method to quantify particle aggregation. The simulations show that the motion configurations and exposed particle surface areas are important due to the patchy nature of the particle surface reactivity. Interesting follow-up studies will be to investigate the (bio)chemical characteristics and amount of patches on different particles and to investigate possible differences in how specific and nonspecific interactions influence aggregation rates.

The single-dimer aggregation experiment can be used for studying superparamagnetic particles of many material types and with different biochemical coatings. The size range of the particles is limited on the lower side by the resolution of the optical microscope and on the upper side by the drag of the secondary particle. The drag can be reduced by decreasing the rotation frequency of the field, but that also reduces the time resolution of the experiment and limits the maximum observable aggregation rate.

In conclusion, the described single-dimer aggregation experiment gives the unique ability to reveal the influence of particle surface heterogeneities on interparticle aggregation. The developed methodology and model description will be valuable for further scientific studies, as well as optimizations of the functional properties of colloids.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.9b02199.

Substrate functionalization; experimental setup and flow system; analysis software; statistics of the time-to-aggregation; varying Ab coverage: histograms of time-to-aggregation; and aggregation rate as a function of particle surface chemistry (PDF)

Microscopy video of the formation of a single dimer, showing multiple aggregation and dissociation events (Video S1) (AVI)

Microscopy video showing immediate aggregation upon secondary particle trapping (Video S2) (AVI)

Microscopy video showing a non-aggregating single dimer (Video S3) (AVI)
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