

Title page

Title: Visualization of the spontaneous emergence of a complex, dynamic, and autocatalytic system

Short title: Visualization of a complex emergent autocatalytic system

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Abstract

Autocatalytic chemical reactions are widely studied as models of biological processes and to better understand the origins of life on earth. Minimal self-reproducing amphiphiles have been developed in this context, and as an approach to *de novo* 'bottom-up' synthetic protocells. How chemicals come together to produce living systems remains poorly understood, despite much experimentation and speculation. Here, we use ultrasensitive label-free optical microscopy to visualize the spontaneous emergence of an autocatalytic system from an aqueous mixture of two chemicals. Quantitative, *in situ* nanoscale imaging reveals heterogeneous self-reproducing aggregates and enables the real-time visualization of the synthesis of new aggregates at the reactive interface. The aggregates and reactivity patterns observed vary together with differences in the respective environment. This work demonstrates how imaging of chemistry at the nanoscale can provide direct insight into the dynamic evolution of non-thermodynamically controlled systems across molecular to microscopic length scales.

Significance statement

Chemical reproduction is central to biology and understanding how chemical systems may give rise to complex systems that form self-reproducing cell-like structures is a leading goal for scientists. Here we use an ultrasensitive optical microscopy technique to directly monitor the formation and dynamics of self-replicating supramolecular structures at the single particle level. As a result, we are able to quantify the kinetics of these systems and changes in nanoparticle distribution in time. Our ability to observe a variety of complex phenomena may contribute to understanding how cell-like systems can emerge from much simpler chemical components and provides a general route to studying assembly and disassembly on the nanoscale.

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Introduction

Autocatalysis is a fundamental class of chemical reactions which drives many biological processes and underpins research into the origins of life on earth (1). Surfactant molecules can self-reproduce through physical autocatalysis, a process in which aggregates of these monomers, in the form of micelles or vesicles, catalyze the formation of additional monomers. Several chemical models of physical autocatalysis have been developed that involve biphasic reaction conditions (2). In these systems, reactants are partitioned between aqueous and organic phases and react to give amphiphilic products, which aggregate into micelles or vesicles. Autocatalysis occurs in these reactions because the product aggregates take up organic precursor molecules into the aqueous phase, allowing more efficient mixing of the reaction components and thereby increasing the rate of reaction. Understanding the dynamics of individual lipid aggregates during growth and division is a long-standing problem in the field of prebiotic chemistry (3–7) because vesicles are widely held to have compartmentalised and catalysed reactions in the prebiotic world (8–10). A full understanding of these dynamics has not yet been achieved in large part owing to analytical limitations.

While physical autocatalysts have been widely studied for 25 years, their behaviour remains poorly understood (2) and direct observation of the behavior of individual lipid aggregates remains elusive. At the single-particle level, division of giant vesicles ($> 1 \mu\text{m}$) has been visualized in real time using

optical microscopy (11). Micelles and sub-micron vesicles can only be imaged directly with electron microscopy, which strongly perturbs the system and precludes real-time analysis (4, 12). Ensemble methods such as dynamic light scattering (12) and fluorescence resonance energy transfer (7) enable the analysis of aggregate populations, and ensemble spectroscopic methods are frequently used to record the concentration of individual molecular species in reaction mixtures. The critical nanometer scale on which physico-chemical self-replication occurs, however, has not been imaged dynamically. As a result, we struggle to understand the dynamics of even the simplest supramolecular aggregates such as micelles and vesicles, and as a corollary we do not fully comprehend how protocells may evolve out of chemical mixtures and ultimately to which degree they are relevant to primitive life. Here, we show that interferometric scattering microscopy (iSCAT) (13–15) can be used to monitor physical autocatalysis *in situ* because it enables the direct observation of the generation of new lipid aggregates at the reactive interface, without the use of labels or any other perturbations to the system, down to single micelles.

Results

Our system consists of a biphasic reaction between aqueous and organic components placed above a microscope cover glass (Fig. 1a). The reaction of thiol **1** with enone **2** at high pH yields a single amphiphilic product, **3**. The product **3** aggregates into micelles at millimolar concentrations and enables the reagents to mix more efficiently, thus behaving as a physical autocatalyst.

Compound **3** is an analogue of a physical autocatalyst that we previously characterized (16). At present we are unable to reliably detect the smaller micelles of the earlier system using iSCAT, and so compound **3**, bearing a longer hydrophobic tail, was selected as it forms larger micelles ($R_H \approx 3$ nm, see Supplementary Figures 8-10), which can reliably be detected by iSCAT. Unsaturation in the alkyl chain was introduced to keep the corresponding thiol **1** a liquid at room temperature for experimental simplicity, allowing the thiol to be used neat rather than as a solution in an organic solvent. The corresponding saturated compound, 1-octadecanethiol, is a solid at room temperature.

To determine the sensitivity limits of iSCAT in visualizing the reaction products directly, we monitored binding of individual micelles to a microscope cover glass from a solution of pure **3**. Binding of micelles to the cover glass changes the local refractive index and thus the scattering properties of the surface, which is detected by the iSCAT microscope. The resulting differential images consist of diffraction-limited spots, with a contrast on the order of 0.1% (Fig. 1b). Given an average micelle hydrodynamic radius of 3 nm determined by DLS (Fig. 1c, inset) an iSCAT contrast of 0.18% for single 500 kDa unlabeled proteins (13) and the unimodal distribution in the detected signal for the aggregates in iSCAT and dynamic light scattering (Fig. 1c) demonstrates that these signatures arise from individual micelles. At this point a quantitative conversion from iSCAT contrast to hydrodynamic radius is not achievable; the contrast depends not only on the particle polarizability and hence volume, but also the effective refractive index, which may vary with particle size. In addition, the detected signal depends in part on the focal position and optical path length, requiring a constant focus position across measurements. Nonetheless, the comparison between iSCAT contrast and the DLS size distribution demonstrates that we can detect the smallest micelles present in samples of **3**.

In order to monitor the synthesis of **3** *in situ* we take advantage of the stochastic binding of micelles to the coverglass surface, analogous to localization-based super-resolution fluorescence microscopy (17). In contrast to fluorescence imaging, light scattering does not saturate or bleach. Thus a surface partially covered by micelles acts as a new scattering background that can be subtracted. Subtraction of consecutive images only reveals changes in surface scattering (18), even though the respective raw scattering images are essentially indistinguishable (Fig. 2a). This is because the rough cover glass surface and any micelles or vesicles already present dominate the signal (15). In our assay micelle binding results in dark spots, while departing/rupturing particles generate a bright, positive contrast.

Prior to the onset of the autocatalytic reaction, consecutive image subtraction (see methods section, Data Processing) reveals no binding to the surface as expected in the absence of micelles in solution (Fig. 2a). Approximately 15 minutes after establishing the interface, we observe binding of micelles to the surface, the rate of which then rapidly accelerates and also involves unbinding events as the surface saturates. We can generate a super-resolution map of binding events (Fig. 2c), since we can detect and localize each particle arriving at or departing from the surface.

A corresponding time-course of binding events allows us to determine the landing rate per unit area as a function of time (Fig. 2b). We observe an exponential increase in the landing rate after an initiation period, which tails off resembling a Langmuir adsorption isotherm as the available binding sites on the coverglass surface become occupied (Fig. 2b, orange). The exact shape of the time-course and final binding is somewhat variable (Supplementary Figs. 1 and 5) but the overall trend is consistent and reproducible.

The observed variation of the saturation point between and within experiments is likely a consequence of multiple factors. The maximum landing rate is given by the availability of binding sites on the substrate. The availability of binding sites itself depends on several competing dynamic processes such as unbinding events, formation of a supported lipid bilayer, deformation of individual aggregate structures, and the size of the aggregate products. Some of these processes have been reported to be dependent on the local density of particles on the substrate (19). Hence variation in the maximum landing rate is not unexpected.

Beyond characterization of the landing rate, we can monitor the particle size distribution as it evolves in time. Under these conditions, the average particle contrast converges around $0.26 \pm 0.02\%$ (Supplementary Figure 2). This is consistent with positive controls carried out in the reaction medium, giving an average particle contrast of $0.28 \pm 0.02\%$ (Supplementary Figure 3). The absolute contrast is sensitive to the refractive index of the solution and the focal position, and consequently is somewhat higher in the reaction mixture than in pure water. Positive controls carried out in the presence of starting material **2** and Cs_2CO_3 reveal a sharp critical micelle concentration between 0.5 and 0.75 mM (Supplementary Figure 4), with an equilibrium binding rate above this concentration in agreement with the saturation binding rate observed in reactions.

In negative control experiments where **2** is omitted, no micelles are formed (Fig. 2b, black). By contrast, inclusion of 0.5 mM of **3** in the aqueous solution to initiate the catalyzed reaction eliminates the lag period and rapidly forms the product upon addition of **2** (Fig. 2b, purple). The final binding rate in this case is close to the average binding rate observed in the unseeded reactions

(Supplementary Fig. 1) and quantitatively distinct from the much lower binding rate observed in a positive control of 0.5 mM **3** in the absence of any thiol **1** (Supplementary Fig. 1).

Given that iSCAT can be used to detect and quantify the autocatalytic synthesis of **3** on the single-particle level, we attempted to directly image the reactive interface. To do so we generated micrometer-sized thiol droplets on the glass coverslip and surrounded them with an aqueous solution of **2** (Fig. 3a). This allowed the direct visualisation of the thiol-water interface and the production of new aggregates.

Here, small lipid aggregates diffuse out of the thiol-water interface (Fig. 3c). Remarkably, there is a clear spatial association of reactivity with the thiol-water interface: near the interface there are high levels of activity and many lipid aggregates form, while far from the interface the rate of binding is lower (Movie S1). These observations can be quantified, demonstrating that the binding rate is negatively correlated to the distance away from the interface (Fig. 3d). This observation agrees with the proposed biphasic reaction mechanism: if the reaction is indeed occurring at the thiol-water interface, the binding rate ought to decrease with distance from the interface. Conversely, if the binding arises from reactivity at a distant interface, or from a homogeneous reaction in the aqueous phase, the rate of binding ought to be independent of the distance from the observed interface. As such the quantification of this correlation supports the proposed mode of reactivity, providing spatial information that would be difficult to obtain by other methods (16).

One hour after the addition of **2**, the interface around these droplets breaks down almost entirely, and complex extended lipid structures emerge (Movies S2-4). These proliferate rapidly, and lead to events consistent with the growth and division of individual nanometer-scale vesicles: new material is seen to rapidly grow and separate from existing vesicles, although it is difficult to isolate individual events owing to the large number of vesicles.

We are also able to generate macroscopic water-thiol interfaces where the interfacial curvature is negligible on the nanometer scale (Fig. 4). Here, the reactive behaviour is rather different. Whereas we previously found proliferation of aggregates around the interface, here we observe the steady retreat of the organic phase and corresponding movement of the aqueous phase across the cover slip. Interestingly, the retreat of the thiol phase was not a continuous process as might be expected. Instead, we could discern the formation of individual aggregates at the interface and merging with an intermediate phase, which pushes the thiol phase back in a series of discrete events. Consecutive image subtraction of these data clearly reveal discrete events (Fig. 4b). By overlaying the differential series on the flat-field images, reveals colocalization of these events with the retreating interface (Fig. 4c, Movie S5). It is likely that these discrete events correspond to the formation of individual vesicles at the interface.

Discussion

We have demonstrated the spontaneous formation of complex aggregates from simple precursors by directly visualizing an autocatalytic reaction on the nanometer scale. Through label-free, super-resolution imaging of individual micelles and thus direct probing of the supramolecular product/catalysts, we can obtain quantitative kinetic data that allows the study of a physical autocatalytic reaction. Further, we are able to directly image the reactive interface and distinguish

between processes occurring at different regions of the multiphasic system, thereby revealing the complexity and diversity of mechanistic behavior of physical autocatalysis on the nanometer scale.

The capability to observe these label-free particles in real time provides the opportunity to study complex non-equilibrium systems so that we may better understand the collective behavior of autocatalytic aggregates. Understanding how complex supramolecular dynamics gives rise to the formation of extended membranes, and the production, growth and division of vesicles is a fundamental problem relevant to the origins of life (1, 2, 20). Here we examine a model bond-forming autocatalytic system, which rapidly generates molecular and supramolecular complexity to demonstrate a general method by which we can directly image and study nanoscopic dynamics with high spatiotemporal resolution.

Materials & methods

iSCAT Setup

The iSCAT experimental setup is not described in complete detail here, but is similar to that described by Ortega-Arroyo *et al.*(13) A 445 nm diode laser was used as the incident light source with an approximate incident power of 10 kW/cm² on the sample. Frames were recorded at 1 kHz with an exposure time of 0.56 ms using a CMOS camera (Photonfocus MV-D1024-160-CL-8). Unless noted otherwise, images were recorded at 333× magnification (31.8 nm/px), corresponding to an 8.1 × 8.1 μm² window.

Focus in the z-axis is maintained using an autofocus system relying on the total internal reflection (TIRF) of a 638 nm beam.(21) Movement in the z axis results in a corresponding movement in the xy plane of a totally internally reflected beam, which is detected and used as the basis for automated correction of the z position. This system can maintain the z position to within 5 nm consistently.

Sample preparation & coverslips

All samples were purified before use, prepared using ultrapure Milli-Q water, and filtered through 0.2 μm PTFE filters prior to analysis by iSCAT.

Borosilicate glass coverslips (no. 1.5, 24 × 50 mm, VWR) were cleaned by sequential rinsing with distilled water, ethanol, and distilled water, and then sonicated for 10 min whilst standing in fresh HCl (approx. 0.4 M). The slips were washed with Milli-Q water and dried under a stream of dry nitrogen.

Silicone wells (4.5 mm diameter, 1.7 mm depth, Grace BioLabs) were prepared by washing sequentially with Milli-Q water and EtOH then drying under a stream of dry nitrogen.

All coverslips and wells were prepared on the same day as analysis using fresh reaction components.

Experiment

A typical reaction was performed as follows. Milli-Q water (15 μL) was deposited into a silicone well and the glass surface inspected to ensure satisfactory cleanliness. Thiol **1** (2 μL, 0.3 eq relative to **2**) was gently deposited atop the aqueous layer and the system was allowed to equilibrate for several minutes. A solution (15 μL) of MPC **2** (1.2 M) and Cs₂CO₃ (400 mM) was injected into the aqueous

layer and mixed gently using a micropipette. One second of data, equivalent to 1000 frames, was then recorded every 6 seconds.

Negative controls were performed by omitting MPC **2** from the second aqueous solution. Seeded experiments were performed by supplementing the initial aqueous solution with a 0.5 mM solution of **3**. Positive controls were performed by measuring the binding rate of pre-equilibrated solutions of **3** in MPC **2** (600 mM) and Cs_2CO_3 (200 mM) in the absence of thiol **1**.

Direct examination of the thiol-water interface was achieved by first depositing thiol **1** (2 μL) on the glass surface and then displacing it by injection of milli-Q water (4 μL). The reaction site of interest was located and then a solution of MPC **2** (1.2 M) and Cs_2CO_3 (400 mM) was injected into the aqueous layer. Data were recorded manually, typically capturing 5000-10000 (5-10 seconds) at a time.

Data Processing

Data were processed and analysed using National Instruments LabVIEW 2011 and the FIJI distribution of imageJ. To correct for illumination inhomogeneity and fixed pattern noise, a flat field image was taken by running a temporal median filter over a sequence of images acquired when the sample was displaced.⁽²²⁾ Differential imaging was achieved by subtracting sets of images temporally offset by a time Δt . The signal to noise ratio was then improved by spatially (2x2 binning) and temporally averaging the differential images (100 images).

For the generation of super-resolution images and quantification of reaction kinetics, a running temporal average was applied to the differential images. By subtracting a running temporal average from the differential images we reduced the rate of false positives and increased the recovery rate of true positives, given that single (un)binding event would be counted multiple times, in contrast to a signal attributed to spurious noise. To avoid repeated counts, single (un)binding events were only identified on the basis of having a trajectory length with at least four localisations and at most twice the size of the temporal average.

Particle detection was performed as described by Spillane *et al.*⁽²³⁾ Briefly, diffraction-limited spots were identified by a combination of the non-maximum suppression algorithm and selecting pixels that exceeded at least two times the standard deviation of the image, estimated by the median absolute deviation. Candidate particles were then segmented into regions of interest corresponding to approximately 1 μm^2 and fit to a 2D Gaussian function. Particle tracks, used for the quantification of the kinetics, were generated by a modified cost matrix method described by Jaqaman *et al.*⁽²⁴⁾ Here assignments within the cost matrix were determined by a greedy approach, namely by minimising the distance between features in consecutive frames found within a search radius of 40 nm, rather than solving the linear assignment problem. Features with the minimum distance exceeding the search radius were classified as having no connectivity.

The possibility of each diffraction-limited spot being multiple micelles is excluded by consideration of the binding rate. If the landing rate of the micelles was very high, corresponding to a high particle density, there would be the likelihood of having more than one particle land within a diffraction limited area simultaneously (i.e. within a single exposure time or effective exposure time given by averaging multiple frames together to enhance the SNR). To estimate how likely this would be the

case, we refer to the observed maximal rates of particle landing (i.e. the saturation points) in the assay which is approximately $4 \text{ particles s}^{-1} \mu\text{m}^{-2}$ (Fig 2). Assuming a diffraction limit area $= \pi(0.250 \mu\text{m})^2 \approx 0.2 \mu\text{m}^2$, we have a landing rate of less than $1 \text{ particle s}^{-1}$ per DLS. Now, considering an effective temporal window (t) of 0.1 s (equivalent to averaging 100 frames taken at 1000 fps), we now have a landing rate per diffraction-limited spot of $0.1 \text{ particle t}^{-1}$. Assuming a Poisson distributed process, the probability than more than one particle lands under such scenario can be estimated to be $<0.5\%$. Under these circumstances this effect can be neglected, however for higher densities, one can minimise this issue by increasing the temporal resolution of the detection.

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Figure Captions

Fig. 1. Visualising physical autocatalysis by interferometric scattering microscopy (iSCAT). (a) Schematic of the biphasic reaction of aqueous **2** with neat water-insoluble **1** carried out on a microscope coverslip. iSCAT relies on illuminating the sample with a coherent light source and imaging the reflected and backscattered light from the sample; (b) Representative differential iSCAT image of single micelles of **3** bound to microscope coverglass after subtraction of the static scattering background. Scale bar: 2 μm ; (c) iSCAT contrast histogram of a sample of **3** in water. Inset: Dynamic light scattering number distribution of **3** (1 mM).

Fig. 2. Quantification of reaction kinetics by label-free super-resolution imaging. The binding of aggregates of **3** to a glass surface is monitored by iSCAT. The binding and unbinding of particles is detected as a change in the local refractive index and counted, allowing quantification of the binding/unbinding rates per unit area. (a) Top: Flat-field images of aggregates of **3** binding to a microscope cover glass over 25 minutes. Bottom: Corresponding background-subtracted images, highlighting binding (dark) and unbinding (white) events of single aggregates to the surface. Images are taken from the same data set as the orange line in panel b. Each image is the average of 150 frames; (b) Characterisation of reaction kinetics by counting the number of binding events per unit time and area. Background-subtracted images (as illustrated by panel a, bottom) are analysed and each binding/unbinding event counted to give the kinetic curves shown here. Data points with error-bars represent the average and standard deviation of three consecutive one-second measurements sampled once every six seconds. Solid lines are fits to: sigmoidal kinetics for the reaction between **1** and **2**, (orange) and the reaction between **1** and **2** seeded with **3** (purple). The seeded reaction features a high rate of reaction immediately upon addition of **1**, without the lag-period required to build-up product/catalyst as observed in non-seeded reactions. The black line and corresponding data points refer to the negative control, consisting of thiol **1** and an aqueous solution of Cs_2CO_3 . Note that solid lines do not represent a detailed kinetic model and are intended only to highlight major trends; (c) Super-resolution map identifying the centre of mass for each binding event over time. Counting each binding event in this map per unit time gives the data shown in panel b. Data are the same as the orange line in panel b. Scale bars: 1 μm . Kinetic curves for these reactions and additional replicates are included in Supplementary Figures 5-7.

Fig. 3. Direct observation of the reactive interface. (a) Illustration of the reaction geometry; (b) Flat-field images demonstrating reaction about the thiol-water interface. Progress of the reaction is from left to right and top to bottom; (c) Super-resolution map of the binding sites within the first seven minutes of the reaction shown in (b). The color and size of the plot markers encode the arrival time and signal intensity of binding events, respectively. (d) Negative correlation between frequency of binding events per unit area and distance from the reactive interface. Dependence of the bound product density, found within the arc-sector depicted by the greyed-out region of (c), on the radial distance away from the droplet interface, where a radial distance of zero corresponds to the interface. Solid line: fit to a linear function. Scale bars: 1 μm .

Figure 4. Discrete movement of thiol-water interface. The interface between the aqueous (light) and thiol (dark) phases moves towards the right in discrete bursts rather than as a continuous process. (a) Flat field images; (b) Consecutive subtraction images highlighting individual bursts of activity; (c) Composite image of panels A and B highlighting colocalisation of activity and the motion of the interface. $\Delta t = 400 \text{ ms}$, scale bars = 1 μm . An animated version of this figure is available in the supplementary materials.

