

Selective amide bond formation in redox-active coacervate protocells

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Abstract

Membraneless compartments, like complex coacervates droplets, are promising protocell models because of their ability to sequester a wide range of guest molecules and their catalytic properties. However, it remains unclear how the building blocks of life, including peptides, could be synthesized from primitive precursor molecules inside such protocells. Here, we develop a new protocell model formed by phase separation of prebiotically relevant small redox-active ferricyanide ($\text{Fe}(\text{CN})_6^{3-}$)/ferrocyanide ($\text{Fe}(\text{CN})_6^{4-}$) molecules and a cationic peptide. The assembly of these coacervate protocells can be regulated by redox chemistry and they act as oxidizing hubs for sequestered metabolites, such as NAD(P)H, and fiber precursors. Interestingly, we show that the oxidizing potential of ferricyanide inside coacervates can be harnessed to drive the selective formation of amide bonds between prebiotically relevant amino thioacids and amino acids or peptides. We demonstrate that aminoacylation is enhanced in $\text{Fe}(\text{CN})_6^{3-}$ /peptide coacervate dispersions compared to the surrounding dilute phase, and selective for amino acids that interact less strongly with the coacervates. We finally use this amide bond formation to create self-

reinforcing coacervates by reacting hydrophobic amino thioacids to amines on the protocell scaffold and show that this significantly enhances their salt resistance. These results provide an important step towards the prebiotically relevant integration of redox chemistry in cell-like compartments.

Introduction

Amide bond formation is an essential chemical reaction in all forms of life that is catalyzed by highly evolved biomolecular machinery. However, before ribosomes and specialized enzymes became capable of protein synthesis,^{1,2} alternative, simple prebiotic routes to create peptide bonds in a spatiotemporally controlled way likely existed. Protocellular compartments provide a promising platform to localize chemical reactions relevant to life.³⁻⁵ What the nature of such protocellular compartments capable of peptide synthesis could be, remains unknown. As plausible precursors to peptides, α -aminothioacids (AA-SH)⁶ and acetylated aminothioacids (Ac-AA-SH)⁷ have been shown to be formed in aqueous conditions at near-neutral pH. They are considered interesting alternatives to biological thioesters for prebiotic peptide ligation, and can be ligated into peptide with the aid of an oxidizing catalyst, such as ferricyanide.⁷⁻¹⁰ However, high concentrations of reactants and catalysts are typically required for these oxidative peptide ligations, which may not have been easy to reach. Recent work has demonstrated that some micro-compartments and protocell systems could compartmentalize (bio)chemical reactions, and act as catalytic microreactors or reaction localization centers with potential for prebiotic peptide ligation.¹¹⁻¹⁶

In particular, membraneless compartments based on complex coacervates have been considered as versatile protocell models in the origins of life research.^{5,16-19} Coacervate droplets

are formed spontaneously by liquid-liquid phase separation, resulting in a polymer- or peptide-rich, cell-sized dense coacervate phase, and a coexisting dilute phase. These membraneless droplets can easily take up and concentrate guest molecules from their surroundings due to charge complementarity or hydrophobicity.^{16,20,21} The ability to up-concentrate and exchange guests make the coacervate droplets capable of supporting biochemical reactions, and sometimes enhancing the activity of catalysts, such as ribozymes,^{12,13} or enzymes in cascade reactions,^{15,22} thereby increasing the overall rate. Recent work has demonstrated that coacervate droplets can be active,^{18,23} and that their formation and dissolution can be regulated by environmental changes, such as pH,^{24,25} temperature,^{26,27} light,²⁸ enzymatic reactions,^{23,29–31} or fuel-driven chemical reaction cycles.¹⁸

To date, research on coacervates as reaction localization centers has mostly focused on enzymatic reactions, or reactions involving complex RNAzymes (ribozymes). However, the fundamental biochemical reactions that lead to the formation of peptides, which themselves are often the building blocks of coacervates, have not been studied. For coacervates to be a plausible protocell model, they must be able to support a prebiotically relevant ligation reaction of peptides, and ultimately, link this reaction to the construction of new coacervates. Here, we show that ferricyanide-containing coacervates can potentially fulfill this role. These coacervates can be used to oxidize not only a variety of common metabolites, but also amino thioacids, which can subsequently be aminoacylated yielding a product with a new amide bond.

Coacervates with ferrocyanide, the reduced form of ferricyanide, as multivalent anion have already been reported by Bungenberg-de Jong and Kruyt in 1929.³² They used gelatin at low pH as a long polycation and observed small punctated droplets upon mixing with potassium ferrocyanide. However, the redox potential of the iron centers inside these coacervates remains unclear. More recently, it was shown that coacervates can be formed from much smaller cationic oligopeptides, such as oligolysine and oligoarginine with as little as five amino acids, complexed

with either small tri- or tetravalent anions, like ADP and ATP.¹⁹ We therefore hypothesized that it should be possible to make coacervates of the redox couple ferri- and ferrocyanide with a cationic polypeptide in such a way that ferrocyanide, the reduced state with a 4⁻ charge, would form stable droplets, but ferricyanide, the oxidized state with a 3⁻ charge, would not. This would enable the selective compartmentalization of guest molecules only under certain redox potential in the environment. In addition, from an origins of life perspective, the environment on early Earth was likely depleted of oxygen, a strong oxidizing agent that is widely used in living systems, for a considerable period. Alternative oxidizing agents, such as ferricyanide ($\text{Fe}(\text{CN})_6^{3-}$), could have been essential in the prebiotic activation of building blocks for peptide ligation,^{7,9,33,34} and oxidation of metabolites.³⁵ By condensing the ferricyanide with an oppositely charged peptide at low ionic strength, it may be possible to localize these reactions in a droplet compartment and enrich the coacervate with the products of ferricyanide-catalyzed oxidation reactions.

Here, we show that both ferricyanide and ferrocyanide ions can be condensed into coacervate droplets with short cationic polypeptides depending on the ionic strength. These droplets are responsive and can be regulated by redox chemistry. They act as prebiotic oxidizing hubs for metabolites and common electron donors, such as NADH, NADPH and GSH, thus taking the role of oxygen as terminal electron acceptor. Besides small metabolites, aromatic thiols such as benzoyl cysteine can be oxidized inside ferricyanide-based coacervates, leading to their assembly into stacked filaments, which further bundle into rigid fibers that resemble a cytoskeletal network inside and around the protocells. Moreover, we show that the oxidizing potential of ferricyanide-based coacervates can be harnessed to drive the formation of peptide bonds between amino acid and amino thioacids which are considered as potential prebiotic precursors of amino acids. We demonstrate that amino thioacid ligation is enhanced in $\text{Fe}(\text{CN})_6^{3-}$ /peptide coacervate dispersions compared to the surrounding dilute phase due to the local high ferricyanide concentration. The

coacervate environment imposes a selection pressure that results in kinetic pathway selection and a strong, preferential incorporation of certain amino acids. When amino acids or short peptides are used as client substrates, the reaction products can leave the coacervate droplets again, but they can also be anchored to the coacervate compartments by using peptides containing amine groups as coacervating scaffolds. We show that this strategy can be used to create self-reinforcing coacervates, in which hydrophobic amino acid residues are ligated to the coacervate building blocks and enhance their stability. In short, our results show that prebiotically relevant ferricyanide-based coacervate protocells are versatile oxidizing hubs that exist in aqueous solution, in which metabolites can be converted, fibrous networks assembled and peptides synthesized.

Results and discussion

Coacervation of ferricyanide/ferrocyanide and polypeptides

Small, multivalent ions can be condensed into liquid coacervate droplets by complexation with an oppositely charged peptide or polymer.⁵ We sought to use this principle to create coacervate protocells that can concentrate a prebiotically relevant redox catalyst to enable localized peptide synthesis. Ferricyanide is a trivalent anion and its reduced form, the tetravalent ferrocyanide, has been shown to be able to form coacervates with gelatin at low pH.³² We used cationic peptides to induce phase separation of ferri- and ferrocyanide. Spherical complex (heterotypic) coacervate droplets were readily formed as a turbid dispersion via spontaneous liquid-liquid phase separation associated with the charge neutralization of a range of peptides ((Lys)₁₀, (Lys)₂₀, (Lys(Me)₃)₂₀, (Lys)₃₀, (Lys(Me)₃)₃₀, poly-L-lysine (pLys) and (Arg)₁₀) in the presence of ferrocyanide or ferricyanide (Fig. 1a, Supplementary Fig. 4). (Arg)₁₀ formed coacervates with both ferrocyanide and ferricyanide, while (Lys)₁₀/(Lys)₂₀ only formed coacervates with tetravalent ferrocyanide in

salt-free solution. The difference between (Lys)₁₀ and (Arg)₁₀ can be explained by the higher pK_a of the basic residue in arginine compared to lysine, which may generate more stable ionic interactions at a given chain length.³⁶ Increasing the length of polylysine to (Lys)₃₀ resulted in the formation of coacervates with trivalent ferricyanide as well (Supplementary Fig. 4c), in agreement with previous studies involving nucleotides.^{19,27}

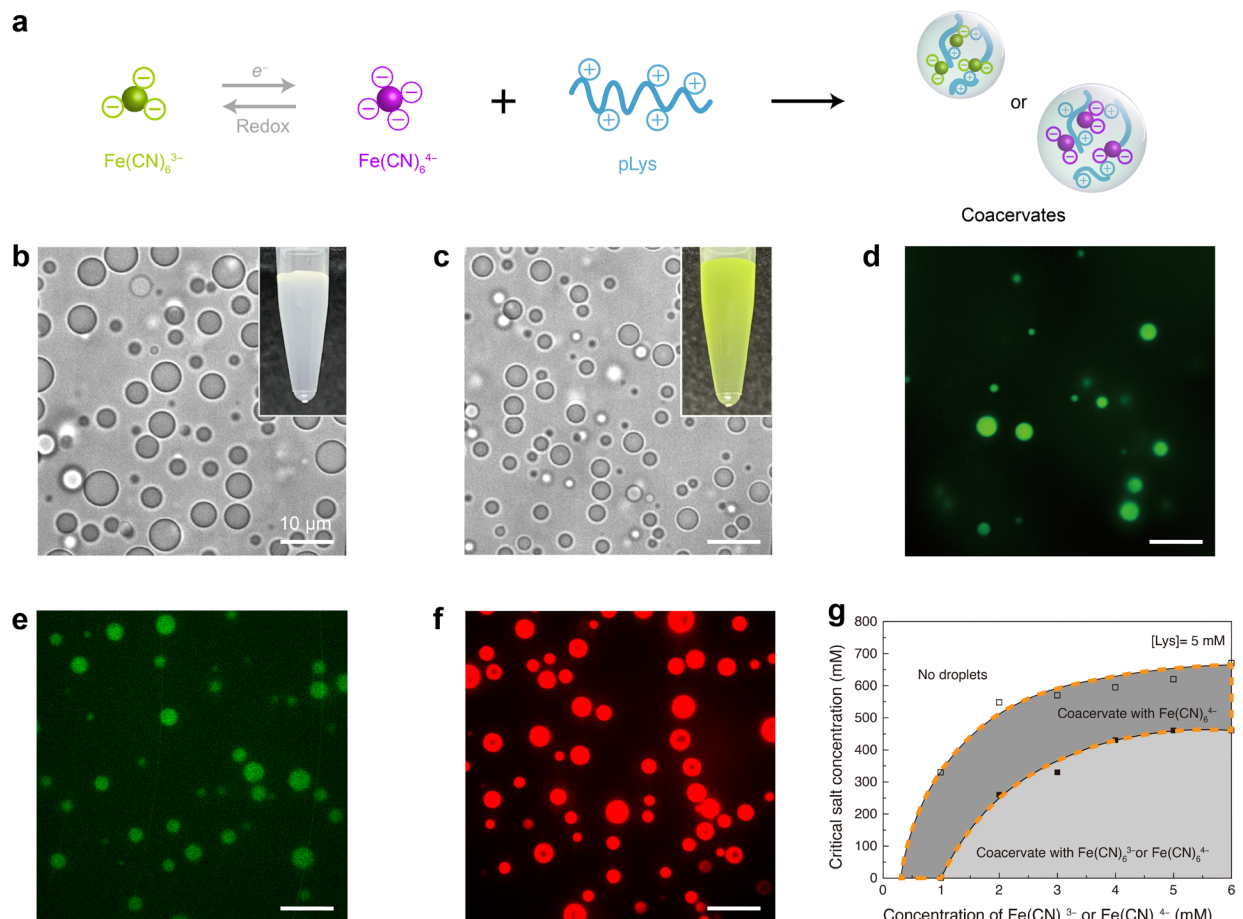


Figure 1. (a) Schematic illustration of associative liquid-liquid phase separation of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ and pLys to produce coacervate droplets. (b) Optical microscope images of $Fe(CN)_6^{4-}$ /pLys droplets prepared at 1 mM $Fe(CN)_6^{4-}$ and 5 mM pLys (monomer basis), and (c) at 2 mM $Fe(CN)_6^{3-}$ and 5 mM pLys (monomer basis). Insets show photographs of the corresponding turbid suspensions. (d-f) Fluorescence microscopy images of $Fe(CN)_6^{4-}$ /pLys droplets with various client molecules: (d) pyranine, (e) NADPH, (f) poly-rU₁₅ (scale bars indicate 10 μ m). (g) Critical salt concentration of $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ coacervates with a fixed concentration of 5 mM pLys, determined from turbidity titrations.

The obtained coacervates with a diameter of about 2 to 3 μm were observed in a PLL-g-PEG functionalized microchamber (Figure 1b-c). These microdroplets exhibited liquid-like properties: they wetted the bottom glass surface and sedimenting coacervates coalesced with coacervates already present at the bottom of the microchambers to form bigger droplets. The droplets sequestered negatively charged client solutes, including pyranine, NAD(P)H, and ribonucleic acids (Figure 1d-f, Supplementary Fig. 4). Determination of the partitioning of ferricyanide and ferrocyanide by Uv-vis spectroscopy (see Methods) showed that both these multivalent scaffolding anions were highly concentrated in the coacervate droplets: the internal concentration in coacervate droplets was 100 times higher than the surrounding dilute aqueous phase. For example, for samples prepared from 2 mM ferricyanide and pLys (5mM lysine monomers) solutions, we found an internal ferricyanide concentration of ~ 30 mM, compared to ~ 0.3 mM in the surrounding aqueous phase. (Supplementary Fig. 5). Surprisingly, the concentration ratio for ferri- and ferrocyanide was the same, despite their different valency. We attribute this to the formation of a tighter complex between ferricyanide and polycations, which results in a more hydrophobic coacervate,²⁷ an effect that has previously been observed with ferricyanide in polyelectrolyte brushes.³⁷ A similar 100x concentration was previously found for a negatively charged bis-carboxylic acid ligand, which can exist in a tetravalent (4-) ring configuration, in coacervates with cationic polymers.³⁸ These initial observations suggest that the spontaneous assembly of peptides and ferricyanide or ferrocyanide could be developed as potential prebiotic membraneless compartments.

Typically, coacervates formed by charge-charge interaction are sensitive to the ionic strength and they exhibit a critical salt concentration (CSC) above which phase separation does not take place. Since ferricyanide and ferrocyanide have a different net charge, their CSC is likely different, even though the previously discussed hydration differences may decrease that effect. A difference in CSC would allow for selective compartmentalization controlled by redox chemistry. To

determine the conditions under which the redox couple ferri- and ferrocyanide could give rise to reversible coacervate formation and dissolution, we evaluated the salt resistance of $\text{Fe}(\text{CN})_6^{4-}$ and $\text{Fe}(\text{CN})_6^{3-}$ -based coacervates. As a model peptide, we focused on pLys ($M_w = 15\text{-}30$ kDa). Supplementary Fig. 6 shows turbidity-based titration curves of pLys (5 mM monomer units), as a function of $\text{Fe}(\text{CN})_6^{4-}$ or $\text{Fe}(\text{CN})_6^{3-}$, and as a function of salt concentration. From plots of the turbidity we determined the critical salt concentration (CSC), the point at which coacervate droplets completely disappear. Figure 1g shows the resulting phase diagram of both $\text{Fe}(\text{CN})_6^{4-}$ and $\text{Fe}(\text{CN})_6^{3-}$ coacervates. As expected, $\text{Fe}(\text{CN})_6^{4-}$ -based coacervates have a higher salt resistance, expressed by their CSC, compared to $\text{Fe}(\text{CN})_6^{3-}$ -based coacervates. Our results are in good agreement with previous studies with nucleotide/pLys-based coacervates, where the ATP/pLys droplets have a higher CSC than ADP/pLys droplets, in line with their valency.^{19,30}

Redox chemistry of ferri/ferrocyanide in coacervates

We next exploited the redox activity of the droplets in the critical salt concentration window highlighted in Fig. 1g. To illustrate the feasibility of the redox cycling proposed in Fig. 2a to induce phase separation and droplet dissolution, we prepared mixtures of $\text{Fe}(\text{CN})_6^{4-}$ with pLys and $\text{Fe}(\text{CN})_6^{3-}$ with pLys under identical conditions within the highlighted region of Fig. 1g between the two binodal lines. The original $\text{Fe}(\text{CN})_6^{4-}$ -containing mixtures and $\text{Fe}(\text{CN})_6^{3-}$ -containing mixtures are white turbid and yellowish transparent, respectively. When observed under the microscope, the $\text{Fe}(\text{CN})_6^{4-}$ -containing mixtures had clearly condensed into droplets, while the $\text{Fe}(\text{CN})_6^{3-}$ -containing mixtures remained a homogeneous solution. Upon oxidation of the $\text{Fe}(\text{CN})_6^{4-}$ -coacervates by $\text{S}_2\text{O}_8^{2-}$, the originally white turbid solution turned yellowish transparent, and no droplets could be observed under the microscope (Fig. 2b). Conversely, after reduction of the $\text{Fe}(\text{CN})_6^{3-}$ -containing mixtures with GSH or NADH (Fig. 2b-d), the original, light-yellow

transparent $\text{Fe}(\text{CN})_6^{3-}$ -containing mixtures became white turbid, and droplets were clearly visible under the microscope.

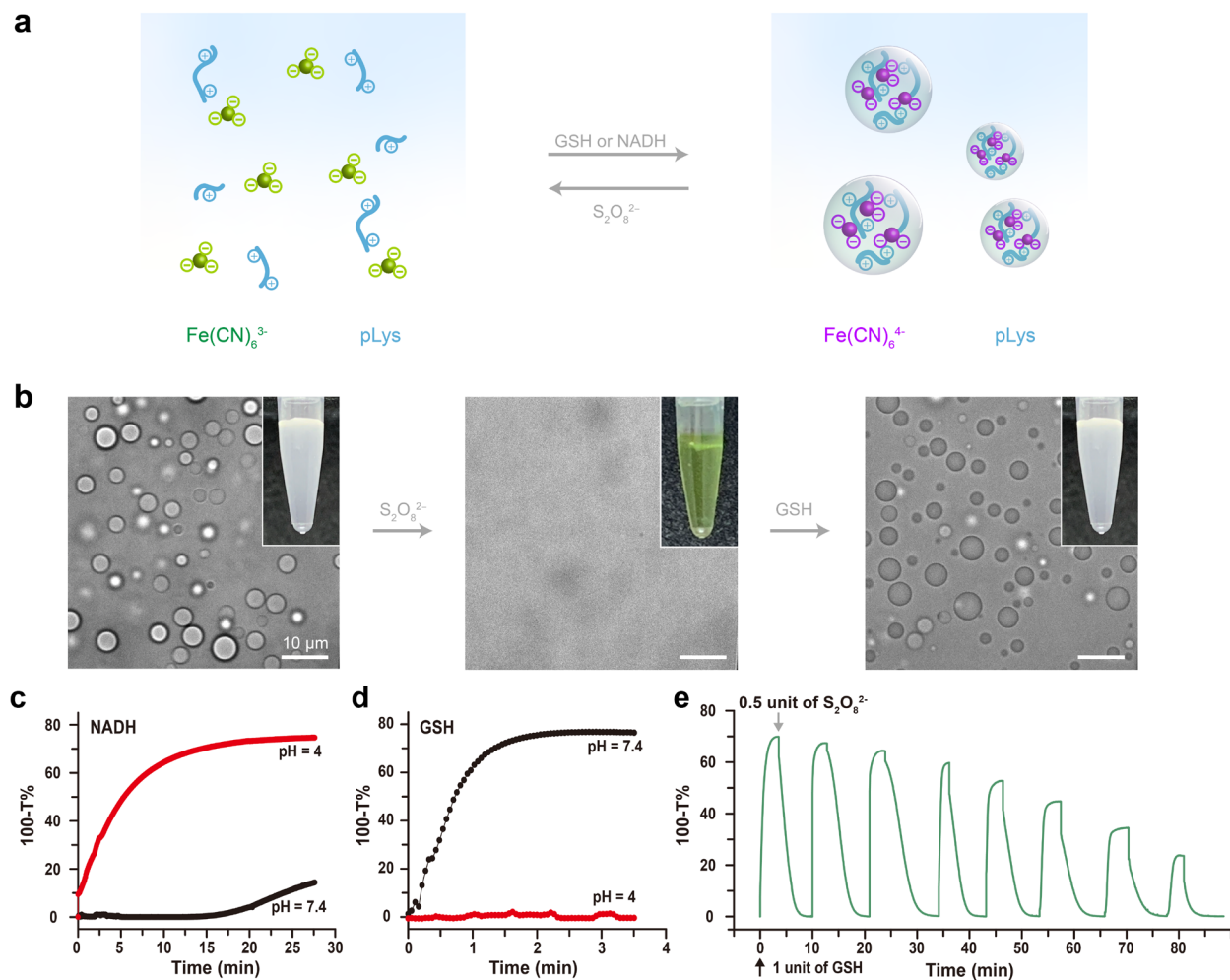


Figure 2. (a) Schematic illustration of the redox reaction network underlying dynamic and reversible formation and dissolution of $\text{Fe}(\text{CN})_6^{4-}/\text{pLys}$ coacervate droplets. (b) Optical observation of droplet dissolution by $\text{S}_2\text{O}_8^{2-}$ addition to $\text{Fe}(\text{CN})_6^{4-}/\text{pLys}$ coacervates dispersion and droplet formation by GSH addition. Scale bars: 10 μm . Insets show photographs of the corresponding turbid suspensions, or the clear solution. (c) Formation of $\text{Fe}(\text{CN})_6^{4-}/\text{pLys}$ coacervate droplets induced by addition of 1 equivalent of NADH at pH 4, but not at pH 7.4. (d) Same as (c), but induced by addition of 1 equivalent of GSH, which proceeds at pH 7.4, but not at pH 4. (e) Alternating additions of GSH and $\text{S}_2\text{O}_8^{2-}$ at pH 7.4 show that condensation and dissolution are both reversible and that the system can be switched multiple times between a compartmentalized droplet state and a single-phase homogeneous solution.

The oxidation reaction showed a clear pH dependence (Fig. 2c,d). We were able to use a stoichiometric amount of GSH to turn a homogeneous ferricyanide solution into a dispersion of ferrocyanide droplets in the presence of pLys within 5 minutes in neutral conditions (pH 7.4, Fig. 2b,d), while no conversion occurred under acidic conditions (pH 4). When using NADH as reducing agent, almost no conversion of the same homogeneous ferricyanide solution with pLys could be observed in neutral conditions (pH 7.4, Fig. 2c), while complete conversion into ferrocyanide droplets was observed within 15 minutes under acidic conditions (pH 4). In both cases, with GSH and NADH, a stoichiometric amount of $\text{S}_2\text{O}_8^{2-}$ completely dissolved a dispersion of $\text{Fe}(\text{CN})_6^{4-}$ droplets, converting $\text{Fe}(\text{CN})_6^{4-}$ back into $\text{Fe}(\text{CN})_6^{3-}$ within ten minutes. Fig. 2e illustrates the remarkable reversibility of this process: droplets could be generated and dissolved up to eight times, and we were able to carry out identical transitions when starting from either $\text{Fe}(\text{CN})_6^{4-}$ or $\text{Fe}(\text{CN})_6^{3-}$. After eight cycles, the system loses its ability to condense into droplets, which is mainly caused by accumulation of the waste products from GSH or NADH and $\text{S}_2\text{O}_8^{2-}$. The level of redox control over droplet generation shown in Figure 2 has not been achieved before and holds great promise for the development of dynamic protocell models.

For use of these ferricyanide coacervates as potential oxidizing hubs in which oxidation reactions could be localized, it is important to know where the redox reactions utilized in Fig. 2 take place. We can take advantage of the fluorescence of common redox-active metabolites such as NADPH, in combination with the observed pH dependence, to monitor the conversion ferri- and ferrocyanide. We incubated ferricyanide/(Arg)₁₀ coacervates (8 mM ferricyanide/24 mM Arg) with 2 mM NADPH at neutral pH and observed clear NADPH fluorescence inside the coacervates, indicating that NADPH is sequestered by the coacervates (Supplementary Fig. 7). At this pH, the reduction of ferricyanide by NADPH is suppressed (Fig. 2c), which allows for equilibration and focusing of the microscope. We then decreased the outer pH by addition of a fixed amount of acid

and monitored how the redox reaction progressed and led to the rapid disappearance of the fluorescence of NADPH within 2 min. (Supplementary Fig. 7). Interestingly, the fluorescence intensity first disappeared from the center of the coacervate droplets (Supplementary Fig. 7), indicating that the oxidation of NADPH to non-fluorescence NADP^+ by ferricyanide took place predominantly inside the droplets instead of in solution,¹⁸ in which case an exchange of NADPH/ NADP^+ and reduction of fluorescence at the droplet interface would be expected. Taken together, these data show our ferricyanide-based coacervates are redox-active compartments that can locally oxidize sequestered metabolites.

Fiber self-assembly inside ferricyanide-based coacervates

We further investigated if the redox activity of ferricyanide-based coacervates could lead to spatially controlled higher order assembly. Self-assembly of filaments inside or at the periphery of (proto)cellular compartments is key to many transport processes, locomotion and division.^{39,40} Spatially controlled assembly of analogous model filaments in cell-like compartments is therefore an interesting goal in protocell and synthetic cell research.^{40–42} We selected an amino acid derivative benzoyl cysteine as precursor for filaments (Fig. 3). *N,N'*-dibenzoyl-L-cystine (DBC) is a well-known redox-active supramolecular gelator,^{43,44} which has been used to make filaments in aqueous solution at low pH. To create the precursor form, we reduced the water soluble DBC^{2-} at pH 7 with 1 equivalent of dithiothreitol (DTT) to give non-active monomer *N*-benzoyl-L-cysteine (BC^-), which is highly soluble at both high and low pH and does not stack to form fibers.

We flushed a solution of BC^- (with dye Nile red) in a microchamber containing ferricyanide/((Arg)₁₀ coacervates (final concentration: 10 mM BC^- , 4 mM ferricyanide, 12 mM (Arg)₁₀ (monomer basis)) at pH 7. Upon addition of the coacervates, we observed a gradual increase in the total fluorescent intensity inside coacervates, as newly formed non-active monomer 2-

charged DBC²⁻, which weakly binds to Nile red, partitioned in the coacervates (Fig. 3a). At this pH, no fibers are formed, and the non-active DBC²⁻ monomers are distributed homogeneously inside the coacervates. We note that ferricyanide/peptide coacervates without DBC²⁻ do not sequester Nile red (Supplementary Fig. 4h). Upon addition of BC to an aqueous dispersion of ferricyanide/(Arg)₁₀ coacervates at pH 3 (below the pK_a of the carboxylate groups of DBC, pK_a ~ 3.6),⁴⁴ we observed oxidized, bright fluorescent DBC filaments assembled into shells around the coacervate droplets, and several bundles of filaments present inside the droplets (Fig. 3b). The bundled filaments inside the coacervates are clearly visible in transmission, and are strongly stained by Nile red. These bundles can be seen to pierce the interface of the coacervate droplets. In control with ferricyanide but without polycations, we observed a fiber dispersion without clear bundling or formation of shells (Fig. 3c).

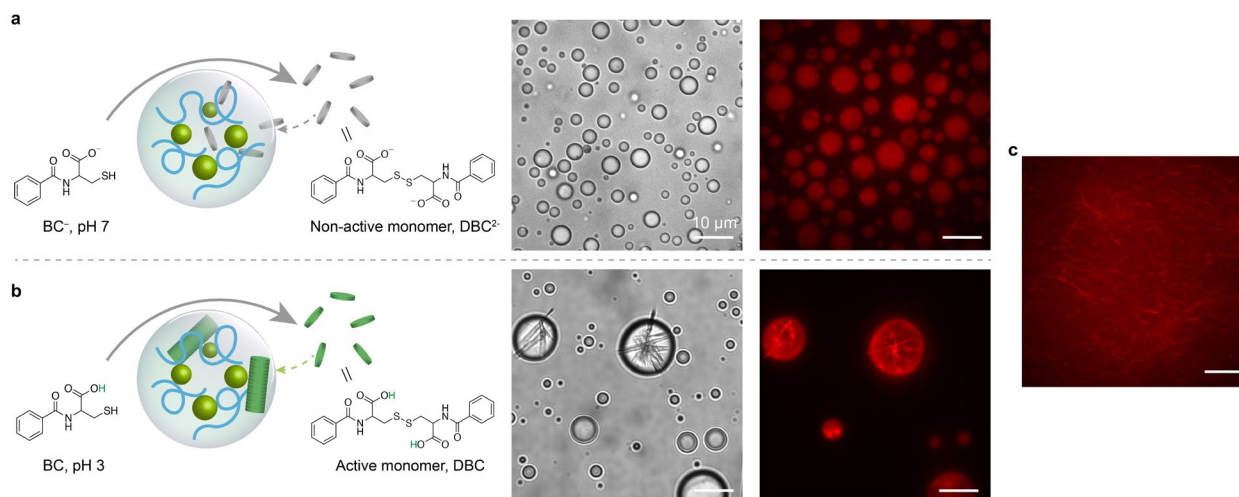


Figure 3. (a) Confocal micrographs of ferricyanide/(Arg)₁₀ protocells (with dye, Nile red), time series of representative protocells after addition of BC⁻. (b) Confocal micrographs of ferricyanide/(Arg)₁₀ protocells (with dye, Nile red) after addition of BC. (c) Confocal micrographs of fiber formation after adding ferricyanide into BC solution.

A similar type of interfacial filament assembly has been observed for actin filaments and peptide-based pLys/pGlu coacervates before.⁴¹ We also observed that different peptides lead to altered fiber assembly: in the case of ferricyanide/(Lys(Me)₃)₂₀ and ferricyanide/(Lys(Me)₃)₃₀ coacervates, the fibers preferentially localized inside and at the interface of the coacervates (Supplementary Fig. 8a-b). Interestingly, in the case of ferricyanide/(Lys)₃₀ coacervates, the fibers did not remain confined inside the coacervates, but grew out into the surrounding environment, giving an aster-like shape (Supplementary Fig. 8c). In short, these findings show that ferricyanide coacervates can drive the formation of self-assembled filaments via oxidation of the filament precursors.

Amide bond formation through amino thioacid oxidation in coacervate protocells

Having established the potential of ferricyanide-based coacervates as oxidizing hubs for redox-active guest molecules, we sought to use the oxidizing potential to synthesize peptides by catalyzing the formation of amide bonds. Ferricyanide has been described as a prebiotically abundant oxidizing agent,³³ and has been used to activate amino thioacids by oxidation to facilitate the formation of an amide bond upon reaction with nucleophilic aminonitriles and amino acids.^{6-9,34} However, the oxidative aminoacylation of thioacids is usually performed with high reactant and ferricyanide concentrations (sometimes close to 100 mM), which could have been difficult to reach everywhere on Early Earth. Therefore, the highly concentrated ferricyanide coacervate droplets could be interesting model compartments for prebiotic amide bond formation (Fig. 4a). As a proof of principle, we first prepared ferricyanide coacervate droplets by direct mixing of aqueous solutions of ferricyanide and trimethylated poly-L-lysine (pLys(Me)₃) at charge stoichiometry. The pLys(Me)₃ was chosen to avoid the ϵ -coupling of Lys-NH₂. To study the amide bond formation in coacervate droplets, we incubated *N*-acetyl-glycine thioacid (Ac-Gly-SH) (8 mM) with Gly (3 eq.)

in ferricyanide (1 eq.)/ pLys(Me)₃ coacervates dispersion at pH 9. The consumption of Ac-Gly-SH and formation of the ligation product Ac-Gly-Gly-OH was monitored with ¹H NMR (Fig. 4b).

Interestingly, we found a yield of Ac-Gly-Gly-OH of up to 80% after 3 days in the presence of coacervates, while in dilute phase at most 5% of ligation product was observed. In controls with peptides but without ferricyanide, we found no ligation product (Supplementary Fig. 9), and also in the presence of ferrocyanide/pLys coacervate droplets, no ligation product was observed (Supplementary Fig. 10). The significantly higher yield in the presence of coacervates can be attributed to the high local ferricyanide concentration inside coacervate droplets and implies that the ligation reaction takes place predominantly inside the coacervates. Ferricyanide-based coacervate droplets can thus act as microreactors that enhance the rates of oxidative aminoacylation. We found that the ligation reaction was most enhanced in droplets formed from shorter peptides, (Lys(Me)₃)₂₀ and (Lys(Me)₃)₃₀, compared to longer pLys(Me)₃ (Fig. 4c). We attribute this to the lower multivalency of the polycation, which results in weaker complexation with the ferricyanide, making it more available for the reaction with the amino thioacids.

From an origins of life perspective, it is interesting to explore the selectivity of aminoacylation when more than one amino acid can react with an available amino thioacid inside coacervate droplets. Instead of only glycine (Gly), stoichiometric (1:1) competition reactions between glycine and glutamic acid (Glu), Alanine (Ala), and Phenylalanine (Phe) were investigated. All competition reactions demonstrated a significant selectivity for one of the ligation products at pH 9 (Supplementary Table 1). For example, when we added both glycine (12 mM) and glutamic acid (12 mM) to ferricyanide-based droplets (8 mM ferricyanide) containing Ac-Gly-SH (8 mM), we observed a strong selectivity for peptide ligation with glycine. Glutamic acid alone yields the Ac-Gly-Glu-OH dipeptide in similar yields as glycine (Fig. 4d, Supplementary Fig. 11a), but when

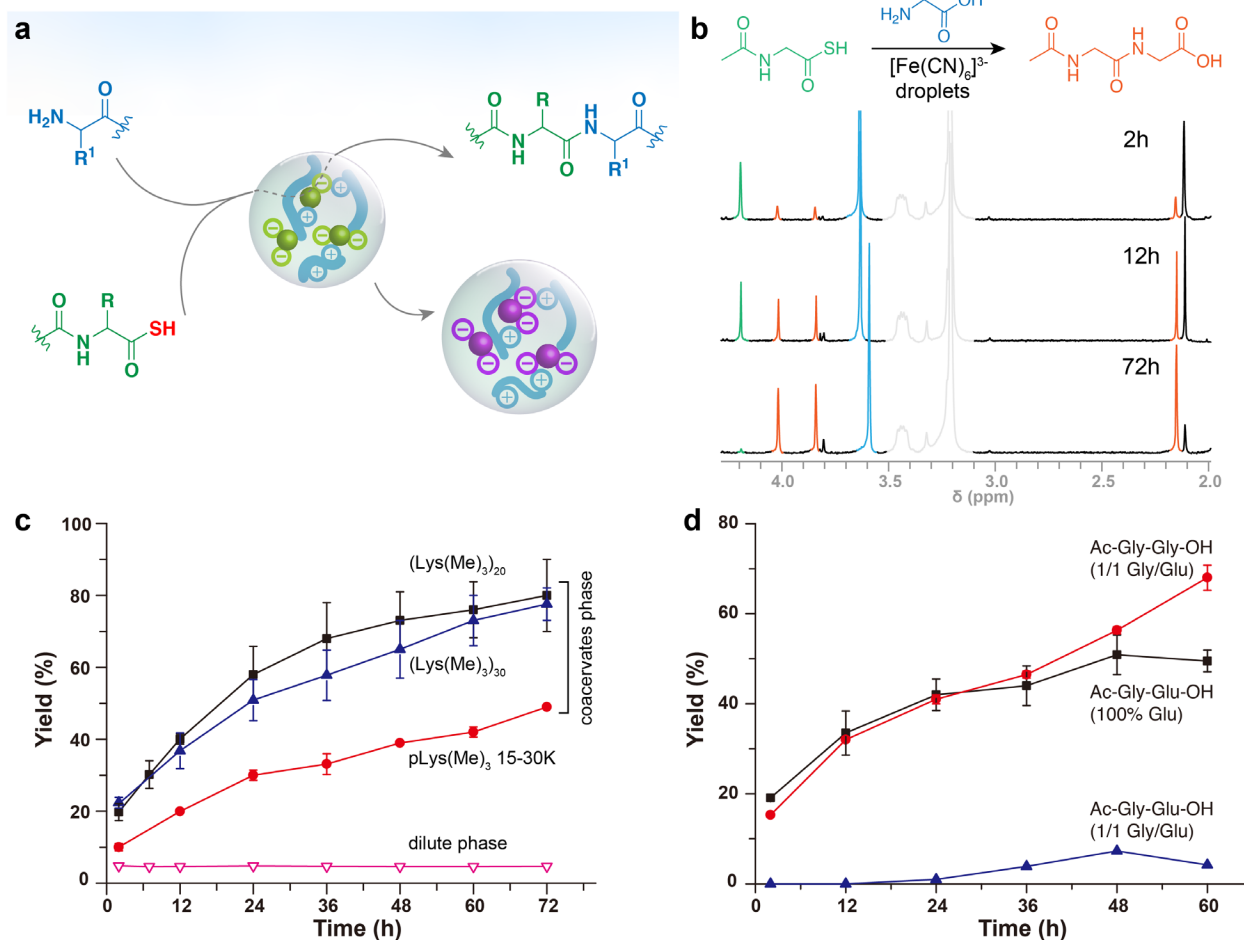


Figure 4. Peptide bond formation in ferricyanide-based droplets. (a) Schematic illustration of ferricyanide-based droplets as microreactors for peptide ligation in water. (b) ^1H NMR spectrum showing the peptide ligation reaction of *N*-acetyl-glycine thioacid Ac-Gly-SH (8mM, green) and Gly-OH (3 equiv., blue) with ferricyanide/plys(Me)₃ (gray) coacervates (1 equiv., pH 9, room temperature) to yield Ac-Gly-Gly-OH (orange). (c) Plot of % ligation products vs time for peptide ligation reaction in dilute phase or ferricyanide-based coacervate droplets. (d) Plot of selective peptide bond formation within coacervate droplets.

glycine and glutamic acid were incubated together in a 1:1 ratio with Ac-Gly-SH in (Lys(Me)₃)₂₀/ferricyanide coacervates, more than 90% of the ligation products was Ac-Gly-Gly-OH (Fig. 4d, Supplementary Fig. 11b). Glycine thus outcompeted glutamic acid very effectively in the ligation process. We reasoned that the activity of glycine inside coacervates is higher than glutamic acid. Although glycine and glutamic acid have similar nucleophilicities,⁴⁵ glutamic acid

is bound more strongly to the cationic lysine residues inside the coacervate and therefore has a lower activity. As a result, glycine reacts faster than glutamic acid inside the coacervate environment and the outcome we observe is an example of kinetic pathway selection, caused by the local protocell environment. Likewise, we observed selective incorporation of glycine in mixtures with alanine (83% Gly/17% Ala) and phenylalanine (78% Gly/22% Phe) (Supplementary Table 1), because the latter have a slightly stronger interaction with the peptide backbone in the coacervates. Alanine is incorporated with slight preference over glutamic acid (60% Ala/40% Glu), but surprisingly, glutamic acid is incorporated more very slightly effectively than phenylalanine (63% Glu/37% Phe).

The products of the above reactions with simple amino (thio)acids are small molecules that interact only weakly with the coacervates. Therefore, they can quickly escape the protocell and the evolutionary advantage for the protocells is limited. To establish a feedback between the formation of amide bonds inside protocells and protocell fitness, we sought to retain the reaction product inside the coacervates. We tested if the scaffold peptides used to form the coacervates could themselves react as nucleophiles with oxidized aminothioacids. We added Ac-Phe-SH or Phe-SH (4 mM) to ferricyanide (4 mM)/ pLys (12 mM) droplets, leading to ϵ -NH₂ ligation of the protocell building blocks (pLys) (Fig. 5a, Supplementary Fig. 12). Anchoring of the aromatic phenylalanine to the pLys scaffold makes the coacervate interior more hydrophobic, and leads to additional cation- π interactions between the coacervate components, both of which result in an expected increase in salt resistance.²⁷ Indeed, we found that ferricyanide/pLys-g-Phe coacervates remain stable far beyond the CSC of the original ferricyanide/pLys coacervates. Fig. 5b shows the turbidity of the coacervates for increasing the salt concentrations. In the control sample, to which we added GSH (4 mM) instead of an amino thioacid to reduce the ferricyanide, the turbidity decreases and

reaches background levels around 180 mM NaCl, where all droplets had disappeared completely (Fig. 5c). In contrast, when Ac-Phe-SH/Phe-SH was added and reacted with the ϵ -NH₂ of pLys, the turbidity transition shifted to a significantly higher salt concentration and the absolute intensity of the plateau at high salt concentration was higher (Fig. 5b). Even at high salt concentrations (220 mM), above the original CSC, microscope images show the presence of gel-like droplets, which do not fuse (Fig. 5c). These condensates have an increased salt resistance and a more gel-like consistency as a result of the attachment of an aromatic residue to the pLys side chains.

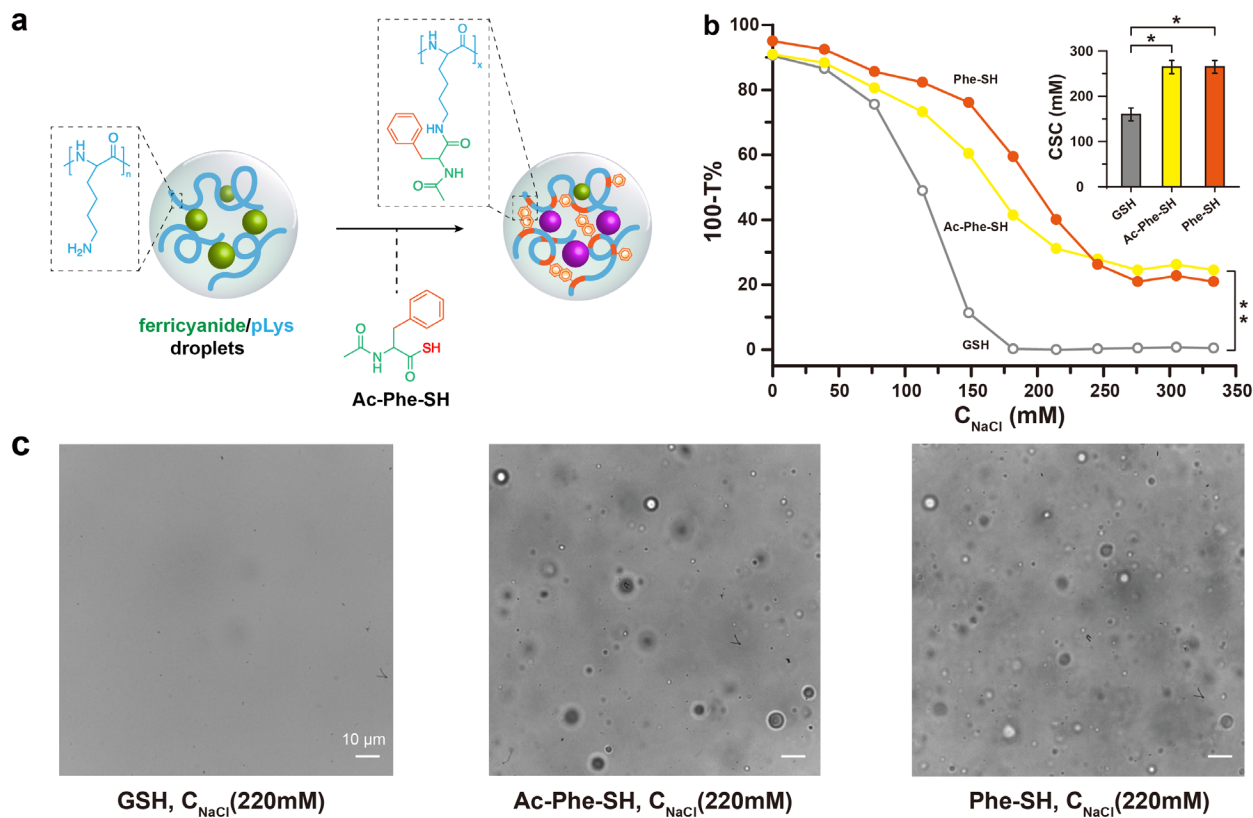


Figure 5. (a) Schematic representation of pLys ϵ -NH₂ ligation in coacervates. (b) Salt resistance of coacervates with Ac-Phe-SH/Phe-SH ligation with droplets building blocks (pLys). Insets show the significantly different CSC, or the turbidity plateau. (c) Optical microscope images illustrating samples corresponding to the high salt concentration (220 mM) points in (b).

Conclusion

In summary, we developed a protocell model based on prebiotically relevant ferricyanide as redox-active species. Membrane-free droplet compartments were spontaneously assembled in the presence of short cationic peptides and the assembly of ferri/ferrocyanide-peptide droplets can be regulated by redox chemistry and salt concentration. Ferricyanide-peptide droplets can act as oxidizing hubs for metabolites, such as NAD(P)H and GSH, filament stacking element like benzoyl cysteine, and amino thioacids as potential prebiotic precursors of amino acids. We demonstrate that the oxidation of amino thioacids by ferricyanide coacervates can be used to drive aminoacylation, resulting in the formation of new peptide bonds. The amino acid ligation is enhanced in coacervate dispersions compared to the surrounding dilute phase due to the local high ferricyanide concentration. The coacervate environment imposes a selection pressure that results in kinetic pathway selection and a strong, preferential incorporation of certain amino acids. Finally, this strategy can be used to create self-reinforcing coacervates, in which hydrophobic amino acid residues are ligated to the coacervate building blocks and enhance their stability. Our results show that prebiotically relevant ferricyanide-based coacervate protocells are versatile oxidizing hubs that exist in aqueous solution, in which metabolites can be sequestered and peptides synthesized. These results provide an important step towards prebiotically plausible integration of chemical processes in cellular compartments.

Methods

A full description of materials and methods used in this work is given in the Supplementary Information.

Acknowledgements

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Additional information

Supplementary information accompanies this paper.

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Competing interests

The authors declare no competing financial interests.

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Supplementary information

Selective amide bond formation in redox-active coacervate protocells

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Materials and methods

Materials

Poly-L-lysine hydrobromide (pLys, 15–30 kDa), potassium ferricyanide, potassium ferrocyanide, glutathione (GSH), DL-dithiothreitol (DTT), tris(hydroxymethyl)aminomethane (Tris), pyranine, and sodium chloride were purchased from Sigma Aldrich and used without further purification. Short polycations (Lys)₁₀, (Lys)₂₀, (Lys)₃₀, (Arg)₁₀ were purchased from Alamanda Polymers. Nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Roche. The fluorescently labeled oligonucleotides poly-A₁₅ (Cy5-A₁₅), Poly-rU₁₅ (rU₁₅-Cy3Sp) were purchased from Integrated DNA Technologies (IDT).

Coacervate formation

Samples for turbidity measurements were prepared directly into 96-well plates, by adding, respectively, Milli-Q water, Tris buffer (pH 7.4, 50 mM), pLys, and ferricyanide or ferrocyanide to a total volume of 100 μ L. Mixing was done by gentle pipetting (3 \times) before each measurement. Samples for the microscopy experiments were prepared in microcentrifuge tubes. After addition of the substrate, a 20 μ L aliquot was immediately taken for imaging on a glass slide.

Turbidity measurements

Turbidity measurements were performed in triplicate using a Berthold Tristar (2) LB 942 microplate reader. The temperature was kept constant at $25 \pm 1^\circ\text{C}$. The absorbance was measured at 520 nm, where none of the mixture components absorbed significantly. The absorbance of a well filled with the same volume of water was used as a blank. Samples were shaken for 5 s before every readout. The critical point was determined by extrapolating the first-order derivative at the inflection point to zero turbidity. Note that this critical salt concentration does not take into account ions from other sources than the added NaCl, and the actual critical ionic strength may be slightly higher.

Ferricyanide and ferrocyanide partitioning

In a typical procedure, the coacervates dispersion was centrifuged in an Eppendorf tube at 3000x g until the dilute phase was transparent under microscope, the coacervate phase had sedimented to the bottom of the cell. The concentration of the ferricyanide/ferrocyanide in the dilute phase was quantitatively analyzed with Uv-vis at wavelength of 320 nm and 420 nm (Supplementary Fig. 5). We measured the volume of the top (dilute) solution and calculated the ferricyanide/ferrocyanide

concentrations of the top solution from the standard curve, the volume and the moles of ferricyanide /ferrocyanide in coacervate phase can be calculated from the total feed, then the ferricyanide and ferrocyanide concentrations inside the coacervate phase can be obtained.

Confocal fluorescence microscopy

Optical and fluorescence microscopy images were recorded on an Olympus UIS2 microscope, equipped with a motorized stage (Prior, Optiscan II). Fluorescent images were recorded with an EMCCD camera (Andor, iXon), using illumination from a mercury lamp, an excitation filter of 482/18 nm (Semrock BrightLine) and an emission filter of 525/45 nm (Semrock BrightLine). Samples were loaded into the wells of PLL-g-PEG-functionalized *Ibidi* μ -slides and closed with a lid (microscopy chambers).

Microscopy chambers preparation

The *Ibidi* μ -slides used for imaging were functionalized with PLL-g-PEG to minimize wetting and spreading of the coacervate droplets. Each slide was first activated by oxygen plasma treatment (Diener electronic, Femto). The PLL-g-PEG (0.01 mg/mL in 10 mM HEPES buffer, pH 7.4) solution was added into each well and incubated at room temperature for 1 h. After that, the glass slides were cleaned by rinsing (3 times with 10 mM HEPS, pH 7.4, 3 times with MQ). The slides were dried by using compressed air.

NMR measurements

Nuclear Magnetic Resonance (NMR) spectra were measured on a Bruker-AVANCE III 400 MHz spectrometer.

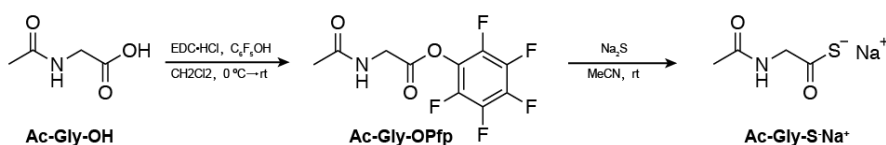
Mass spectrometry

Mass spectra were obtained from a Thermo Scientific™ LCQ Fleet™ ion trap mass spectrometer with Gemini-NX C18 110A 150 x 2.0 mm column and JEOL Accurate Time of Flight (ToF) instruments, both using linear ion trap electrospray ionisation (ESI).

Synthesis of trimethylated oligolysine and poly-L-lysine

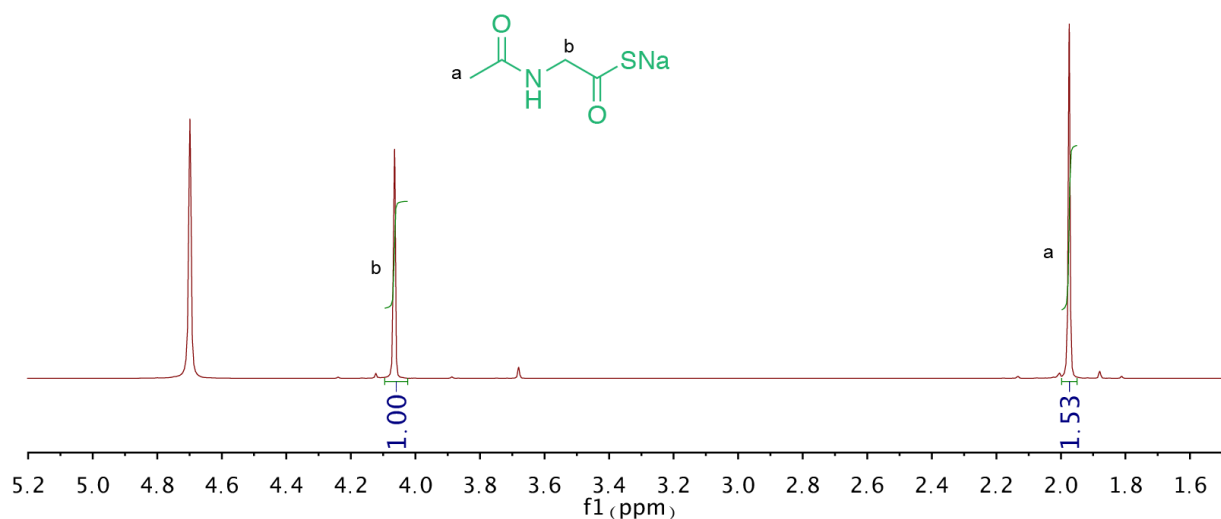
Trimethylated oligolysines (Lys(Me)₃)_n and poly-L-lysine (pLys(Me)₃) were prepared from (Lys)_n and pLys, according to literature methods.³ Briefly, 2 mL DMS was added to 50 mg (Lys)_n, in 20 mL H₂O and 6 mL ethanol. The pH was adjusted to 9.5 and maintained by the addition of 1 M NaOH. The reaction was considered complete when the pH remained nearly constant. The pLys(Me)₃ was further purified by dialysis against 2 times 1 L of a 2 M NaCl aqueous solution and subsequently, 3 times 1 L of water in a dialysis membrane (MWCO, 3500). After that, the residue was freeze-dried to yield a white powder.

Synthesis of sodium 2-acetamidoethanethioate Ac-Gly-S⁻Na⁺

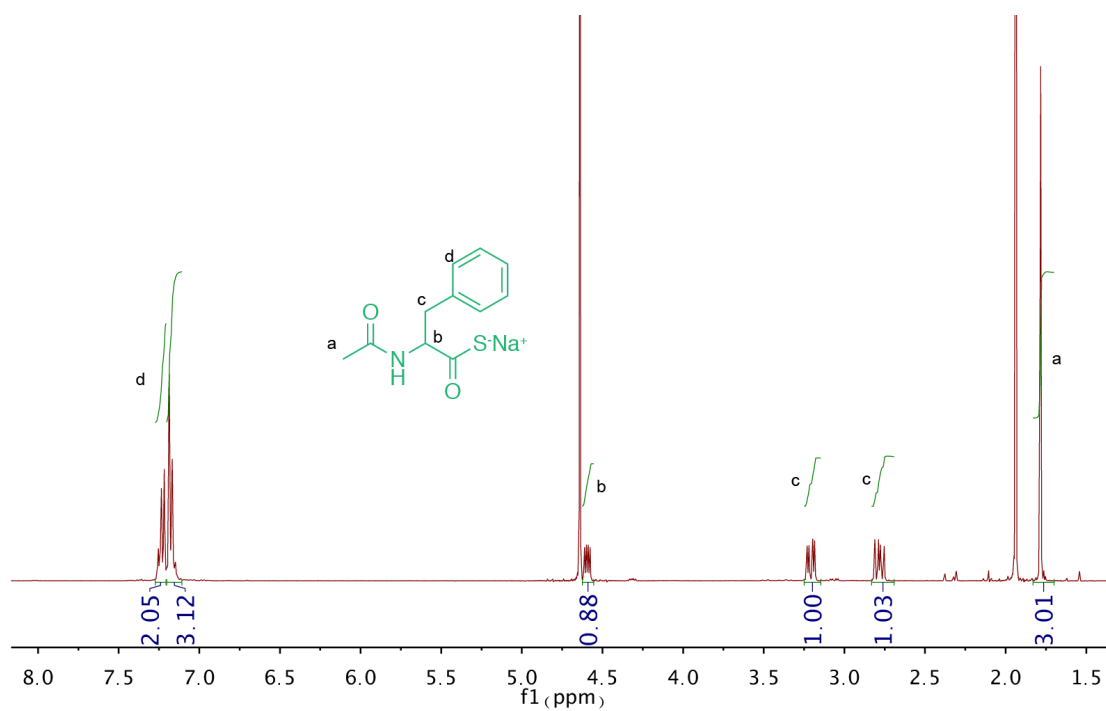


Ac-Gly-S⁻Na⁺, Ac-Phe-S⁻Na⁺ and Phe-SH were synthesized according to literature methods (Supplementary Fig. 1-3).¹⁻² In brief, for **Ac-Gly-S⁻Na⁺**, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl) (3.45 g, 18.00 mmol) was added to a stirring solution of *N*-acetylglycine **Ac-Gly-OH** (943 mg, 6.00 mmol) and pentafluorophenol (1.22 g, 6.60 mmol) in CH₂Cl₂ (25 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 16 h. The resultant homogenous solution was diluted with CH₂Cl₂ (20 mL) and washed

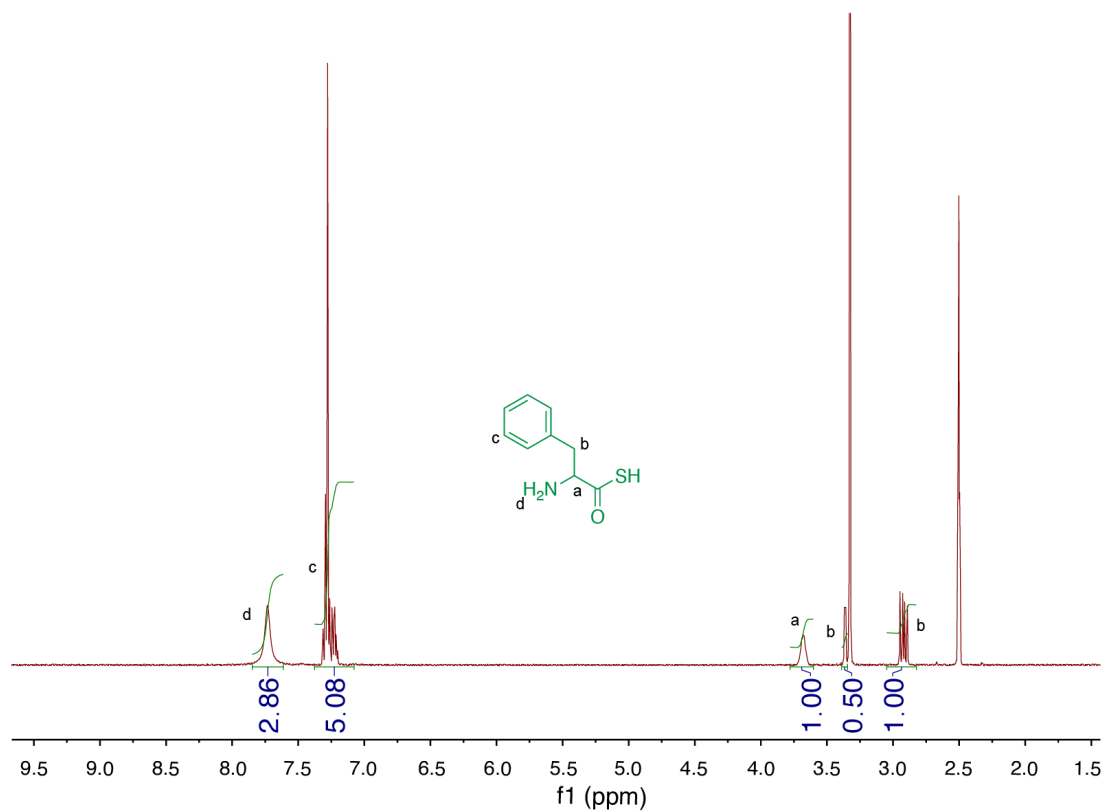
with water (2 x 20 mL), NaHCO₃ (sat., 2 x 20 mL) and brine (20 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated in vacuo to give *N*-acetylglycine pentafluorophenyl ester **Ac-Gly-OPfp**, which was used immediately without further purification. The crude **Ac-Gly-OPfp** was resuspended in anhydrous acetonitrile (15 mL) and stirred vigorously with anhydrous sodium sulfide (1.1 equiv) under argon atmosphere for 6 h at room temperature. The resultant precipitate was isolated by centrifugation and washed with diethyl ether (3× 10mL) and lyophilized to yield **Ac-Gly-S⁻Na⁺** as a white solid (350 mg). ¹H NMR (400 MHz, D₂O): d 4.08 (s, 2H, (C2)–H₂), 1.97 (s, 3H, (COCH₃)). HRMS-ESI [M–H][–] calcd. for [C₄H₇NO₂S–H][–]: 132.0119; observed: 132.0117.



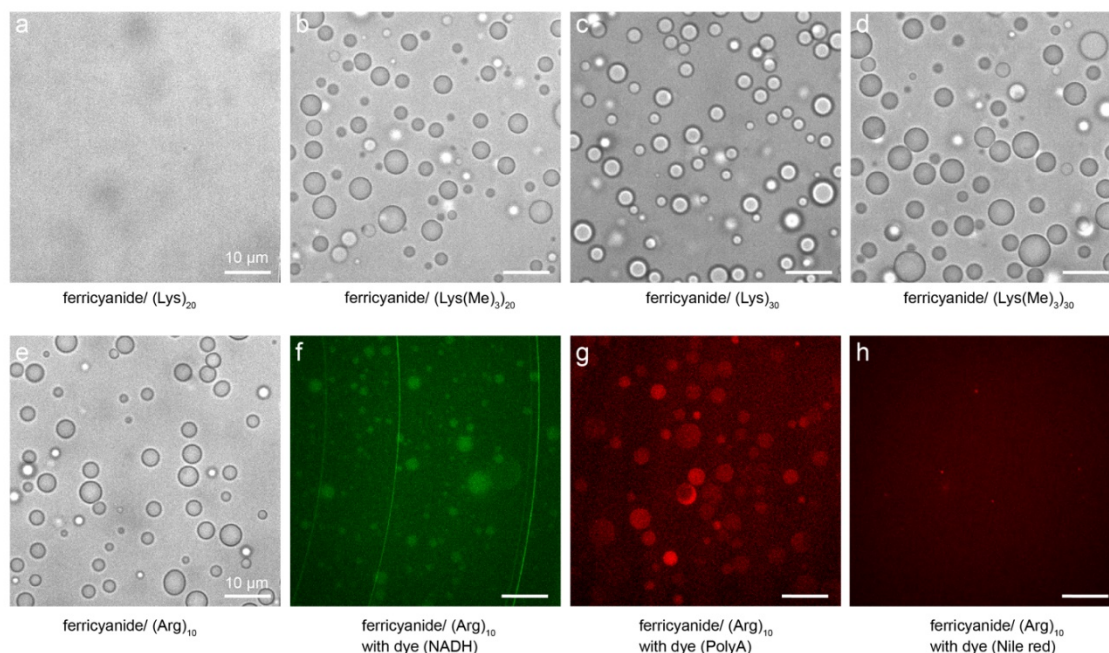
Supplementary Figure 1. ¹H-NMR spectrum of Ac-Gly-S⁻Na⁺ (400 MHz, D₂O).



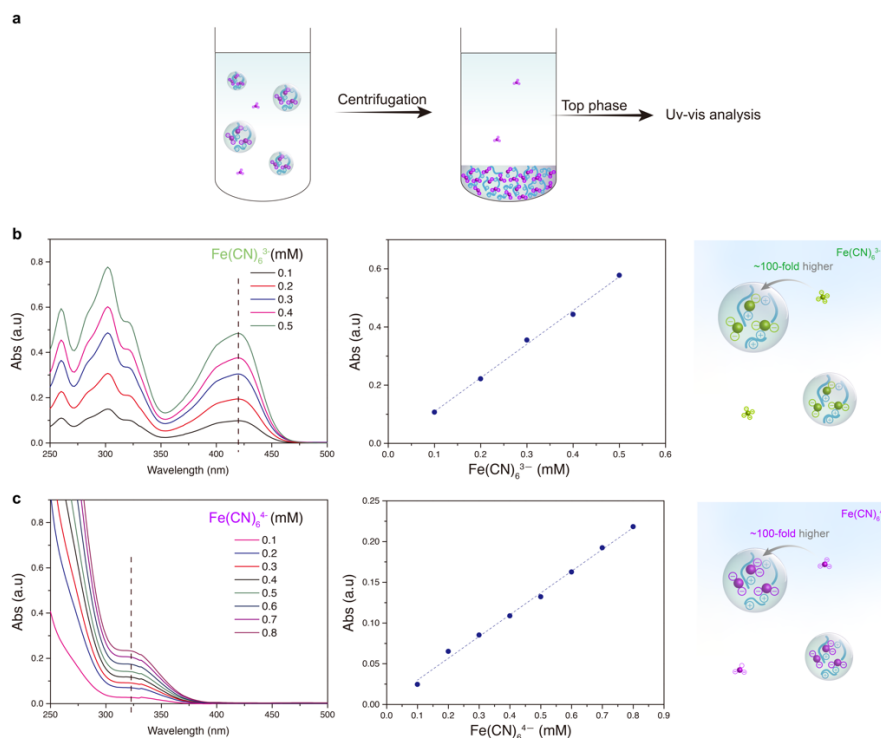
Supplementary Figure 2. ¹H-NMR spectrum of Ac-Phe-SNa⁺ (400 MHz, D₂O)



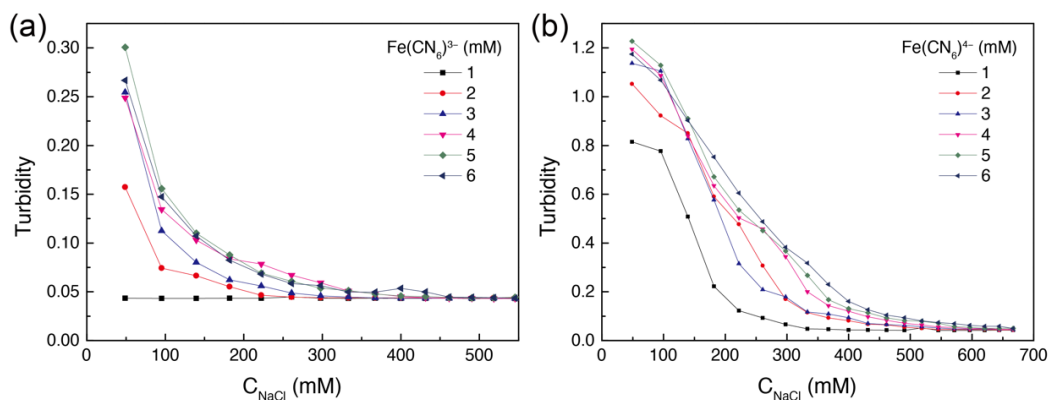
Supplementary Figure 3. ¹H-NMR spectrum of Phe-SH (400 MHz, DMSO-D₆).



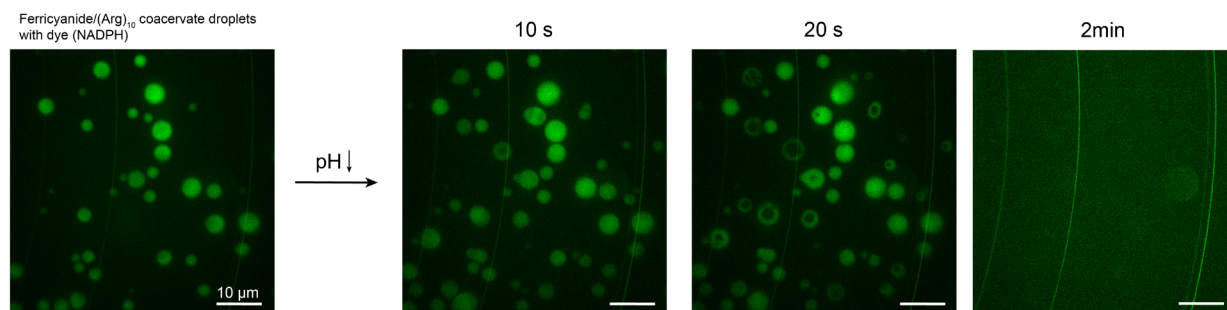
Supplementary Figure 4. Optical microscope images of ferricyanide/(Lys)₂₀ (a), ferricyanide/(Lys(Me)₃)₂₀ (b), ferricyanide/(Lys)₃₀ (c), ferricyanide/(Lys(Me)₃)₃₀ (d), and ferricyanide/(Arg)₁₀ (e) coacervates. Fluorescence images of ferricyanide/(Arg)₁₀ coacervate droplets selectively uptake of NADH (f), Poly-A₁₅ (Cy5-A₁₅) (g), and Nile red (h).



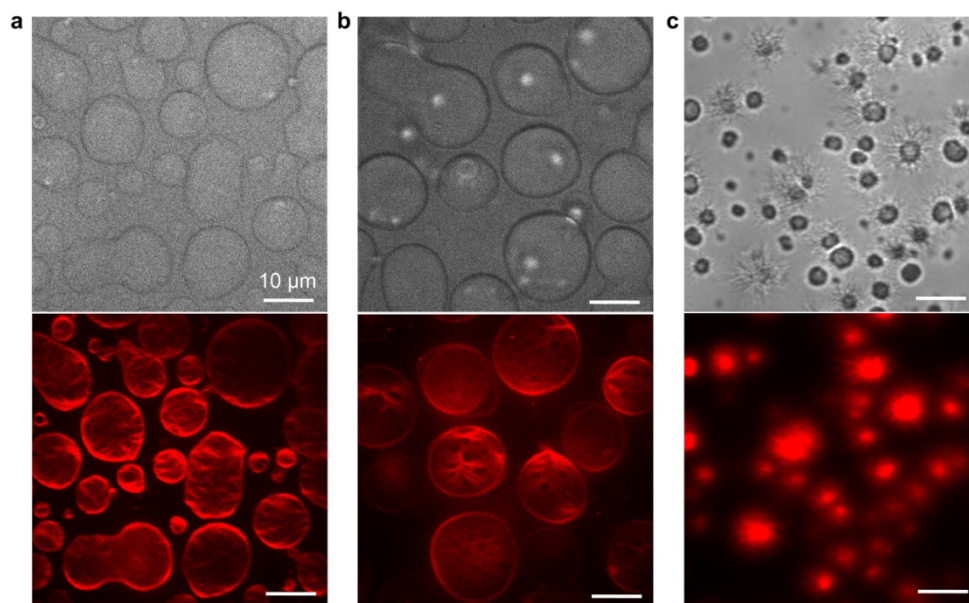
Supplementary Figure 5. (a) Scheme of the method to determine the ferricyanide concentrations inside and outside the droplet phase. (b, c) UV-vis spectra of the ferricyanide or ferrocyanide concentration in top phase at different concentrations, and the corresponding standard curve of the ferricyanide/ferrocyanide absorbance. We calculated the ferricyanide and ferrocyanide concentrations of the top solution from the standard curve, and then the ferricyanide and ferrocyanide concentrations inside the coacervate phase can be known.



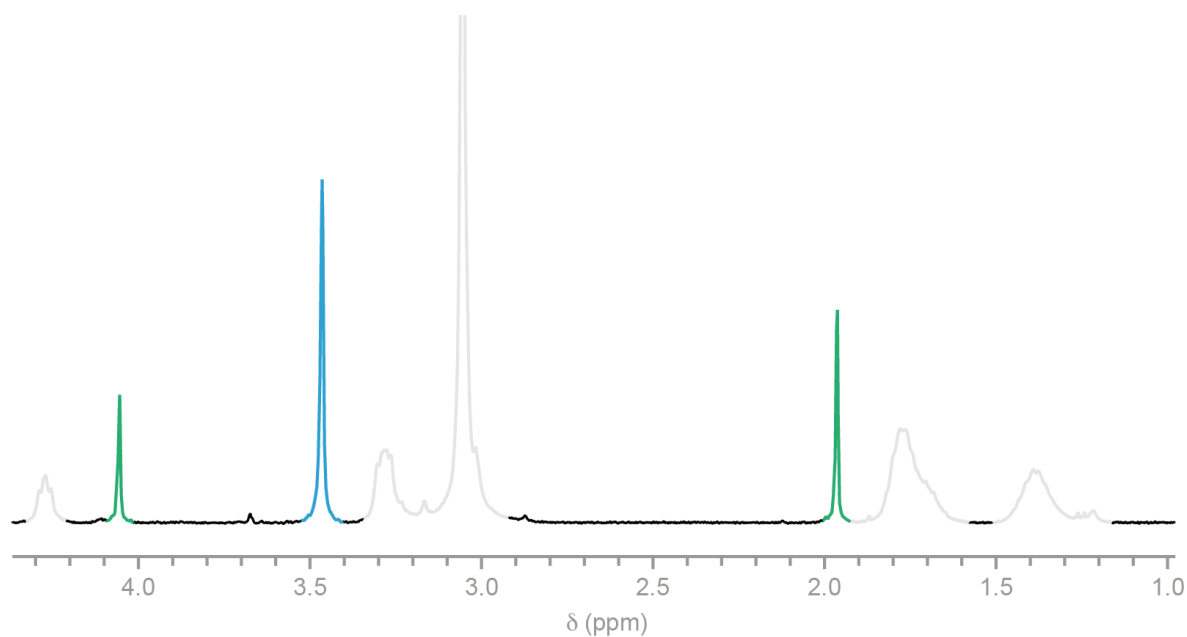
Supplementary Figure 6. Turbidity of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ -pLys mixtures as a function of concentration of added NaCl (a and b). The mixtures contained a fixed concentration of 50 mM Tris buffer, 5 mM pLys (monomer units). The mixtures containing ferricyanide were titrated with NaCl 0.5 M, while the mixtures containing Ferrocyanide were titrated with NaCl 2 M.



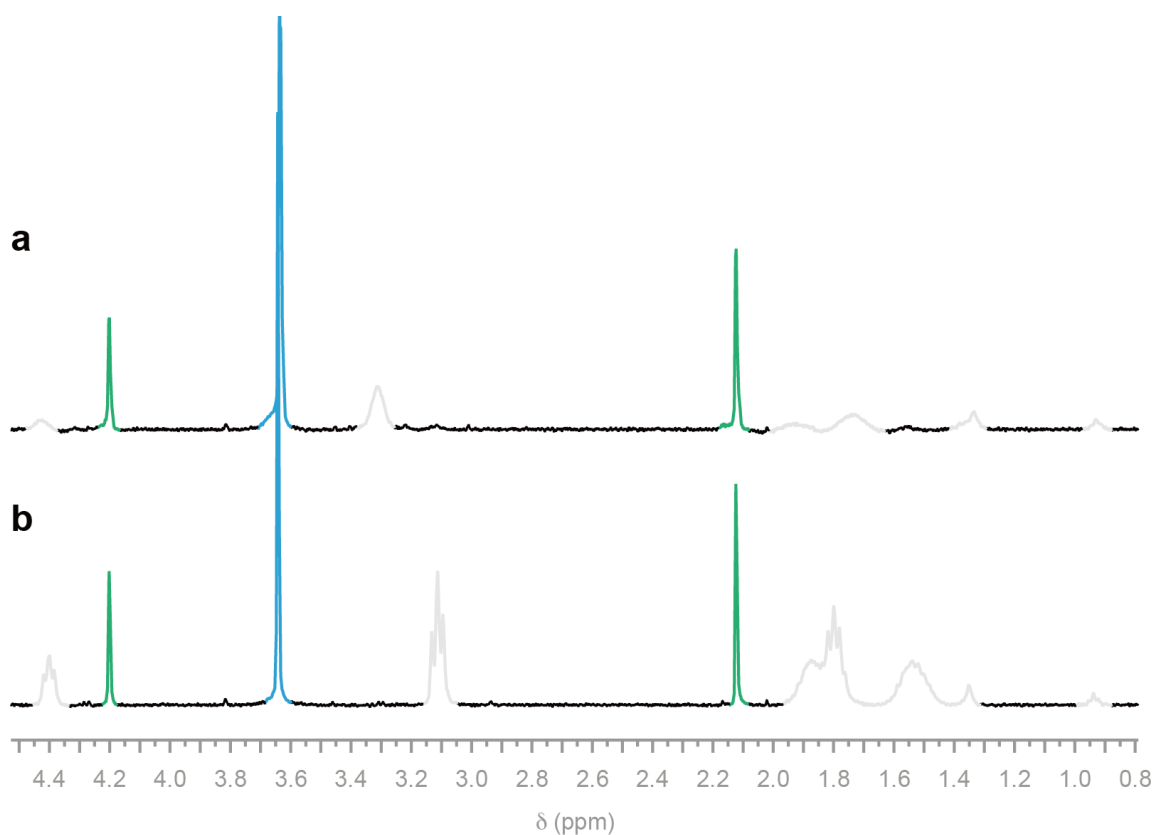
Supplementary Figure 7. Confocal micrographs of ferricyanide/(Arg)₁₀ protocells (stained with NADH), time series of representative protocells after pH-triggered redox reaction.



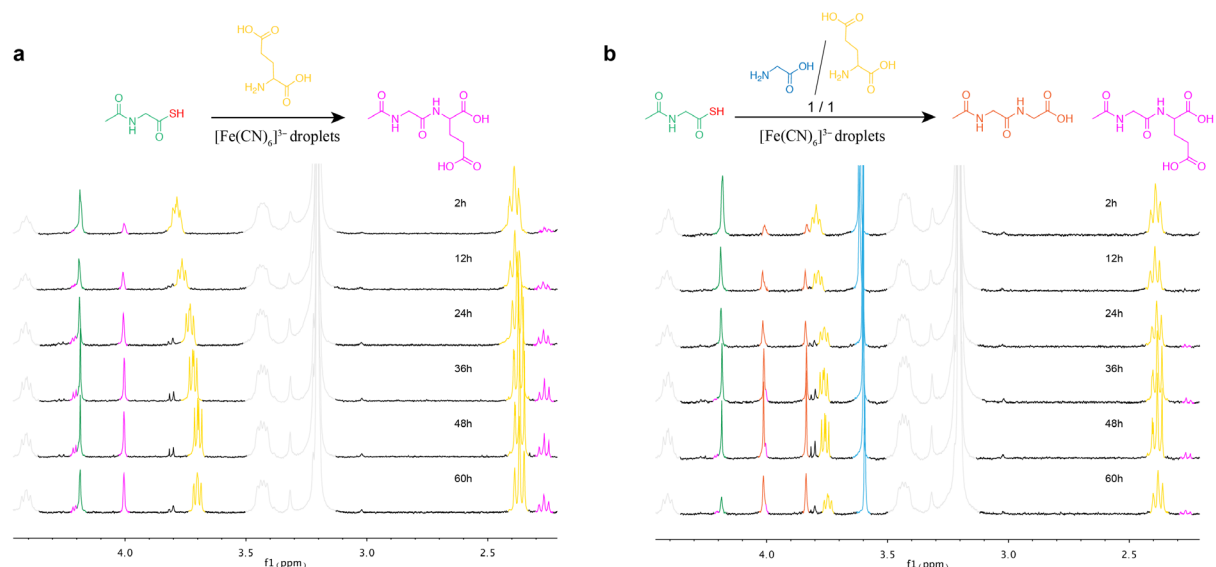
Supplementary Figure 8. Confocal micrographs of ferricyanide/Lys(Me)₃₂₀ (a), ferricyanide/Lys(Me)₃₃₀ (b), and ferricyanide/Lys₃₀ (c) protocells (with dye, Nile red) after addition of BC. Scale bar, 10 μm .



Supplementary Figure 9. ^1H NMR spectrum of peptide ligation reaction in control solution without ferricyanide, no ligation product observed after 3 h. (Ac-Gly-SH (8mM, green), Gly (3 equiv., blue), (ferrocyanide, 6mM), ((Lys(Me)₃)₃₀, gray)).



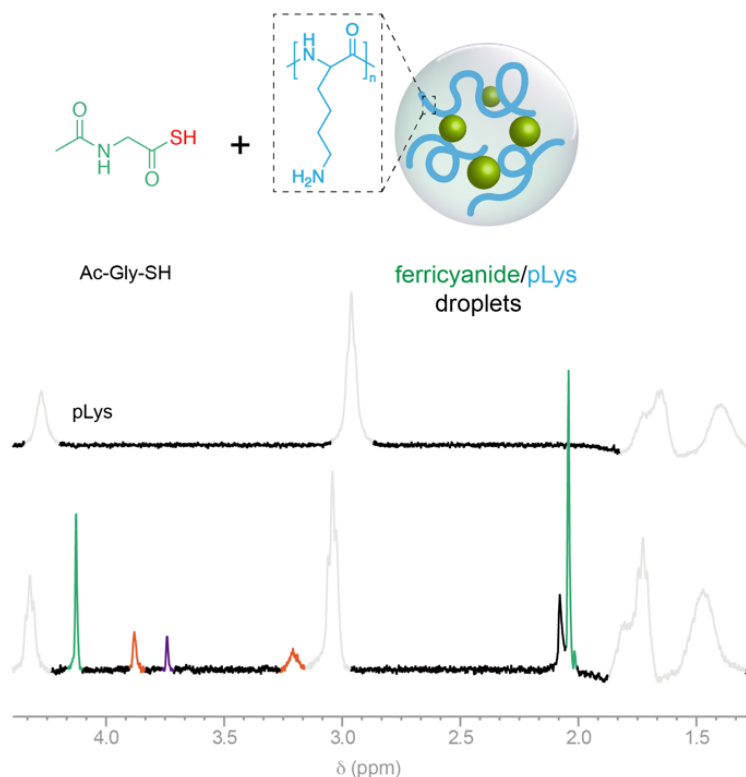
Supplementary Figure 10. ^1H NMR spectrum of peptide ligation reaction in ferrocyanide/(Arg)₁₀ (a) and ferrocyanide/(Lys)₂₀ protocells, no ligation product observed after 3 h. (Ac-Gly-SH (8mM, green), Gly (3 equiv., blue), (ferrocyanide, 6mM), (polycations, gray)).



Supplementary Figure 11. (a) Time series of ^1H NMR spectrum of peptide ligation reaction in ferricyanide/ $(\text{Lys}(\text{Me})_3)_{20}$ protocells. (Ac-Gly-SH (8 mM, green), Glu (24 mM, yellow), (ferricyanide, 8 mM), (polycations, gray)). (b) Time series of ^1H NMR spectrum of peptide ligation reaction in ferricyanide/ $(\text{Lys}(\text{Me})_3)_{20}$ protocells. (Ac-Gly-SH (8 mM, green), Glu (12 mM, yellow), Gly (12 mM, blue) (ferricyanide, 8mM), (polycations, gray)).

Supplementary Table 1. Yields for the products of prebiotic oxidative coupling of α -aminoacetyl thioacid **Ac-Gly-SH** (8 mM) with amino acid mixture **AA₁/AA₂** (ratio 1:1, 12 mM: 12 mM) and Ferricyanide (8 mM)/ $(\text{Lys}(\text{Me})_3)_{20}$ (Lys monomer 24 mM) coacervates dispersion, pH 9, unless stated otherwise.

Amino Acid mixture AA ₁ :AA ₂ 1:1	Yield ratio Ac-Gly-AA ₁ -OH(%) : Ac-Gly-AA ₂ -OH(%)	
	Time (h)	
	4 h	60 h
Gly:Glu	10:0	10:1
Gly:Ala	6:1	5:1
Gly:Phe	4.8:1	3.5:1
Ala:Glu	1.7:1	1.5:1
Glu:Phe	3.5:1	1.7:1



Supplementary Figure 12. ^1H NMR spectrum showing the thioacid ligation reaction of Ac-Gly-SH (8 mM, green) with ferricyanide (8 mM)/pLys (15-30K, Lys monomer 24 mM, gray) coacervates to yield pLys ϵ -NH $_2$ ligation (orange).

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