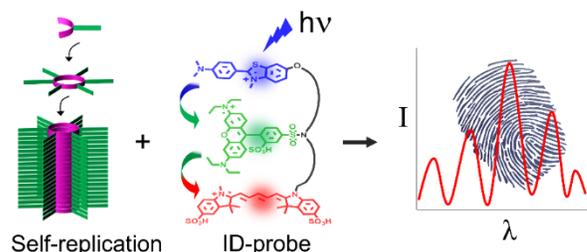


An Optical Probe for Real-Time Monitoring of Self-Replicator Emergence and Distinguishing between Replicators

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ABSTRACT: Self-replicating systems play an important role in research on the synthesis and origin of life. Monitoring of these systems has mostly relied on techniques such as NMR or chromatography, which are limited in throughput and demanding when monitoring replication in real time. To circumvent these problems, we now developed a pattern-generating fluorescent molecular probe (an ID-probe) capable of discriminating replicators of different chemical composition and monitoring the process of replicator formation in real time, giving distinct signatures for starting materials, intermediates and final products. Optical monitoring of replicators dramatically reduces the analysis time and sample quantities compared to most currently used methods and opens the door for future high-throughput experimentation in protocell environments.



INTRODUCTION

The question how life can emerge from an abiotic chemical mixture is among the grand challenges in contemporary science.¹⁻⁴ Self-replicating systems play a key role in addressing this question.⁵⁻⁷ In the last decades, different self-replicating systems have been reported based on synthetic molecules⁸⁻¹⁰ or biology-inspired motifs like nucleobases¹¹ or peptides¹²⁻¹⁶ or combinations thereof.¹⁷⁻¹⁹ Self-replication can be achieved through a template-based mechanism, modelled after the replication of nucleic acids in nature, or driven by self-assembly, as shown schematically in Figure 1a.²⁰

The field of self-replication is now gradually entering the next phase in which systems of replicators are extended to capture additional essential ingredients for life, including proto-metabolism and compartmentalization.^{7,21-22} Also efforts directed at achieving Darwinian evolution of these systems are imminent. This shift in focus in research on self-replicating systems will put new demands on analytical tools.

Until now the study of self-replicators has relied on analytical methods such as NMR or chromatography (HPLC/UPLC) coupled to mass spectrometry. While these techniques have proven very powerful in unravelling the behavior of systems of individual replicators in solution, they are less suitable for parallel screening, sampling of small volumes and low concentrations (in the case of NMR) and in-situ monitoring (in the case of chromatography).

Thus, for further development of self-replicators in the direction of life, additional analytic tools are required that would ideally allow real-time and non-destructive monitoring of self-replicators at low concentrations and in small sample volumes.

Fluorescent molecular probes appear particularly suitable for monitoring self-replication and have been used in a few instances. Philp et al. have labelled a replicator precursor with a fluorophore to monitor a propagating replication-diffusion front.²³ We have used thioflavin T (ThT) to monitor β -sheet formation in our peptide-based replicators.²⁴ However, none of the probes used so far allow for discrimination between different replicators.

To track the dynamic formation of several molecular species in solution, a new class of fluorescent molecular probes²⁵⁻²⁹, termed ID-probes²⁸ was recently developed. Unlike conventional small molecule-based probes, which generally bind a single analyte and produce a single fluorescence output, ID-probes combine several fluorophores and non-specific (or partially specific) recognition elements that enable them to interact with various different molecular species in a mixture and generate unique identification (ID) fingerprints for different analytes and their combinations. This differential sensing mode is similar to that underlying the function of the olfactory system, or artificial nose/tongue analogues.³⁰⁻³⁵

It occurred to us that the ID-probe strategy could provide an important new tool to study self-replicating systems; one that would complement the current analytical techniques.

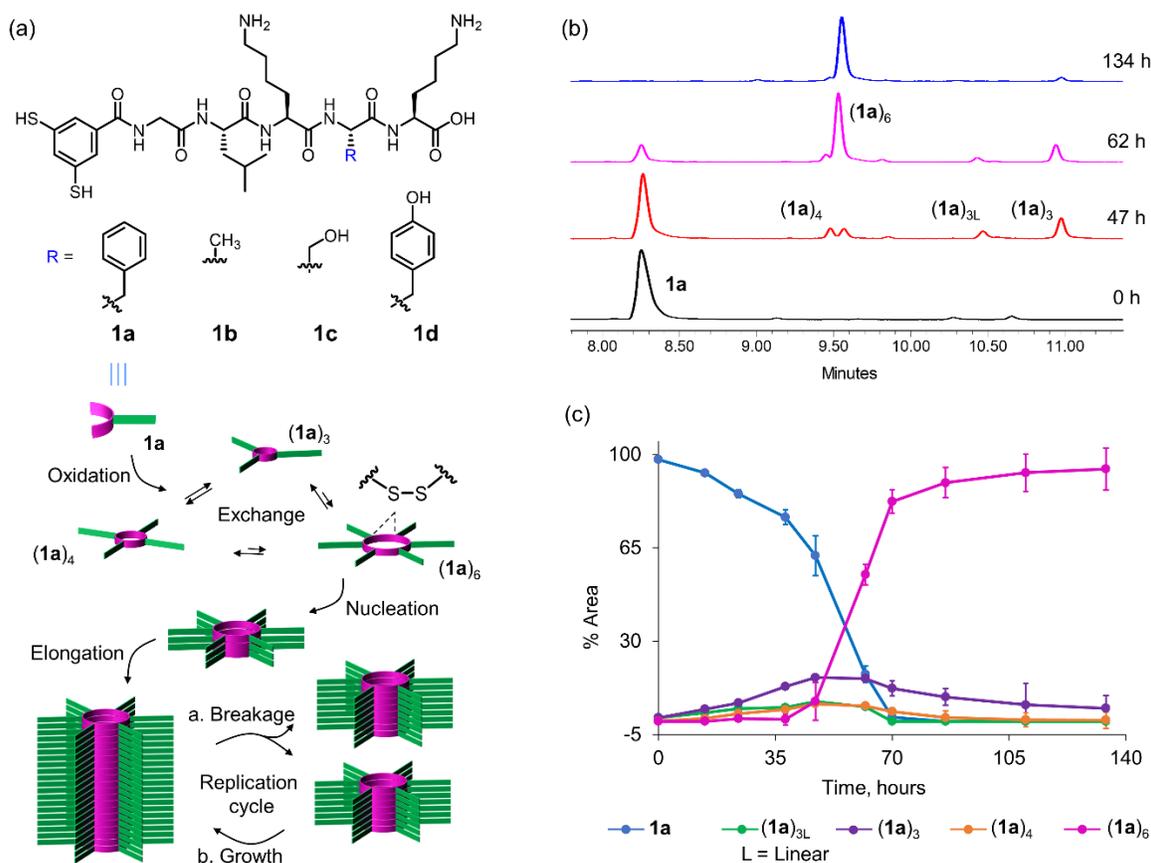


Figure 1: (a) Mechanism of self-assembly driven self-replication. Air-oxidation of dithiol building block **1a** initially produces a mixture of disulfide macrocycles of different ring size that interconvert through thiol-disulfide exchange. Assembly of, in this case, the cyclic hexamer (**1a**)₆ into fibers results in the autocatalytic production of more hexamer. Fiber formation is driven by a combination of pi-stacking interactions and the assembly of the peptide tails into β -sheets. Agitation-induced fiber breakage liberates more growing fiber ends enabling exponential growth³⁶; (b) Selected ultra-performance liquid chromatography (UPLC) traces (monitored at 254 nm) recorded at different stages during the emergence of replicator (**1a**)₆; (c) Kinetic profile (average of three independent experiments) of the emergence of (**1a**)₆. Samples were made from 30 μ M building block **1** in 50 mM (in boron atoms) borate buffer, pH 8.2 stirred at 1200 rpm at 30 °C.

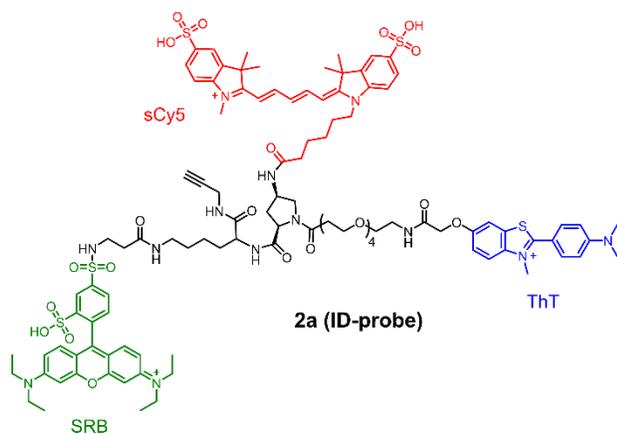


Figure 2: Structure of the pattern-generating ID-probe **2a** carrying three fluorescent reporters: ThT (blue, Ex: 440 nm, Em: 490 nm), SRB (green, Ex: 530 nm, Em: 595 nm) and sCy5 (magenta, Ex: 630 nm, Em: 675 nm).

We now report the design and synthesis of an ID-probe **2a**, developed to study the self-replicating systems made from a family of dithiol-containing peptides (**1a-d**, Figure 1a). We first show that this pattern-generating probe can be used to discriminate between building blocks, intermediates and final replicators prepared separately. We subsequently used the sensor to monitor the process of replicator-formation in-situ in real time. Finally, we show that the sensor can discriminate between replicators of subtly different chemical nature.

Results and Discussion

Probe design

We designed ID-probe **2a** to monitor systems of self-replicators made from the family of peptide-containing building blocks (such as **1a-d**) that we developed recently.³⁷⁻³⁸ Replication is partially driven by assembly of the peptides into β sheets (see Figure 1a). We exploit this feature by including ThT in the design of the sensor, which

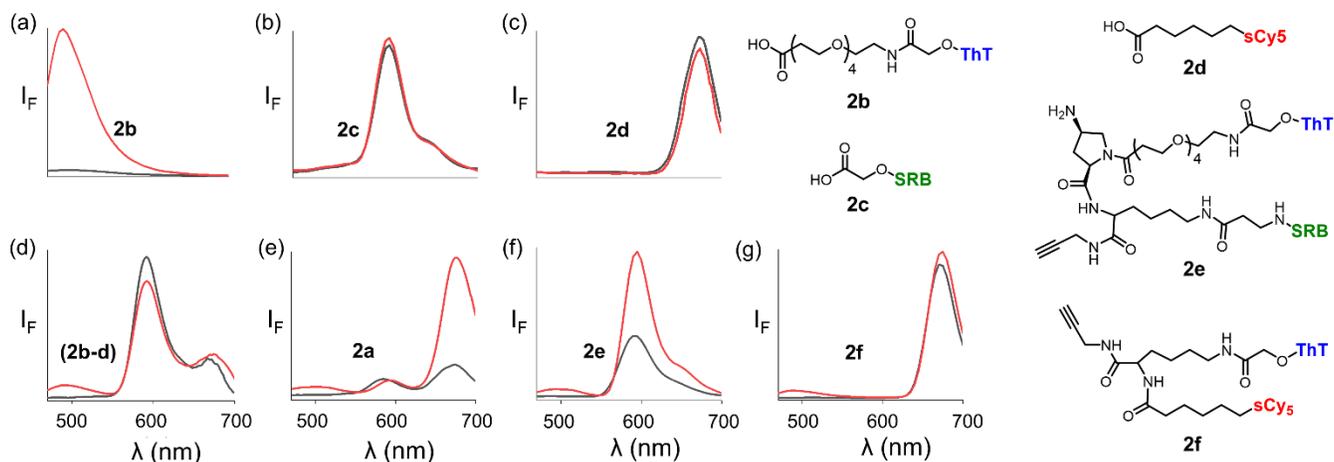


Figure 3. Emission spectra of sensor **2a** and different control molecules **2b-f** (2.0 μ M, 50 mM borate buffer, pH 8.2), in the absence (black line) and presence (red line) of fibers of replicator (**1a**)₆ (30 μ M in building block **1a**, $\lambda_{\text{ex}} = 440$ nm for all experiments).

is well-known to interact with β sheets, resulting in a marked increase in its fluorescence that depends only little on the exact nature of the β -sheet assembly.³⁹⁻⁴⁰ In order to enhance the discriminating ability of the sensor we also introduced two additional dyes: sulforhodamine B (SRB) and sulfo-Cy5 (sCy5). These dyes were selected based on their successful use in a previously developed ID-probe that was used to study amyloid beta (A β) aggregates (like our replicators, these aggregates involve extensive β -sheet formation).²⁷ The dyes can engage in Förster resonance energy transfer (FRET) with each other (see Figure S1 for the absorption and emission spectra of the individual dyes). They are also likely to bind to the replicator systems through electrostatic and hydrophobic interactions. We expect that the exact geometry of binding should be different for different peptide assemblies causing differences in the optical properties of the individual dyes as well as in the efficiency of FRET, which is highly dependent on the distance between the fluorophores. The three dyes were attached on a *cis*-aminoproline core that projects them in the same direction which should benefit FRET. Flexible spacers between core and dyes allow the sensor to adopt different binding geometries when bound to different analytes, which should result in different optical signatures. Finally, the sensor was equipped with an alkyne group to enable easy future structural elaboration using click chemistry.

The ID-probe response involves FRET

A key hypothesis underlying the design of **2a** is that the combination of the three dyes on a single molecular platform would result in FRET between the dyes. This optical communication should enable sCy5 and SRB to respond to the presence of replicators, despite the fact that the emission of these two dyes, when used in isolation, is unlikely to be affected by the presence of the self-replicators. To test this hypothesis, we first measured the fluorescence emission generated by a derivative of

each dye (Figures 3a-c) and by a mixture containing the three derivatives (Figure 3d) in the absence (black line) and presence (red line) of replicator (**1a**)₆. The resulting spectra were compared to one generated by **2a** under the same conditions (Figure 3e). Inspecting the fluorescence responses of the individual dyes (Figures 3a-c) revealed that, as expected, only the ThT responded with a marked (26-fold) increase in fluorescence (Figure 3a, Em: 490 nm) upon irradiation at 440 nm, whereas the emission of SRB (Figure 3b, Em: 595 nm) or sCy5 (Figure 3c, Em: 674 nm), remained unchanged. Moreover, when the individual dyes were combined in a single solution (Figure 3d) fluorescence enhancement was almost exclusively observed in the ThT channel, precluding the possibility of FRET between the dyes in the absence of a covalent linkage between them. In contrast to the strong, single-channel response of the dye mixture (Figure 3d, ThT channel), in the presence of the (**1a**)₆ fibers, **2a** exhibited notable changes both in the ThT and sCy5 channels (Figure 3e), indicating FRET between the three dyes.

The prominent increase in the emission of sCy5 (Ex: 630 nm, Em: 675 nm) upon excitation of ThT (Ex: 440 nm, Em: 490 nm) suggests that FRET is mediated via the SRB dye, which serves as a FRET acceptor for ThT and as a donor for sCy5. This assumption was validated by comparing the fluorescent response of compound **2e**, which combines ThT and SRB (Figure 3f), to the response of **2f** that bears ThT and sCy5 (Figure 3g). In agreement with a SRB-mediated FRET process, compound **2e** exhibited a large increase in the emissions of both ThT and SRB in the presence of (**1a**)₆, whereas in compound **2f**, mainly the emission of ThT was enhanced. This indicates that the SRB is essential for obtaining efficient optical communication between the three dyes and that their integration on unimolecular platform is required for the generation of fluorescence patterns.

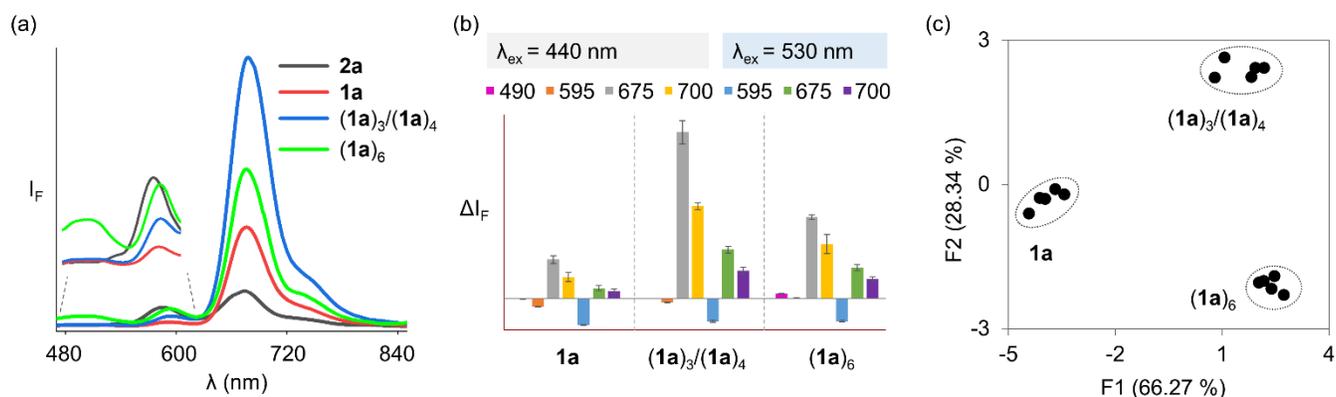


Figure 4: (a) Emission spectra of **2a** (2.0 μ M, 50 mM borate buffer, pH 8.2) in the presence of monomer **1a**, a mixture of trimers and tetramers $(\mathbf{1a})_3/(\mathbf{1a})_4$ or replicator fibers $(\mathbf{1a})_6$, all at a concentration of 30 μ M in units of **1a**; (b) Change in fluorescence intensity of **2a** at seven different emission channels ($\lambda_{\text{ex}} = 440$ nm and 530 nm, respectively) upon being exposed to **1a**, $(\mathbf{1a})_3/(\mathbf{1a})_4$ or replicator fibers $(\mathbf{1a})_6$; (c) Principle component analysis (PCA) of the fluorescence data in b showing five repeats for each sample.

The sensor can discriminate between different replicators and their precursors

To test the discriminatory ability of **2a** we first subjected it to separately prepared samples that represent different phases during the emergence of the self-replicators: the starting monomers **1a**, a mixture dominated by trimers and tetramers $(\mathbf{1a})_3/(\mathbf{1a})_4$, which are intermediates in replicator formation (see Figure 1a), and replicator fibers $(\mathbf{1a})_6$ (prepared as described in methods section). The individual samples were characterized by liquid chromatography–mass spectrometry (UPLC-MS; **Figure S2**). Analysis of replicator $(\mathbf{1a})_6$ by transmission electron microscopy (TEM), and ThT assay confirmed the expected presence of fibers and β -sheets, respectively (**Figure S3**). Fluorescence was measured using a microplate reader in 384-well microplates. The fluorescence spectra were recorded by mixing **2a** (2.0 μ M) with different samples (e.g., monomers, mixtures of trimers-tetramers, and fibers; 30 μ M in units of building block) prepared from building block **1a** in borate buffer (50 mM in boron atoms, pH 8.2).

Inspecting the fluorescence responses of **2a** (2.0 μ M) to the different samples upon excitation of the ThT dye ($\lambda_{\text{ex}} = 440$ nm) revealed a markedly different pattern for each sample (Figure 4a). In the presence of intermediates $(\mathbf{1a})_3/(\mathbf{1a})_4$, we observed a decrease in the emission of the SRB (595 nm) channel and an increase in the emission of sCy5 (675 nm). Unexpectedly, sCy5 fluorescence was also enhanced in the presence of monomers **1a**. Fluorescence of sCy5 most likely comes about through FRET which seemed unlikely to be promoted by binding to molecularly dissolved **1a** (given the relatively small size of this molecule), and suggests that this building block (and potentially also the $(\mathbf{1a})_3/(\mathbf{1a})_4$ mixture) forms aggregates. Indeed, titration of **2a** (2.0 μ M) with **1a** or $(\mathbf{1a})_3/(\mathbf{1a})_4$ revealed the typical signature of a critical aggregation concentration (CAC) where $\text{CAC}_{1a} = 9.2$ μ M (**Figure S4a**). Previous studies on the $(\mathbf{1a})_3/(\mathbf{1a})_4$ mixture had already revealed that these molecules also

aggregate.⁴¹ Studies with our ID-probe confirm this conclusion ($\text{CAC}_{(\mathbf{1a})_3/(\mathbf{1a})_4} = 5.1$ μ M in units of **1a**, **Figure S4b**).

In contrast to the $(\mathbf{1a})_3/(\mathbf{1a})_4$ intermediates, which induced a decrease in the SRB channel and an increase in the sCy5 channel, in the presence of $(\mathbf{1a})_6$ the emission of **2a** was increased both in the ThT (440 nm) and the SRB (595 nm) channels, resulting in a unique fluorescence fingerprint for the replicator fibers.

To enhance the differentiation ability, we measured the optical response of **2a** to the different oligomers of **1a** at four different wavelengths (490, 595, 675, and 700 nm), upon excitation of both the ThT (440 nm) SRB (530 nm) dyes (Figure 4b). The resulting patterns were subjected to principal component analysis (PCA; Figure 4c). PCA is a linear transformation data processing technique that is commonly used to reduce the dimensionality of multidimensional datasets enabling its visualization. It chooses a linear combination of data (the principal components; here a selection of fluorescence intensities at specific wavelengths) that maximizes the spread of the data in a two-dimensional graph⁴². The PCA map clearly shows that **2a** can discriminate between the monomers, trimer/tetramers, and fibers made from **1a**. Using this PCA map an untrained student was able to identify with 100% accuracy 12 samples of which the student did not know the composition (Figure S5 and Table S3).

In order to probe the sensitivity of the technique we repeated the PCA at different analyte concentrations (10 μ M, 3.0 μ M and 300 nM in units of **1a**). The corresponding data is shown in Figure S6 and indicate that the sensor's discriminatory ability gradually diminishes as analyte concentrations are lowered, but is still present even at high nanomolar concentrations.

These experiments were run in 384-well microplates using sample volume of 45 μ L per well, substantially increasing sample throughput and reducing sample volumes, compared to previously used methods for monitoring self-replicators.

The sensor enables monitoring self-replicator formation in-situ

As a first test of the suitability of the ID-probe to track the emergence and growth of replicators in real time, where the molar ratio of precursors $(\mathbf{1a})_3/(\mathbf{1a})_4$ and replicators $(\mathbf{1a})_6$ dynamically changes, the emission of $\mathbf{2a}$ was recorded upon exposure to mixtures containing different molar ratios (specified in Table 1 in the methods section) of separately prepared $(\mathbf{1a})_6$ and $(\mathbf{1a})_3/(\mathbf{1a})_4$. The compositions of these mixtures represent different stages of replicator emergence (Figure 1c). PCA of the resulting data shows that $\mathbf{2a}$ can discriminate between different molar ratios of precursors and replicators (Figure S7).

Encouraged by these results we investigated whether ID-probe $\mathbf{2a}$ could be used to track the spontaneous emergence of replicators from $\mathbf{1a}$ in situ and in real time (Figure 5). To this end, we co-incubated building block $\mathbf{1a}$ (30 μM) and sensor $\mathbf{2a}$ (2.0 μM) and followed the changes in the emission of the ID-probe over time (Figure S8). To confirm that the presence of the ID-probe does not affect the dynamic formation of the different replicators in the mixtures we analyzed the replication process and its kinetics by previously established techniques (UPLC-MS, TEM). The UPLC-based kinetic profile obtained in the presence of $\mathbf{2a}$ (Figure 5a) is comparable to one acquired without the ID-probe (Figure 1c). Similarly, TEM images (Figure 5b) revealed that similar $(\mathbf{1a})_6$ fibers were obtained in the absence (top) and presence (bottom) of the probe.

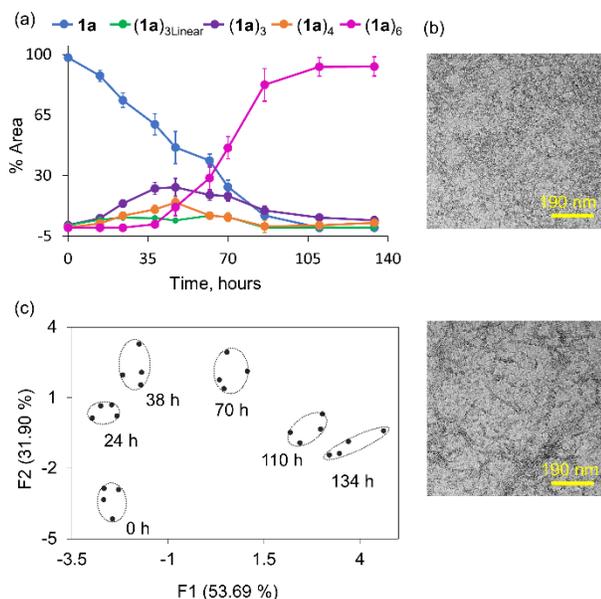


Figure 5. (a) UPLC analysis of the change in product distribution (average of three independent experiments) in a mixture made from building block $\mathbf{1a}$ (30 μM in building block $\mathbf{1a}$) co-incubated with sensor $\mathbf{2a}$ (2.0 μM) in borate buffer (50 mM, pH 8.2, stirred at 1200 rpm at 30 $^{\circ}\text{C}$); (b) TEM images of fibers of replicator $(\mathbf{1a})_6$ in the absence (top) and presence (bottom) of sensor $\mathbf{2a}$; (c) PCA of the emission data recorded for the same sample at various time points.

Analyzing the patterns generated by $\mathbf{2a}$ over time (Figure S8) by PCA (Figure 5c) shows that the ID-probe allows for real-time in-situ tracking of the growth of replicators (data for additional time points is shown in Figure S9). Control experiment in the absence of replicators revealed that the emission of sensor $\mathbf{2a}$ remained unchanged over time (Figure S10), confirming that the observed changes in fluorescence patterns resulted from changes in the composition of the mixture.

The ID-probe can discriminate between different self-replicators

We tested whether ID-probe $\mathbf{2a}$ could also differentiate between replicators that are generated from distinct peptide monomers: $\mathbf{1b}$, $\mathbf{1c}$, and $\mathbf{1d}$ (Figure 1a). The only structural difference between these monomers and $\mathbf{1a}$ is that phenyl alanine in $\mathbf{1a}$ was replaced with another amino acid: alanine ($\mathbf{1b}$), serine ($\mathbf{1c}$) or tyrosine ($\mathbf{1d}$). Another difference concerns the self-assembled structures that are formed during the replication process. Unlike $\mathbf{1a}$ that yields hexamers, $\mathbf{1b}$ and $\mathbf{1c}$ form octamers $(\mathbf{1b})_8$ and $(\mathbf{1c})_8$, respectively, whereas $\mathbf{1d}$ generates pentamers $\mathbf{1d}_5$.³⁷⁻³⁸ The formation of these pentamers was unexpected, given that in previous work (conducted at a higher building block concentration of 3.8 mM and RT instead of 2.0 mM and 45 $^{\circ}\text{C}$) different sized replicators were formed. However, as we noted in our previous work, $\mathbf{1d}$ -based replicators show an unusual plasticity in ring size.³⁸

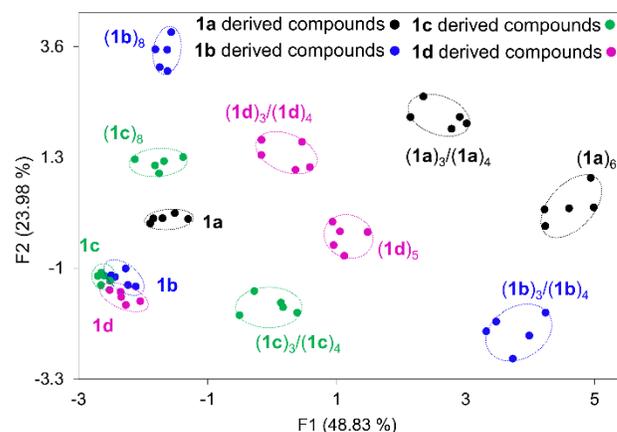


Figure 6: PCA of the emission patterns generated by $\mathbf{2a}$ (2.0 μM) at seven different emission channels ($\lambda_{\text{ex}} = 440$ nm and 530 nm, respectively) in the presence of monomers, a mixture of trimers-tetramers, and replicator fibers prepared from building blocks $\mathbf{1a-d}$ (30 μM in building block). The fluorescent data for each sample consists of five repeats.

Fibers of self-replicators derived from $\mathbf{1b-d}$ (2.0 mM) were prepared by agitating solutions that were subjected to slow air oxidation at 30 $^{\circ}\text{C}$ (for $\mathbf{1a}$)₆ or fast partial oxidation (50%) by sodium perborate and followed by air oxidation at 45 $^{\circ}\text{C}$ ³⁷⁻³⁸ (see Figure S2, S11-S13 for UPLC-MS characterization). Analysis by ThT emission and TEM (Figure S3, S14-S16) confirmed the formation of β -sheets and fibers, respectively (as observed previously for these

systems³⁷⁻³⁸). Samples dominated by trimers and tetramers (as mixture) of these building blocks were also prepared by rapidly oxidizing the individual monomers (2.0 mM) with sodium perborate (40 mM, Figure S2, S11-S13 for UPLC-MS characterization). The different replicators and their small-ring precursors made from building block **1a-d** (30 μ M each) were then incubated with **2a** (2.0 μ M) and its fluorescence emission spectra were recorded (Figure S17). The PCA of this data (Figure 6) clearly shows that **2a** can discriminate between the differently sized macrocycles (trimers and tetramers) and replicators made from the different building blocks. Yet, the probe has difficulty in discriminating between the monomers from which these systems are built up. Using the PCA map shown in Figure 6 an untrained student was able to correctly identify 26 samples out of 28 of which the composition was unknown to the student (Figure S18, Table S4), demonstrating the utility of the ID-probe for the rapid analysis of samples of replicators in a non-destructive way.

CONCLUSIONS

We have demonstrated the use of a pattern-generating fluorescent molecular probe for straightforwardly detecting, discriminating between, and real-time tracking of self-replicators. The sensor is able to discriminate between replicators with different macrocycles sizes (e.g. hexamers, octamers, pentamers) and amino-acid composition. It is also able to differentiate macrocycles with the same size but subtly different peptide sequences. Furthermore, the conversion of building block into replicator could be monitored in situ and in real time without interfering with the replication process. This new technology for the optical analysis of self-replicating systems opens the door for continuous monitoring of parallel experiments in high-throughput ways in small volumes (i.e. in microdroplets or other protocell environments), enabling, for example, the study of stochastic effects, which may prove important in the emergence and evolution of new forms of life. The work also builds one of the first bridges between the still poorly connected fields of systems chemistry and differential sensing, demonstrating the power of such optical tools for rapid fingerprinting of complex reaction networks in terms of composition and dynamics.

METHODS

Preparation of monomer solutions Building block **1a**, **1b**, **1c** or **1d** (stored at $-20\text{ }^{\circ}\text{C}$) was added (around 2 mg) to an HPLC vial (12 \times 32 mm) and around 1.0 mL freshly prepared 50 mM (in boron atom) borate buffer (pH 8.2) was added. The pH of the solution was measured and if necessary, adjusted using 1M NaOH solution. The final concentration of stock solution was 2.0 mM and the monomer solutions were used immediately for the fluorescence experiments. For UPLC and LC-MS analysis, 10 μ L and 5 μ L of sample was injected, respectively, from the solution prepared by mixing 10 μ L of stock solution (2.0 mM) and 80 μ L of UPLC grade water (Figure S2, S11-S13).

Preparation of mixture of trimers-tetramers (**1a**)₃/(**1a**)₄, (**1b**)₃/(**1b**)₄, (**1c**)₃/(**1c**)₄, (**1d**)₃/(**1d**)₄. Building block **1a**, **1b**, **1c** or **1d** (stored at $-20\text{ }^{\circ}\text{C}$) was added (around 2 mg) to an HPLC vial (12 \times 32 mm) and around 1.0 mL freshly prepared 50 mM (in boron atoms) borate buffer (pH 8.2) was added. The pH of the solution measured and if necessary, adjusted using 1M NaOH solution. The final concentration of stock solution was 2.0 mM in borate buffer (50 mM, pH 8.2). A sodium perborate solution (40 mM) was prepared freshly in borate buffer. To prepare a mixture dominated by trimers and tetramers, individual monomer solutions (500 μ L, 2.0 mM) were fully oxidized using sodium perborate (25 μ L, 40 mM) solution. The sample was thoroughly mixed using a micropipette and stored at room temperature for one hour. Purity and composition of the samples were determined by UPLC and UPLC-MS, respectively. The resulting stock solutions can be stored at 25 $^{\circ}\text{C}$ for two to three days without observing any notable changes in sample composition. For UPLC and LC-MS analysis, 10 μ L and 5 μ L of sample was injected, respectively, from the solution prepared by mixing 10 μ L of stock solution (2.0 mM) and 80 μ L of UPLC grade water (Figure S2, S11-S13).

Preparation of replicator fibers (**1a**)₆, (**1b**)₈, (**1c**)₈, (**1d**)₅. Building block **1a**, **1b**, **1c** or **1d** (stored at $-20\text{ }^{\circ}\text{C}$) was added (around 2 mg) to an HPLC vial (12 \times 32 mm) containing a Teflon-coated magnetic stirring bar (5 \times 2 mm, VWR) and around 1.0 mL freshly prepared 50 mM [in boron atoms] borate buffer (pH 8.2) was added. The pH of the solution was measured and, if necessary, adjusted using 1M NaOH solution. The final concentration of stock solution was adjusted to 2.0 mM. A sodium perborate solution (40 mM) was prepared in freshly prepared borate buffer (50 mM, pH 8.2) solution. To prepare fibers, individual monomer solutions (500 μ L, 2.0 mM) were partially oxidized (50%) using sodium perborate (12.5 μ L, 40 mM) solution. The sample was thoroughly mixed using a micropipette and the vial was subsequently closed with a Teflon septum screw cap. The mixture was then placed in a metallic HPLC vial holder and stirred at 1200 rpm at 45 $^{\circ}\text{C}$ for seven days. The composition of the mixture was periodically analyzed by UPLC and UPLC-MS. For UPLC and LC-MS analysis, 10 μ L and 5 μ L of sample was injected, respectively, from the solution prepared by mixing 10 μ L of stock solution (2.0 mM) and 80 μ L of UPLC grade water (Figure S2, S11-S13). To confirm the formation of fibers, emission spectra of ThT (2 μ M, 50 mM borate buffer in boron atoms) in the presence of different samples (30 μ M building block **1**) prepared from peptide building block **1a**, **1b**, **1c** or **1d** were recorded (Figure S3, S14-S16).

Fluorescence measurements differentiating among the monomers 1a-d, mixtures of trimers-tetramers (1a)₃/(1a)₄, (1b)₃/(1b)₄, (1c)₃/(1c)₄, (1d)₃/(1d)₄, and replicator fibers (1a)₆, (1b)₈, (1c)₈, (1d)₅. Fluorescence was measured using a BioTek Synergy H1 microplate reader, in black flat-bottom polystyrene 384-well microplates (Greiner). A 200 μ M stock solution of **2a** was prepared in anhydrous DMSO. The fluorescence spectra were recorded within 1 h incubation of **2a** (2.0 μ M) individually with different samples (30 μ M in units of building block, e.g., monomers, mixtures of trimers-tetramers, and fibers) prepared from building block **1a**,

1b, **1c** or **1d** in borate buffer (50 mM in boron atoms, pH 8.2) at 25 °C. These experiments were performed in five replicates where each sample was freshly prepared starting from the weighing step. The spectra before addition of samples were subtracted from those recorded after the addition of different samples. Principle component analysis (XLSTAT 2020.5.1) was applied to discriminate the emission patterns obtained at the following excitation and emission wavelengths: λ_{ex} : 440 nm, λ_{em} : 490, 540, 570, 595, 640, 675, 700 nm; and λ_{ex} : 530 nm, λ_{em} : 570, 595, 640, 675, 700 nm (Figure 4c and Figure 6).

The discrimination efficiency of the system was further validated by identifying (Figure S5, Table S3 and Figure S18, Table S4) unknown samples which were freshly prepared from building block **1a**, **1b**, **1c** or **1d** on different days. Unknown samples were analyzed using the XLSTAT (version 2020.5.1) prediction mode according to the posterior probabilities for each blind test.

Analyte samples were prepared by mixing different molar ratios of fibers (**1a**)₆ to the mixture of trimers and tetramers (**1a**)₃/**(1a)**₄ prepared from **1a**. As described above fibers of (**1a**)₆ were prepared from monomers of building block **1a** (500 μ L, 2.0 mM) upon partial oxidation (50%) using sodium perborate (12.5 μ L, 40 mM) in borate buffer (50 mM, pH 8.2). The sample was thoroughly mixed using a micropipette and the vial was subsequently closed with a Teflon septum screw cap. The mixture was then placed in a metal HPLC vial holder and stirred at 1200 rpm at 45 °C for four days. To prepare a mixture of trimers and tetramers (**1a**)₃/**(1a)**₄ building block **1a** (500 μ L, 2.0 mM) was fully oxidized using sodium perborate (25 μ L, 40 mM) in borate buffer (50 mM in boron atoms, pH 8.2). The sample was thoroughly mixed using a micropipette and stored at room temperature for around one hour.

Table 1. Composition of samples prepared by mixing different molar ratios of fibers (1a**)₆ to trimers - tetramers (**1a**)₃/**(1a)**₄.**

Sample	Sample composition (%) ^a	
	Trimers-tetramers (1a) ₃ / (1a) ₄	Fibers (1a) ₆
1	100	0
2	70	30
3	40	60
4	20	80
5	0	100

^aThe total concentration of each sample was 30 μ M in units of building block **1a**. These samples represent different stages of replicator emergence.

To test the ability of the sensor to track the emergence of replicators, we prepared libraries having different ratios (shown in Table 1) of trimers-tetramers (**1a**)₃/**(1a)**₄ and fibers (**1a**)₆, which represent different stages of replicator emergence. Fluorescence patterns were recorded following excitation at 440 nm and 530 nm, respectively. Principle component analysis was applied to discriminate among the emission patterns obtained at the following excitation and emission wavelengths: λ_{ex} : 440 nm, λ_{em} :

490, 540, 570, 595, 640, 675, 700 nm; and λ_{ex} : 530 nm, λ_{em} : 570, 595, 640, 675, 700 nm (Figure S7).

Real-time study of the emergence of replicators based on building block 1a using ID-probe 2a. Building block **1a** (stored at -20 °C) was added (around 1 mg) to an HPLC vial (12 × 32 mm) and around 0.5 mL freshly prepared borate buffer (50 mM in boron atoms, pH 8.2) was added. The pH of the solution was measured and if necessary, adjusted using 1.0 M NaOH solution. The final concentration of the stock solution was 2.0 mM. Then 7.5 μ L of this stock solution and 2.0 μ L of a solution of **2a** (0.5 mM in DMSO) was individually transferred to an HPLC vial (12 × 32 mm) containing a Teflon-coated magnetic stirring bar (5 × 2 mm, VWR) to obtain the desired concentration of **1a** (30 μ M in units of building block) and **2a** (2.0 μ M) in borate buffer (50 mM, pH 8.2; final DMSO concentration <0.5 %) (vial A).

For the control experiments, 7.5 μ L of stock solution of **1a** (2.0 mM) and 2.0 μ L of fresh DMSO solution was transferred to an HPLC vial (12 × 32 mm) containing a Teflon-coated magnetic stirring bar (5 × 2 mm, VWR) to obtain the desired concentration of **1a** (30 μ M in units of building block) in borate buffer (50 mM, pH 8.2; final DMSO concentration <0.5 %) (vial B).

For the second control experiment, 2.0 μ L of **2a** (0.5 mM in DMSO) was transferred to an HPLC vial (12 × 32 mm) containing a Teflon-coated magnetic stirring bar (5 × 2 mm, VWR) to obtain the desired concentration of **2a** (2 μ M) in borate buffer (50 mM, pH 8.2) solution (final DMSO concentration <0.5 %) (vial C).

After initial fluorescence measurements, UPLC and LC-MS measurement vials were subsequently closed with Teflon septum screw caps and placed in a metal HPLC vial holder and stirred at 1200 rpm at 30 °C in the dark. Fluorescence readings from the mixture of **1a** and **2a** at various time points were recorded using excitation at 440 nm and 530 nm, respectively. At the same time, the composition of the mixture was periodically analyzed by UPLC and the formation of fibers was analyzed by TEM measurements. All these experiments were repeated four times on independent days using freshly prepared samples starting from the weighing step. Principle component analysis was applied to discriminate among the emission patterns obtained at various time points using the following excitation and emission wavelengths: λ_{ex} : 440 nm, λ_{em} : 490, 540, 570, 595, 640, 675, 700 nm; and λ_{ex} : 530 nm, λ_{em} : 570, 595, 640, 675, 700 nm (Figure 5b, S9).

Transmission Electron Microscopy (TEM). To confirm the formation of replicator fibers from different building blocks as described above, samples (5.0 μ L) were applied to carbon-coated copper TEM grids (400 mesh) for 40 seconds at room temperature. Excess liquid was removed with filter paper and the grids were negatively stained with saturated uranyl acetate (5.0 μ L) and blotted on filter paper after incubation for 20 seconds and air dried. Transmission electron micrographs (TEM) were recorded on a Philips CM120 electron microscope operating at 120 kV. TEM images (Figures 5c, S3b, S14b, S15b and S16b) were recorded on a slow-scan charge-coupled device camera (Gatan).

Ultra-performance liquid chromatography (UPLC) analysis. UPLC analyses were performed on a Waters

Acquity UPLC H-class system equipped with a PDA detector. A reversed-phase UPLC column (Aeris 1.7 μm XB-C18 150 \times 2.10 mm, purchased from Phenomenex) was used for the analyses of all samples, while the UV absorbance was monitored at 254 nm. Note that the different sized macrocycles formed from the different building blocks have comparable extinction coefficients at 254 nm. The column temperature was equilibrated at 30 $^{\circ}\text{C}$ prior to injections. The elution phases consisted of UPLC grade water with 0.1% TFA (eluent A) and acetonitrile with 0.1% TFA (eluent B) at a constant flow rate of 0.3 mL min^{-1} . All UPLC samples were injected using the solvent gradients shown in Table 2. For the analysis of the samples, peaks were assigned (using LC-MS data) then integrated and the resulting peak areas were used to construct the kinetics plots.

Table 2: Gradient used for UPLC analysis

Time (minutes)	Eluent A (%)	Eluent B (%)
0.00	90	10
1.00	90	10
1.30	75	25
3.00	72	28
11.00	60	40
11.50	5	95
12.00	5	95
12.50	90	10
17.00	90	10

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge at <http://pubs.acs.org>. Synthesis of **2a-e** and figures S1-S18, Table S1-S4

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Notes

The authors declare no competing financial interest.

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Table of Contents artwork

