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Programmable On-Chip Artificial Cell Producing Post-Translationally Modified Ubiquitinated Protein

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In nature, intracellular microcompartments have evolved to allow the simultaneous execution of tightly regulated complex processes within a controlled environment. This architecture serves as the blueprint for the construction of a wide array of artificial cells. However, such systems are inadequate in their ability to confine and sequentially control multiple central dogma activities (transcription, translation, and post-translational modifications) resulting in a limited production of complex biomolecules. Here, an artificial cell-on-a-chip comprising hierarchical compartments allowing the processing and transport of products from transcription, translation, and post-translational modifications through connecting channels is designed and fabricated. This platform generates a tightly controlled system, yielding directly a purified modified protein, with the potential to produce proteoform of choice. Using this platform, the full ubiquitinated form of the Parkinson's disease-associated α-synuclein is generated starting from DNA, in a single device. By bringing together all central dogma activities in a single controllable platform, this approach will open up new possibilities for the synthesis of complex targets, will allow to decipher diverse molecular mechanisms in health and disease and to engineer protein-based materials and pharmaceutical agents.

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In living cells proteins function together as the basic machinery of life. The production of functional proteins can be partitioned to specific hierarchical steps known as central dogma activities, comprising transcription, translation, and post-translational modifications (PTMs). These highly coordinated biochemical processes rely on the subdivision of the reactions into different cellular compartments, allowing precise control over the final products. In particular, site-specific PTMs, such as phosphorylation, glycosylation, and ubiquitination, allow to further extend the functionality of proteins by yielding a wide range of protein variants consisting of the same amino acid sequences. While this set of discrete reactions has naturally evolved over millions of years, synthetic approaches aimed to allow such control have been recently developed to generate cell-free artificial cell platforms.[1–4]

One of the main advantages of cell-free systems (CFSs) is the reduction of the intricate cellular environment into its essential components.[5,6] Since their original impact in elucidating the genetic code,[7] CFSs have been proven as an effective method to address fundamental questions in biology.[8] Moreover, CFSs have been used as a powerful synthetic biology tool allowing the production of complex molecules beyond those produced by purely synthetic chemistry, leading to the development of unprecedented therapeutic and diagnostic applications.[9–14] However, utilizing artificial cells as fully compartmentalized and controlled platforms, allowing to spatially and temporally segregate central dogma activities, remains challenging. Specifically, current artificial cell systems lack the ability to perform PTMs. When PTMs are synthetically introduced in an indiscriminate manner, their effect on a single protein is highly difficult to profile and characterize.[15] Moreover, PTMs vary between different organisms, and while human proteins can be exogenously expressed in various in vivo models, such modifications may alter the expressed proteins characteristics compared to their original form.[16]

Here, we present an artificial-cell-on-chip engineered in a modular and programmable manner to control each of the central dogma activities. The new compartmentalized central dogma activities artificial cell (CONTRALL) system allows the production of modified proteins by programming transcription,

translation, and PTMs in a discrete and highly precise manner (**Figure 1**). Using this approach, we demonstrate decoupling of transcription from translation and control over the translation time of specific RNA transcripts. In addition, decoupling these processes allows selective and simple control over transcription activation, thus making screening of synthetic promotor libraries or designing complicated genetic circuits redundant. By integrating simple biosynthesis techniques, we further extend this approach to purify desired proteins on-chip, allowing dynamic modulation of PTMs of choice. We explored the ubiquitination of α-synuclein, a protein with notable aggregation propensity that is associated with Parkinson's disease (PD), characterized the reaction products and identified the ubiquitination sites. Our results reveal that by modulating the substrates used in the reaction, the CONTRALL platforms allows for precise PTMs to be incorporated within the protein sequence in a selective manner. This dynamic approach shows promise in revolutionizing current CFS methods currently used and could be applied to a wide range of protein-based systems.

To allow hierarchical implementation of transcription, translation, and PTMs processes, we utilized a microfluidics-based approach to reduce reagent usage and generate highly controlled microcompartments comprising unique engineered features. In the CONTRALL biochip (Figure 1), each process takes place within a different microcompartment and the product is isolated or allowed to move into the subsequent compartment by a series of engineered microfluidic valves, used as ON/OFF switches, and size secluding pillars, which act as physical barriers for targeted elements (**Figure 2**a). First, aiming to recapitulate the natural eukaryotic cell process, we sought to decouple transcription from translation.[17] We developed a biochip comprising of two confined parallel transcription compartments with a set of controlled mechanical promotor-like valves (Figure 2a), thereby allowing control of transcription initiation and strength. The compartments were followed by a detection channel where mango light-up RNA aptamer could be detected (Figure 2a). To localize and segregate transcription in a confined environment, we used PDMS based lithographically defined elements to trap Ni beads bound to His-tagged T7 RNA polymerase (T7RP), thereby immobilizing the enzyme and preventing its transfer to downstream compartments (Figure 2a; Figure S1, Supporting Information). To verify sufficiently effective and accurate transcription by the immobilized T7RP, we employed the biochip to transcribe mango light-up RNA aptamer, which generates a fluorescent signal only in the presence of a specific fluorogene when its secondary structure remains active.[18] Following transcription, we have been able to detect a fluorescent signal from the reaction flow-through, demonstrating correct transcription by immobilized T7RP (Figure 2b). Expanding on this approach, we have been able to transcribe two different RNA light-up aptamers in two parallel compartments. By alternatively modulating the flow rate and valve shuttering of the two compartments, we developed an oscillator switch of RNA transcripts (Figure 2c). By selectively transferring different volumes of the RNA transcript solution, we demonstrated the controlled accumulation of specific RNA transcripts in the collected fraction, thus mimicking mechanically promotor strength (Figure 2d). The immobilization of T7RP holds great potential for re-using the transcription compartment with different genes.

Following the establishment of confined and controlled transcription processes, we aimed to mimic nuclear transport, a key feature in all eukaryotic cells, comprising the tightly regulated and highly selective mobilization of RNA transcripts from the nucleus to the cytoplasm.[17] First, we verified the ability of the immobilized T7RP to transcribe from both biotinylated and nonmodified DNA template molecules (Figure 2b). Following transcription, the solution containing RNA molecules, biotinylated DNA, and other essential components was flown into a downstream channel comprised of immobilized streptavidin (SA) beads, which bound the biotinylated DNA and allowed RNA transcripts to continue flowing into the translation compartment (**Figure 3**a).

To avoid introducing additional background noise during the translation process, we used cell-free protein synthesis kit, PUREfrex, which comprises untagged purified translation machinery components of *Escherichia coli* with reduced contaminations.[19] These components were directly introduced into the translation compartment via an inlet (Figure 3a). Following transcription and RNA mobilization into the translation compartment, RNA molecules encoding His-tagged green fluorescent protein (eGFP) encountered the PUREfrex components, facilitating the translation process. The newly translated His-eGFP was immobilized using Ni beads trapped inside the compartment. Following translation, the compartment was washed to remove any remaining RNA molecules and translation components, while the His-eGFP remained trapped. His-eGFP was then eluted from the CONTRALL biochip using an imidazole-based solution. Using fluorescence spectrum and Western blot analysis His-eGFP was detected in both the translation and elution solutions, but not in the flow-through fraction, indicated that most of the translated protein was trapped within the CONTRALL biochip (Figure 3b,c; Figure S2, Supporting Information). The CONTRALL protein yield is $\approx$24 μg mL^{-1} per cycle.

After establishing the ability to both translate and immobilize a protein of choice in a controlled environment, we proceeded to perform site-specific PTMs by enzymatic means. PTMs allow to further extend the functionality of proteins by yielding a wide range of protein variants consisting of the same amino acid sequences. Implementing PTMs compartment as part of the CONTRALL platform will potentially allow to produce different proteins caring various PTMs. As a model protein, we used α-synuclein, a natively unfolded protein, shown to be linked to Parkinson's disease (PD).[20] Understanding the molecular mechanisms underlying α-synuclein aggregation and identifying α-synuclein's modification sites could result in more effective therapeutic strategies. α-synuclein has been shown to be mono- and poly-ubiquitinated by the E3 ubiquitin ligase, Hsp70-interacting protein (CHIP).[21] CHIP E3 ligase was shown to reduce α-synuclein oligomerization and mediate the degradation of misfolded proteins associated with Alzheimer disease, Huntington and PD.[22] However, the ubiquitination sites in which CHIP E3 ubiquitinates α-synuclein have not been identified to date.[21,22] Here, His-α-synuclein was translated and immobilized onto Ni beads in the translation compartment of CONTRALL as detailed above. Following a washing step, we introduced in vitro ubiquitination

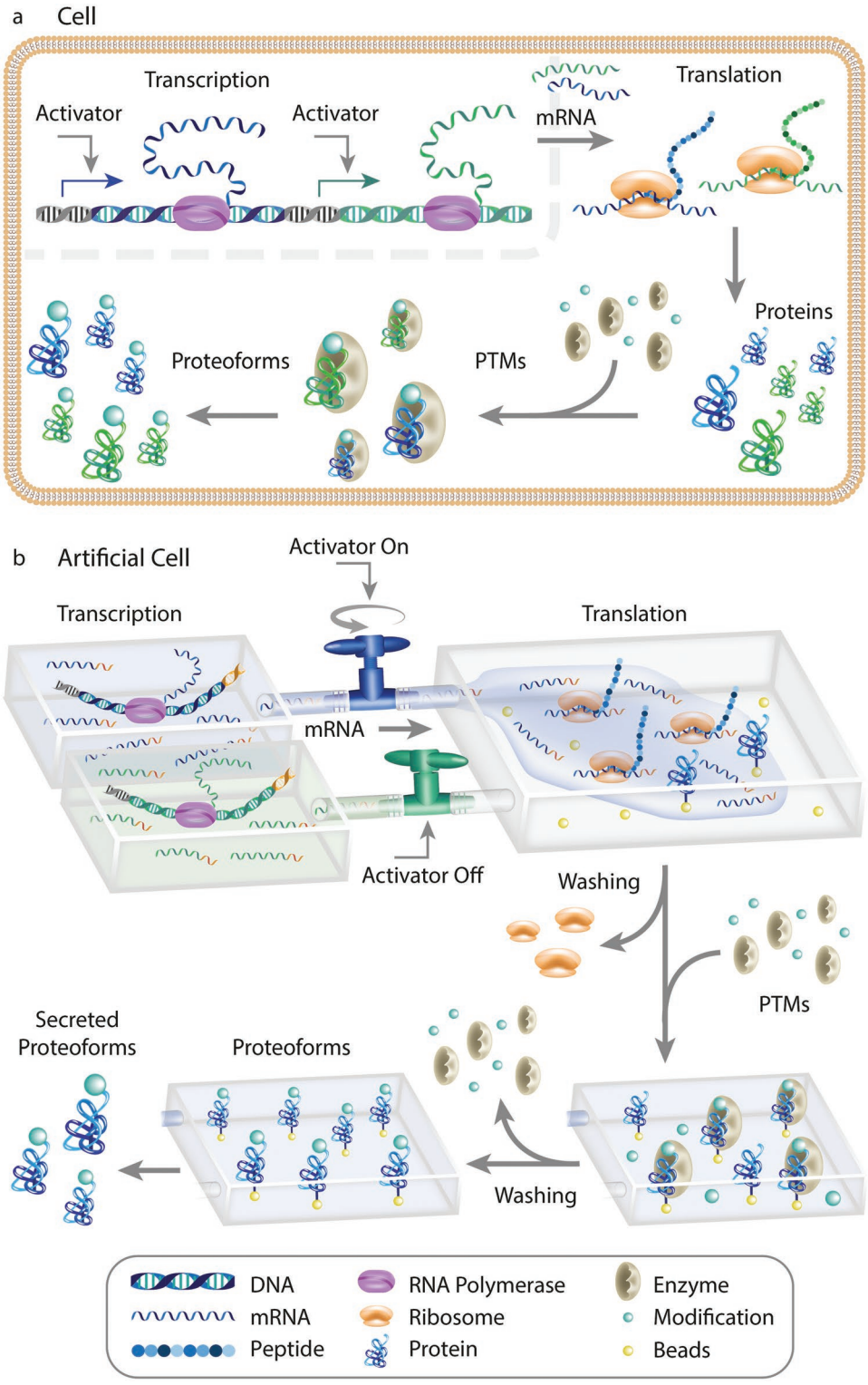

Figure 1. Cellular processing of transcription, translation and PTMs and their compartmentalization in the CONTRALL platform. a) Illustration of eukaryotic cell compartmentalized expression process of two genes in response to promotor activation. Following transcription in the nucleus (dashed grey line), RNA transcripts are transported to the cytoplasm, where they undergo translation and PTMs. b) Schematics of the CONTRALL platform showing parallel transcription of two genes encoding for His-tagged proteins at two spatially separated compartments. Transcription is performed by an immobilized T7RP trapped in the compartments. Closing the mechanical valve following transcription allows RNA transcripts from either one compartment or both to flow into a downstream compartment. DNA molecules are then immobilized, while RNA molecules encounter cell-free translation components. The resulting translated His-protein is immobilized using trapped Ni beads, allowing the removal of translation components and the subsequent introduction of PTMs enzymes of choice. Following incubation, PTMs enzymes are washed and the purified proteoform is eluted.

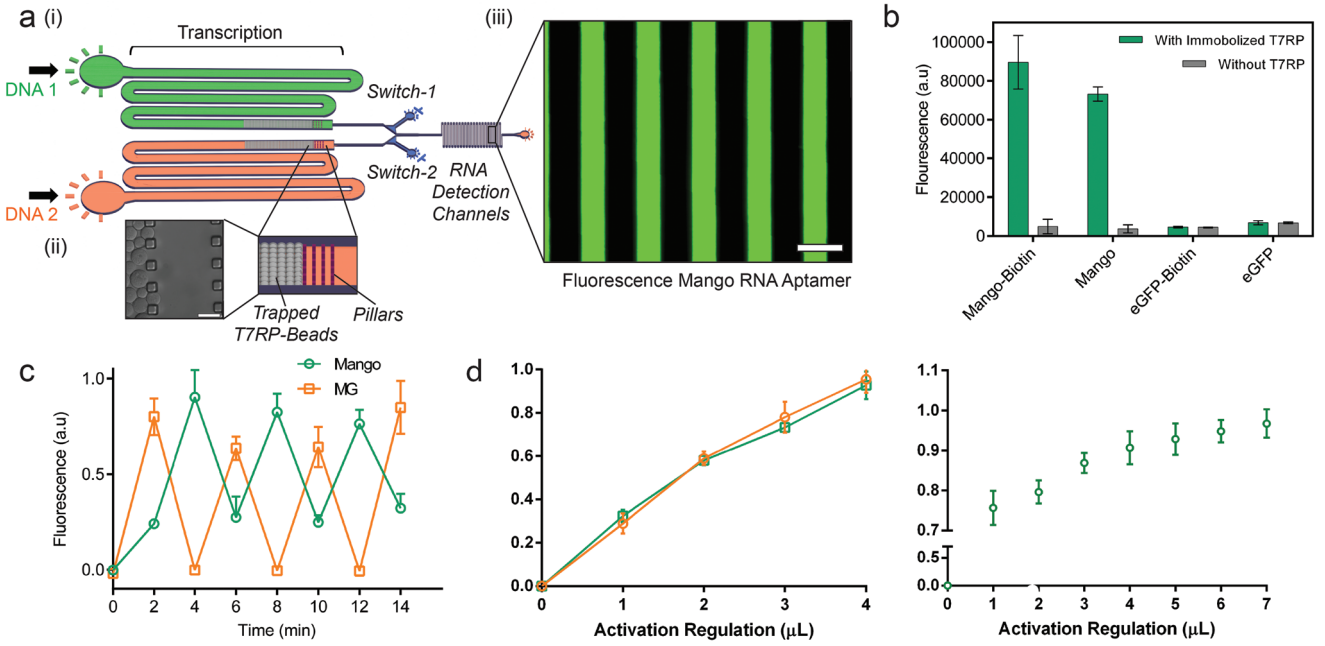

Figure 2. Decoupling of transcription and translation by mechanical promotor-like valves. a): i) Schematics of the two parallel transcription compartments. ii) Schematics and bright field imaging of His-T7RP immobilized onto Ni beads trapped by pillars in the transcription compartments. Scale bar, 50 μm. iii) Fluorescent image of mango light-up RNA aptamer in the detection channel. Scale bar, 100 μm. b) Fluorescence measurements of mango RNA aptamer and eGFP transcripts utilizing immobilized T7RP, using both biotinylated and nonbiotinylated PCR products as templates. c) Alternations in two controlled mechanical promotor-like valves resulting in an oscillating switch. d) Gradual accumulation of RNA transcripts demonstrating control over promotor strength of either a single (right) or two different (left) RNA aptamer transcripts.

components including CHIP E3 ligase. Following increased incubation times, the compartment was washed, yielding purified immobilized α-synuclein, which was eluted in a similar manner to His-eGFP. To validate the formation of ubiquitinated α-synuclein, Western blot analysis was applied, indicating that the newly formed α-synuclein proteoforms were both mono- and polyubiquitinated (Figure 3c). Using mass spectrometry, we were able to identify, for the first time, the CHIP E3 ligase ubiquitination sites on α-synuclein proteoforms (Figure 3d). In parallel, microfluidic diffusional sizing was used to determine directly in the solution state under native conditions the average hydrodynamic radius of the resulting ubiquitinated α-synuclein. The results demonstrate a time-dependent increase in the hydrodynamic radius of the ubiquitinated α-synuclein, compared to pure ubiquitin or α-synuclein (Figure 3e), directly reporting on the increased ubiquitination over time. To the best of our knowledge, this is the first artificial cell mimicking the production of a purified post translationally modified protein of choice.

In conclusion, the artificial cell-on-a-chip presented here allows to perform central dogma activities in a confined and precisely regulated manner, thereby overcoming some of the major limitations of current synthetic biology approaches, such as controlled transcription and translation. We have successfully decoupled and confined transcription from translation, allowing regulation of transcription initiation and established an oscillating switch. In the future, the parallelization of multiple compartments can be applied for other central dogma activities, including translation and PTMs. In addition, we were able to translate α-synuclein, modify it with specific PTMs and characterize the resulting proteoforms, thereby identifying, for the first time, the ubiquitination sites of CHIP E3 ligase. The newly developed CONTRALL platform has thus been shown to allow tight regulation of the central dogma activities, which has previously been unattained using living systems. This platform can expand the current biological tool kit for the identification of PTMs sites. In addition, the possibility to add additional compartments to CONTRALL can potentially lead to the integration of other eukaryotic processes such as RNA splicing, thus further advance the field of artificial cells. We applied microfluidics technology for the miniaturization and parallelization of experimental methodologies, granting significant advantages to control a wide range of in vitro processes and to study the effect of individual steps in protein expression and modifications.[23] Microfluidic approaches have been shown to be highly flexible in their design, allowing the adaptable coupling of a wide range of strategies into a single device. Thus, currently available approaches, such as on chip separation based on protein hydrodynamic radii[24] could be readily integrated within the CONTRALL platform to first allow the generation of a wide range of molecular species[25] and the subsequent on-chip characterization of their interactions in solution. Similarly, the aggregation process of proteins related to amyloid disorders can be studied in detail. Specifically, controlling PTMs and exploring the aggregation kinetics of different proteoforms by utilizing microdroplet-based approaches.[26] Furthermore, the CONTRALL platform facilitates the previously unattainable specific PTMs. By systematically introducing PTMs at specific sites, the products of the CONTRALL platform will allow us to

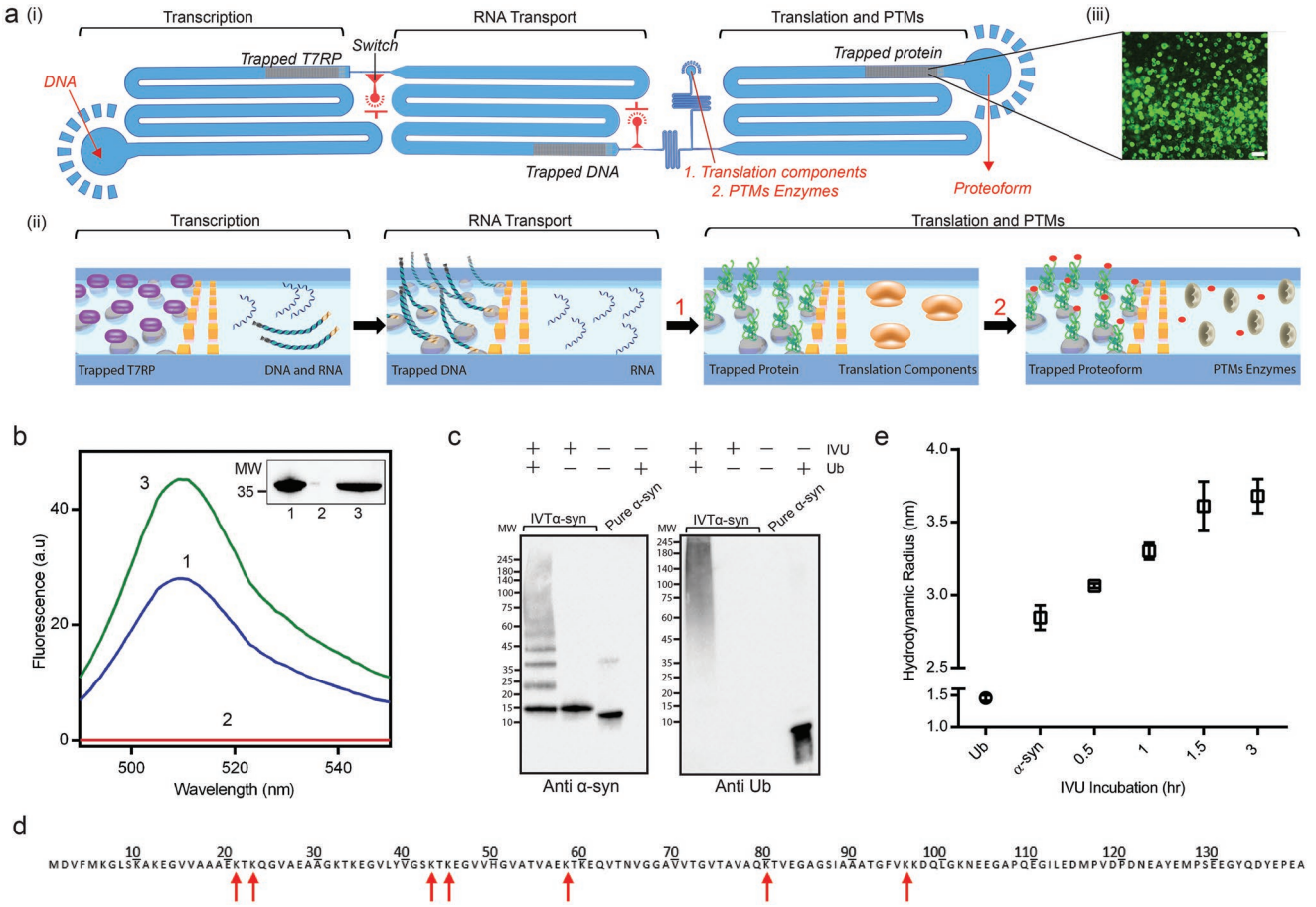

Figure 3. Translation and characterization of purified His-eGFP and α-synuclein proteoforms. a): i) Schematics of the CONTRALL platform combining three hierarchical compartments. Confinement of processes in each compartment is secured by valves (red). Separate inlets allow the introduction of additional reagents. ii) Biotinylated DNA template encoding for His-tagged protein is injected into the transcription compartment containing immobilized T7RP. Following transcription, biotinylated DNA molecules and RNA transcripts are flown to the RNA transport compartment where biotinylated DNA molecules are immobilized on trapped SA beads, allowing RNA transcripts to continue to the translation compartment. Next, newly translated His-protein is immobilized onto Ni beads trapped in the translation and PTMs compartment, allowing the removal of translation components and PTMs enzymes. iii) Fluorescent image of His-eGFP bound to Ni beads in the translation compartment. Scale bar, 100 µm. b) Fluorescence spectrum and Western blot (inset) of 1) purified His-eGFP, 2) collected flow-through, and 3) elution fraction. c) Western blot analysis of ubiquitinated α-synuclein produced by CONTRALL. Left: anti α-synuclein. Right: anti-ubiquitin. d) The newly identified CHIP E3 ligase ubiquitination sites denoted on the α-synuclein protein sequence (red arrows). e) Hydrodynamic radius measurements of ubiquitinated α-synuclein following different incubation times, compared to pure α-synuclein and pure ubiquitin.

expand the currently available protein- and peptide-based molecules, explore the hierarchical code of PTMs, their modulation of protein-protein interactions and the resulting aggregation propensity.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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aptamers, cell free, microfluidics, post-translation modifications, synthetic biology

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