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Expanding One-Pot Cell-Free Protein Synthesis and Immobilization for On-Demand Manufacturing of Biomaterials

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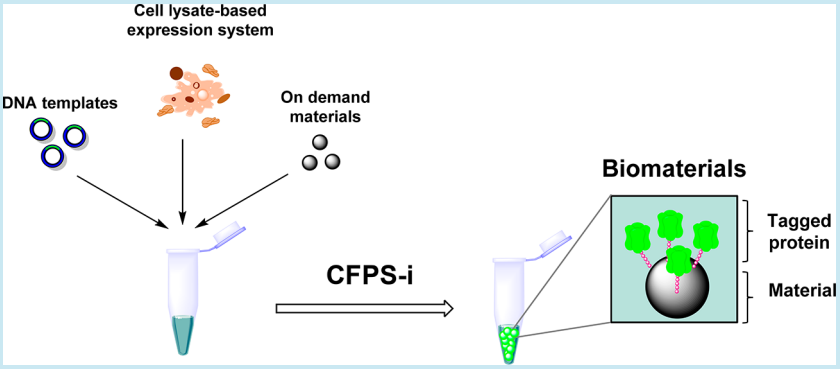
Supporting Information



ABSTRACT: Fabrication of protein-based biomaterials is an arduous and time-consuming procedure with multiple steps. In this work, we describe a portable toolkit that integrates both cell-free protein synthesis (CFPS) and protein immobilization in one pot just by mixing DNA, solid materials, and a CFPS system. We have constructed a modular set of plasmids that fuse the N-terminus of superfolded green fluorescent protein (sGFP) with different peptide tags (poly(6X)Cys, poly(6X)His, and poly(6X)Lys), which drive the immobilization of the protein on the tailored material (agarose beads with different functionalities, gold nanorods, and silica nanoparticles). This system also enables the incorporation of azide-based amino acids into the nascent protein for its selective immobilization through copper-free click reactions. Finally, this technology has been expanded to the synthesis and immobilization of enzymes and antibody-binding proteins for the fabrication of functional biomaterials. This synthetic biological platform has emerged as a versatile tool for on-demand fabrication of therapeutic, diagnostic, and sensing biomaterials.

KEYWORDS: *cell-free protein synthesis, site-selective protein immobilization, biomaterials, synthetic biology, point of care*

The utilization of biomaterials for biomedical purposes has been growing exponentially in the past decade.[1] Consequently, a large variety of biomaterials functionalized with proteins have emerged as therapeutic agents[2] diagnostic devices,[3] biosensors,[4] and contrast agents for molecular imaging.[5] In terms of biological activity, proteins (antibodies, cytokines receptors, ligands, enzymes, *etc.*) are among the most interesting biomolecules to functionalize solid materials with.[6] Generally, the manufacture of protein-based biomaterials includes (1) synthesis of suitable materials for the particular application, (2) expression and purification of the protein, and (3) immobilization of the protein on the solid material.[7] This multistep workflow is designed for bulk production of biomaterials in sophisticated and centralized production facilities, but it fails when the manufactured products need to be deployed at the point of care or are economically nonviable at small scales like orphan drugs.[8]

The major restriction for rapid and efficient protein-based biomaterial manufacturing relies on the stability of the biological element. The production and storage of proteins are tied to their labile nature, which limits their delivery to remote areas where the cold chain cannot be assured. Another hurdle for rapid manufacturing of this type of biomaterials is the biosafety regulations when proteins are produced in living organisms, which require specific skills and specialized facilities, limiting the operation in low-resource areas. Finally, once the protein is conjugated to the material, the greatest challenge is preserving the biological function of the biomaterial during storage and distribution.

On-demand synthesis of protein-functionalized biomaterials at the point of care requires rapid synthesis and immobilization

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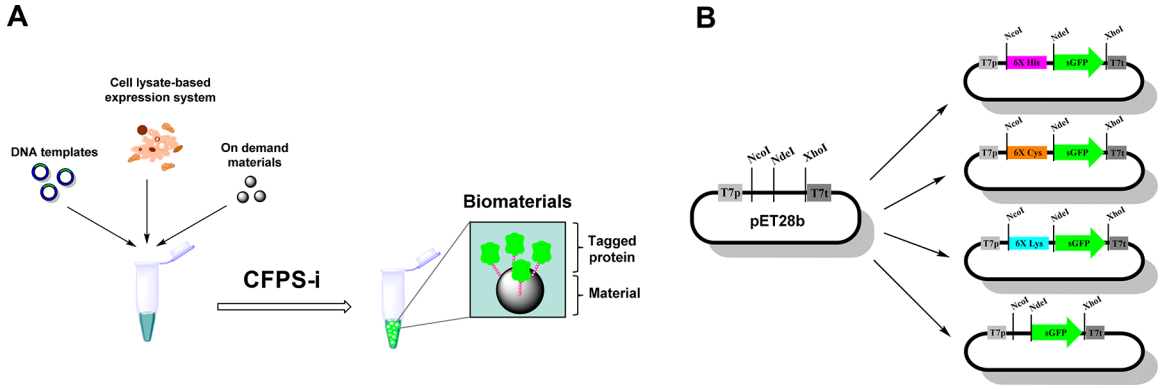

Figure 1. CFPS-i reaction with the toolkit of plasmids encoding the sGFP gene. (A) One-pot CFPS-i process for biomaterials manufacturing. (B) Modular plasmid toolkit engineered for biomaterials manufacturing. The plasmid architecture shows the restriction enzymes needed to modularly exchange the target protein and the tag.

of the protein in one pot. However, multipot *in vivo* protein synthesis and purification are time-consuming and hardly miniaturizable. Alternatively, cell-free protein synthesis (CFPS) is a promising technology to readily synthesize well-folded proteins[9] on demand since crude extracts have been successfully engineered to maximize the productivity and sustain the energy levels in order to assure competitive protein yields compared to *in vivo* systems.[10,11] Hence, incorporating CFPS into biomaterial manufacturing allows simultaneous protein synthesis and immobilization in the same test tube. Now, the fabrication system relies only on the stability of the materials and the freeze-dried cell-free extract, which have demonstrated their longevity under standard storage conditions.[12−14]

To this aim, the immobilization chemistry must be orthogonal to the protein synthesis, avoid unspecific interactions between the protein synthesis machinery and the solid material, and correctly orient the protein to guarantee the functionality of the resulting biomaterial. A previous work showed cell-free synthesis and immobilization (CFPS-i) of membrane proteins tagged with green fluorescent protein (GFP) at their C-termini and immobilized on polyvinylidene difluoride (PVDF) membranes through hydrophobic interactions.[15] Unfortunately, this kind of hydrophobic interaction is not very selective to expand this technique for new applications. Protein tagging with polypeptide or protein domains is the most widespread strategy to purify proteins for biochemical and structural studies. Furthermore, the tag serves to control the orientation of the protein when it is immobilized on a solid material.[16,17] Nowadays we have access to a large variety of peptide tags and domains that selectively bind silver,[18] gold,[19] nickel oxide,[20] and iron oxide[21] through coordination and covalent bonds, silica particles[22] through ionic interactions, biopolymeric particles such as agarose beads[23] through multivalent interactions based on hydrogen and Van der Waals bonds, and even oil droplets[24] through hydrophobic interactions.

In several pioneering works, CFPS was utilized for the fabrication of self-assembling protein microarrays as NAPPA technology,[25] the PISA method,[26] and other protein-array-based approaches.[15,27,28] This concept was further extended to protein synthesis and selective immobilization by using His tag.[29−31] These systems have been successfully applied for drug discovery and protein engineering.[14,30] Likewise, a recent report has described the synthesis and immobilization of single-span membrane proteins on oil drops as a therapeutic biomaterial for killing cultured cancer cells.[32] This one-pot concept may improve the on-demand fabrication of biomaterials overall in the prototyping phase of the manufacturing.

Here we expand the synthetic biological platform coupling CFPS-i of proteins in a single test tube for biomaterials manufacturing (Figure 1A). This portable system may work at the point of care just by addition of DNA templates, CFPS systems, and on-demand materials. The toolbox of polypeptide tags and their different immobilization chemistries open a wide range of possibilities for the on-demand fabrication of biomaterials in one pot. Moreover, coexpression and co-immobilization of several proteins may improve the scope of this approach. Finally, we extend this technological platform to the production of biomaterials based on enzymes and antibodies, demonstrating the biological functionality of the synthesized and immobilized proteins.

RESULTS AND DISCUSSION

Construction of a Plasmid Toolkit for CFPS-i: A Versatile and Modular Genetic System. We fabricated a set of plasmids that encode superfolded green fluorescent protein (sGFP) with different polypeptide tags fused to the N-terminus. In this construction, both the sGFP and the tag sequences can be easily exchanged with others just by enzymatic digestion (Figures 1B and S1A). Hence, the modular genetic toolbox design enables any target protein to be easily fused with any polypeptide tag. To prove the modularity of this architecture, we constructed three plasmids expressing sGFP harboring at its N-terminus poly(6X)His (His-sGFP), poly-(6X)Lys (Lys-sGFP), or poly(6X)Cys (Cys-sGFP) peptides that selectively immobilize the protein on different solid materials activated with metal chelates, negative charges, or disulfide groups, respectively (Figure 1B). After transformation in *Escherichia coli*, all of these plasmids similarly expressed *in vivo* sGFP tagged with the different tags (Figure S2), in accordance with previous results demostrating that sGFP expression remains unaltered regardless of the position (N- or C-terminus) and the presence of the tag.[33] Nevertheless, the *in vitro* expression of the untagged sGFP was significatly more efficient than the expression of the tagged variants, which presented different expression levels among them (Figure S3). The *in vitro* expression of Lys-sGFP was lower than that of His-sGFP and significantly lower than that of Cys-sGFP. These data correlate with the content of amino acids in the sGFP primary

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sequence (20 lysines, 11 histidines, and 2 cysteines), which suggests that the amino acid or tRNA pools may be the limiting factor to achieve high protein yields with tagged proteins.

Cell-Free Synthesis and Immobilization of His-sGFP in One Pot. To demostrate that protein synthesis and immobilization can occur orthogonally and simultaneously, we synthesized His-sGFP with commercially available S30 cell-free extract from *E. coli* in the presence of porous agarose particles activated with cobalt chelates (AG-Co). Expectedly, protein synthesis was not observed when empty pET28b was used as a negative control. On the contrary, when the CFPS system was incubated with the plasmid encoding the untagged sGFP and the beads, the fluorescence appeared and remained in the reaction bulk without selectively colonizing the beads (data not shown). Finally, only when the plasmid expressing His-sGFP and AG-Co were incubated with the cell-free extract did we observe the selective confinement of the fluorescence on the bead surface. Under these conditions, we were able to load up to 2.8 ± 0.2 mg of protein per gram of carrier after 12 h (see Methods for more information). This experimental evidence demostrates that the *in vitro* synthesis and concurrent selective immobilization of the protein occurred only when the protein tag/bead pair was properly selected. To better understand the dynamics of this two-step/one-pot process, we performed the synthesis of His-sGFP and its immobilization on AG-Co by measuring sGFP fluorescence as a function of time (Figure 2).

The online monitoring of the process revealed that the sGFP gene under the control of the T7 promoter is transcribed and translated to produce a His-tagged nascent protein that is subsequently immobilized on the AG-Co. As can be seen from the time-course images, fluorescence is barely accumulated in the bulk during the entire process (Figure 2A and Movie S1). This fact suggests that the immobilization rate is higher than the synthesis rate, converting the latter into the rate-limiting step. Despite the fact that CFPS reactions in solution normally reach the maximum protein yields in 2−4 h,[34,35] the accumulation of the nascent proteins inside the porous beads continued for more than 12 h and never reached a plateau (Figure 2A). Furthermore, we observed that larger beads are more efficient in recruiting the nascent proteins because of their higher surface areas (Figure 2A). Finally, the online monitoring of the CFPS-i process also revealed a uniform distribution of His-sGFP within the microstructure of the beads (Figure 2B). A similar uniform distribution was found in a similar setup but using agarose beads activated with nickel chelates.[14] Once the CFPS-i system was demonstrated to be functional, we reused the cell extracts by separating the loaded beads and incubating the cell extracts with fresh empty beads, cofactors, and reagents added to the cell-free solution. Unfortunately, the protein synthesis yield diminished 96.0% after the second use (Figure S5). Finally, we freeze-dried the cell extracts to improve the portability and robustness of this system according to recent studies.[12,13,16] We were pleased to achieve the same protein synthesis yield after 12 h of reaction with both freeze-dried and non-freeze-dried cell extracts, although the synthesis was 2.63 times slower using the freeze-dried cell extracts compared with the commercial solution (Figure S6).

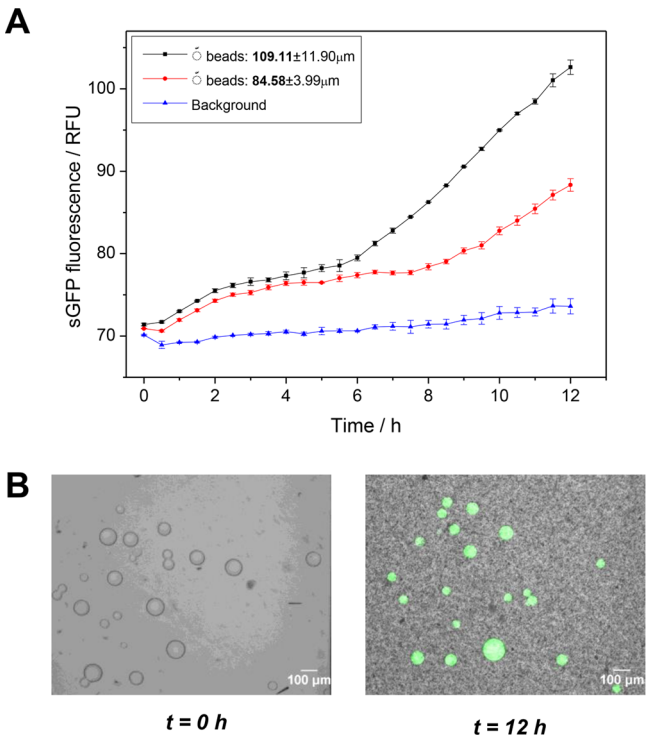

Figure 2. Online monitoring of CFPS-i by fluorescence microscopy. (A) sGFP fluorescence inside the beads and in the background (the reaction medium outside the beads) as a function of time. Beads were grouped according to their sizes. The mean fluorescence value at each time point corresponds to the average fluorescence of three beads. (B) Fluorescence microscopy images overlaying bright-field and sGFP signals (left) before and (right) after the CFPS-i reaction was completed.

In order to demonstrate that all of the His-sGFP was immobilized on AG-Co at a loading of 2.8 mg/g, we also performed an immobilization time course of His-sGFP (*in vivo*-expressed) (Figure S7). Under these conditions, the immobilization was monitored by measuring the sGFP fluorescence outside the AG-Co using a multiplate fluorimeter, a method with a lower limit of detection. After 12 h of immobilization reaction, we detected more than 96% of sGFP on AG-Co, whereas less than 4% of the initial solution of *in vivo*-expressed sGFP was detected outside beads. These results sustain a high efficiency of the CFPS-i platform, at least for a protein loading of 2.8 mg/g.

On-Demand Synthesis of Different Biomaterials Functionalized with sGFP Tagged with Different Tags. First of all, we tested the high selectivity of CFPS-i producing Cys-sGFP in the presence of two different materials, AG-Co and methacrylate beads activated with epoxide groups (Pu-E). Nascent Cys-sGFP was exclusively immobilized on Pu-E (Figure 3) as result of the specific interaction between the thiol groups of the Cys tag and the epoxide groups of the carrier.

To expand the scope of the CFPS-i methodology, we coupled the synthesis of Cys-sGFP and Lys-sGFP to their selective immobilization on gold nanorods (AuNRs) and silica nanoparticles (SiO_2NPs), respectively (Figures 4 and S8). Cys-sGFP was efficiently immobilized on the AuNRs through the formation of thiol−gold covalent bonds (Figure 4A); this chemistry has been extensively exploited in the biofunctionalization of gold-based nanomaterials.[36] Likewise, Lys-sGFP was selectively and stably absorbed on SiO_2NPs through the ionic interactions between the positive charges of the protonated ε-NH_3^+ groups of the Lys tag and the negatively charged surface of these nanoparticles (Figure 4B), in accordance with the results obtained with other basic domains and tags fused to the

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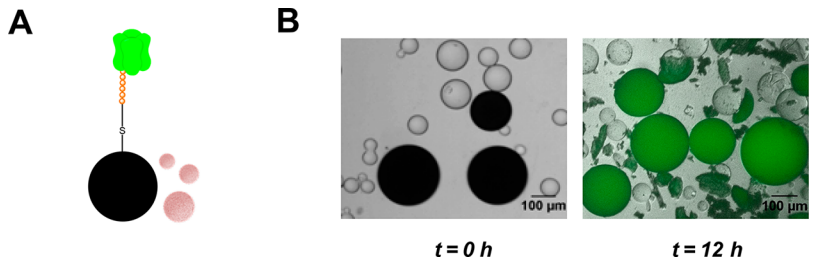

Figure 3. CFPS-i of Cys-sGFP in the presence of AG-Co and Pu-E. (A) Scheme of the selective binding between the Cys-GFP and Pu-E (black) in the presence of AG-Co (pink). (B) Fluorescence microscopy images overlaying bright-field and sGFP signals before and after the CFPS-i reaction was accomplished.

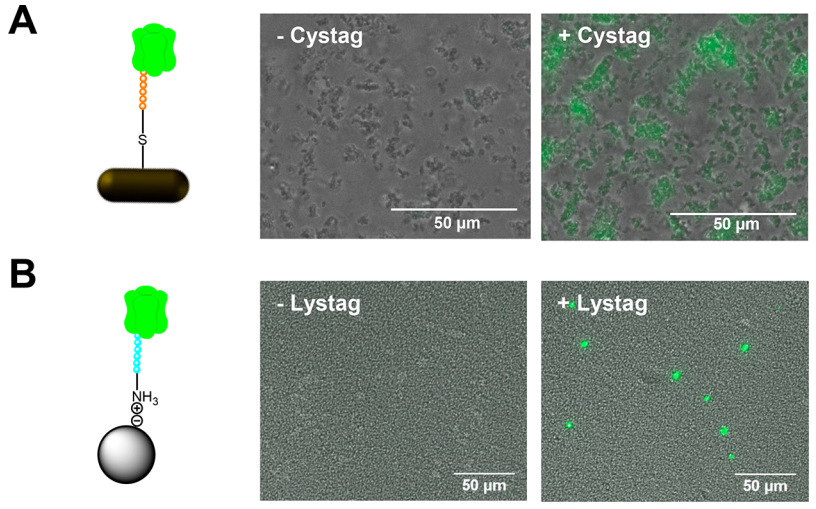

Figure 4. CFPS-i of sGFP onto on-demand materials by selective polypeptide tags: (A) Cys-sGFP on AuNRs; (B) Lys-sGFP on SiO_2NPs. Shown from left to right are a scheme of the selective binding between the polypeptide-tagged sGFP and the nanoparticle and fluorescence microscopy images overlaying bright-field and sGFP signals for the control reaction with untagged sGFP (− Cystag/Lystag) and the reaction with polypeptide-tagged sGFP (+ Cystag/Lystag).

protein of interest.[22] In both cases, the untagged sGFP was synthesized but not immobilized on both nanoparticles (Figure 4). The interaction between the Lys tag and the silica surface is very selective, although we observed some unspecific interactions between proteins from the CFPS machinery and the SiO_2NPs (Figure S9). In addition, we also demonstrated that free amino acids contained in the S30 Premix Plus did not compete with the polypeptide-tagged sGFP for the reactive groups, as similar protein immobilization yields were achieved with and without free amino acids in the bulk. We observed similar protein immobilization kinetics in both cases (Figure S4).

The CFPS-i reaction was also carried out by drop-casting the corresponding plasmid and cell-free protein synthesis machinery on glass slides, letting the reaction mixture react for 12 h, and then intensively washing the glass surface with buffer solution. Herein, the protein synthesis was successfully accomplished since fluorescence was detected in drops containing either tagged or untagged proteins after the reaction time (Figures 5 and S10). After the washing step, the drops containing the plasmid encoding Lys-sGFP displayed fluorescent spots on the glass, while no fluorescence was detected in those drops that *in vitro*-expressed the untagged protein, since it was removed from the surface during the washing step (Figure 5). These experimental data corroborate that the Lys tag drives protein immobilization on silica-based materials and expand its utilization to other solid architectures. This

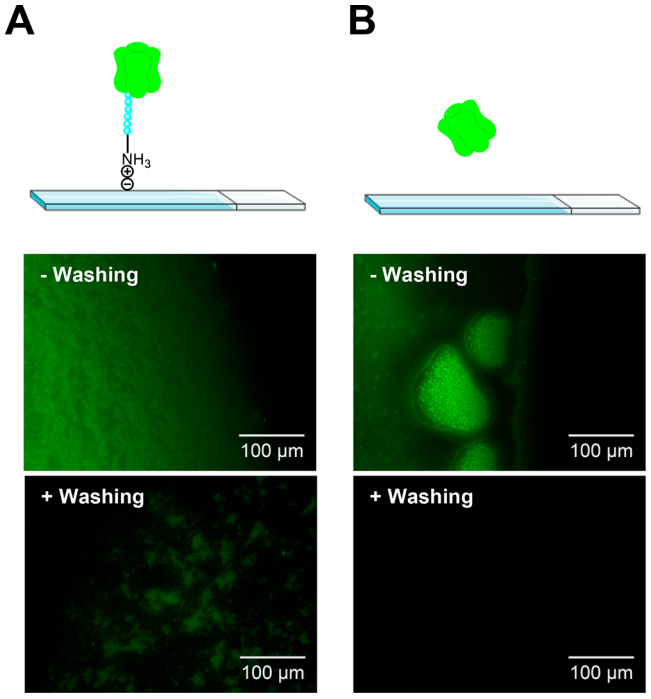

Figure 5. CFPS-i of Lys-sGFP onto glass slides. Systems expressing (A) Lys-sGFP and (B) untagged sGFP are shown.

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technology skips the chemical functionalization steps of glass slides required for conventional procedures to prepare protein-based microarrays.[37]

Incorporation of Non-natural Amino Acids through CFPS To Enable Bio-orthogonal Immobilization Mediated by Copper-Free Click Chemistry. In order to go beyond the chemistry offered by nature, we also expanded the CFPS-i to copper-free click chemistry reactions by incorporating non-natural amino acids harboring azide groups into the nascent proteins and functionalizing materials with cyclooctyne groups. To this aim, we first activated agarose beads with cyclooctyne groups through a new surface chemistry protocol starting from commercially available agarose beads activated with cyanogen bromide (Figure S11). Figure 6 shows that, as expected, protein synthesis fails and fluorescence can be detected neither in the bulk nor inside the beads when neither methionine nor L-azidohomoalanine (L-AHA) is included in the amino acid mixture. Furthermore, when the protein is synthesized with methionine, the protein is produced but immobilization on cyclooctyne-activated agarose beads does not occur, with the nascent protein remaining in the reaction bulk. Likewise, when the protein is synthesized with L-AHA but incubated with agarose beads activated with hydroxyl groups instead of cyclooctyne ones, sGFP fluorescence is detected only in the reaction bulk.

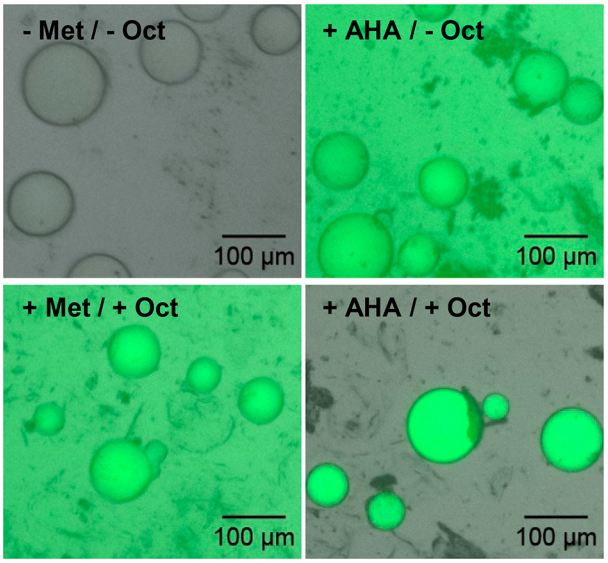

Figure 6. CFPS-i of sGFP incorporating non-natural amino acids by copper-free click chemistry. Fluorescence microscopy images overlaying bright-field and sGFP signals after the CFPS-i was completed show the results for reactions in the absence or presence of each amino acid (methionine (Met) or L-azidohomoalanine (AHA)) with agarose beads activated or not activated with cyclooctyne (Oct) groups.

These experimental data demonstrate that the synthesis and copper-free click immobilization of sGFP is possible only when azide side chains are displayed on the protein surface and the agarose surface is activated with cyclooctyne groups (Figure 6). According to the sGFP X-ray structure (PDB entry 2B3P), L-AHA can be introduced at positions 1, 78, 88, and 216 of the protein primary sequence. Positions 1, 78, and 88 are clustered at the bottom part of the β-barrel, where the N- and C-termini are located, while position 216 is located at the hollow of the barrel facing the chromophore (Figure S12).

Although the incorporation of non-natural amino acids for protein-based biomaterials manufacturing has been described previously,[38−40] here we have coupled the protein synthesis with a cyclooctyne/azide click immobilization in a one-pot concurrent process, unlike two-pot conventional methods in which first the azide- or alkyne-containing amino acids are incorporated either *in vivo* or *in vitro* into the protein and then the modified protein is purified and incubated with solid particles functionalized with either alkyne or azide groups, respectively.[41,42] For a more precise site-selective immobilization, the CFPS-i developed here can be coupled to the cell-free AMBER system by using specific tRNAs to insert non-natural amino acids at unique positions.[43] Therefore, we have demonstrated that copper-free click immobilization based on cyclooctyne/azide pairs is compatible with the protein synthesis according to the broad bio-orthogonality of this chemistry.[44]

***In Vitro* Synthesis and Selective Co-immobilization of Two Proteins Fused to Different Tags on the Same Carrier.** Co-immobilization of different enzymes on the same carrier is a frequently used strategy to assemble complex

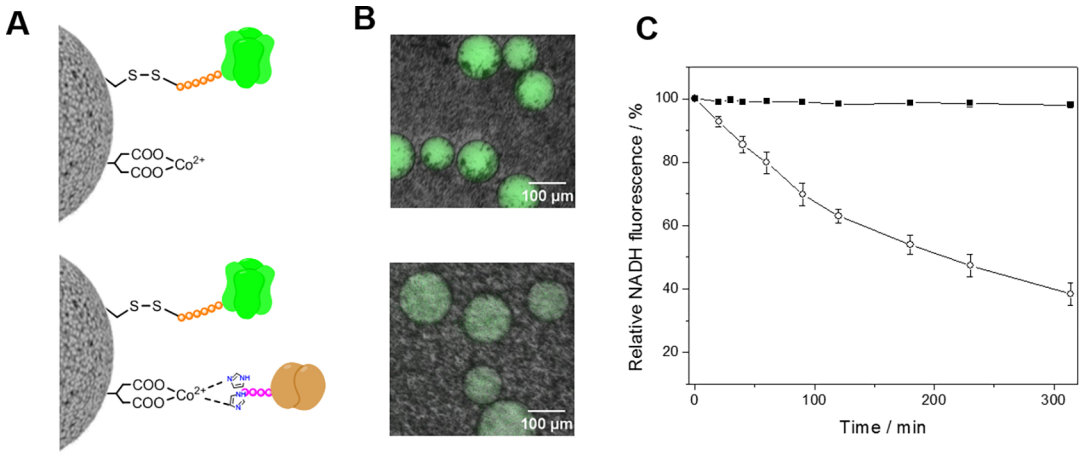

Figure 7. Coexpression and co-immobilization of two different proteins by two different chemistries. (A) Schemes showing the immobilization of Cys-sGFP (top) and the co-immobilization of Cys-sGFP and His-ADH*Bs* (bottom) on agarose beads activated with cobalt chelates and disulfide groups. (B) Overlay of bright-field and sGFP signal images. (C) Relative NADH consumption catalyzed by co-immobilized His-ADH*Bs* and Cys-sGFP (white circles) and the control reaction with empty beads (black squares).

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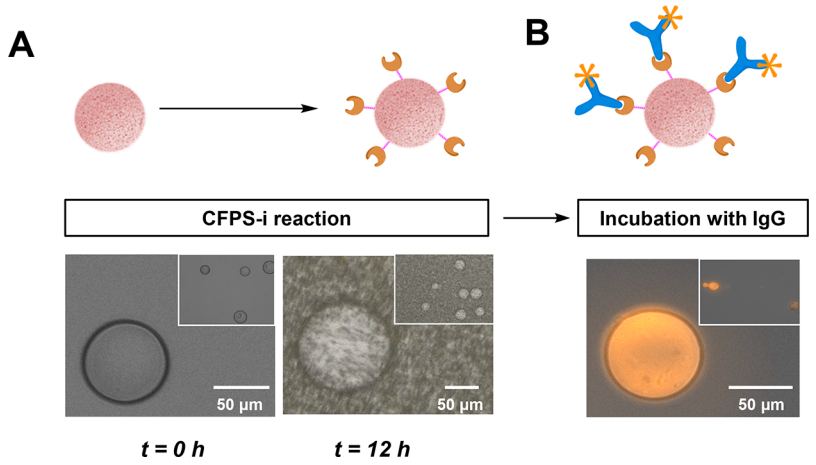

Figure 8. Two-pot synthesis of immunoconjugates by using CFPS-i. (A) Scheme and fluorescence microscopy imaging of CFPS-i of His-ProA (brown) on AG-Co (pink). (B) Scheme and fluorescence microscopy imaging of the capture of IgG (blue) labeled with rhodamine (orange star) by protein A on AG-Co.

biological machineries like multienzyme systems.[45,46] In order to gain selectivity during the co-immobilization protocol, we applied the toolbox of polypeptide tags developed herein to simultaneously synthesize two different proteins and selectively co-immobilize them on the same carrier through two different immobilization chemistries. As a proof of concept, we synthesized *in vitro* Cys-sGFP and an alcohol dehydrogenase (ADH) from *Bacillus stearothermophilus* tagged with six histidines at its N-terminus (His-ADH*Bs*). In order to evaluate the orthogonality of our toolbox for CFPS-i, we prepared a new agarose-based heterofunctional carrier activated with both cobalt chelates and disulfide groups (Figure S13). This new surface theoretically should allow the co-immobilization of Cys- and His-tagged proteins simultaneously through two different immobilization chemistries based on reversible covalent disulfide and reversible metal coordination bonds, respectively (Figure 7A). This concept was demonstrated by incubating the heterofunctional carrier with the corresponding DNA plasmids and the cell-free extract for the one-pot coexpression and co-immobilization of His-ADH*Bs* and Cys-sGFP. As a control reaction, we also expressed only Cys-sGFP in the presence of the heterofunctional carrier.

As a readout to demonstrate the coexpression and co-immobilization of these two proteins, we visualized the fluorescence of Cys-sGFP by fluorescence microscopy (Figure 7B) and spectrophotometrically measured the activity of His-ADH*Bs* attached to the carrier after washing steps to remove the protein synthesis machinery (Figure 7C). These experiments provide evidence that Cys-sGFP and His-ADH*Bs* are coexpressed and co-immobilized on the same beads. However, when we performed the CFPS-i using only the plasmid that encodes Cys-sGFP, the fluorescence intensity per bead was 4.22 times higher than when the two different plasmids were used (Figure S14). This dissimilarity points out a possible competition between the two transcribed mRNAs for the ribosomal machinery that may diminish the specific final yields for both Cys-sGFP and His-ADH*Bs*. Karim and Jewett[47] observed a similar decreasing effect on the protein expression levels when they tried the cell-free coexpression of several enzymes. According to Park et al.,[48] mRNA species must compete for a finite pool of ribosomes and aminoacyl-tRNAs when CFPS reaction mixtures are primed with several DNA templates, leading to unequal expression levels of different proteins. Indeed, the sGFP fluorescence was reported to be inversely proportional to the translation rates of the coexpressed genes. Besides the fluorescence of the immobilized Cys-sGFP, which reveals its proper folding and structurally innocuous immobilization, His-ADH*Bs* synthesized *in vitro* and immobilized was catalytically active after CFPS-i (Figure 7C). The multimeric nature of this enzyme was not a hurdle for its successful expression and *in situ* immobilization using the CFPS-i system, validating the feasibility of our technology for the fabrication of solid platforms harboring more complex biological systems. Additionally, the orthogonality and selectivity of these two immobilization chemistries were demonstrated by selective elution of His-ADH*Bs* in the presence of EDTA, while the Cys-sGFP remained attached to the surface. Likewise, the Cys-sGFP was eluted to the solution by the use of thiolated compounds (Figure S15).

Two-Pot Fabrication of Immunoconjugates by CFPS-i.

The demonstrated plasticity of our methodology encouraged us to manufacture biomaterials for inmunosensing assays. In order to conceive a wide-ranging device, we decided to synthesize and immobilize protein A (ProA) by CFPS-i. Functionalizing materials with ProA provides a universal platform for binding to the Fc region of any immunoglobulin G (IgG).[49] We utilized a previously reported plasmid that encodes ProA tagged with poly(6X)His (His-ProA) at its N-terminus under the control of the T7 promoter.[50] In a first attempt, we incubated the plasmid DNA, the cell-free extract, cobalt-activated beads, and an IgG chemically conjugated with both rhodamine and alkaline phosphatase (AP-IgG-Rh) in one test tube to perform the CFPS-i of His-ProA and the subsequent IgG capture in one pot. Unfortunately, the antibody could not be bound to the beads functionalized with the nascent ProA under the protein synthesis and immobilization conditions. In the light of this unsatisfactory result, we first expressed and immobilized His-ProA on cobalt-activated agarose beads in one pot (Figure 8A), and then AP-IgG-Rh was incubated with those beads in a second pot (Figure 8B). The fluorescence and activity of the enzyme conjugated to the IgG enabled tracing of the antibody capture reaction. When AP-IgG-Rh was incubated with AG-Co lacking His-ProA, we neither visualized fluorescence inside the beads nor detected enzyme activity after sample washing (Figure S16).

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Table 1. Description of the Synthesized Biomaterials

tagged protein	binding material	binding interaction	elution reagent
His-sGFP	cobalt-chelate-activated agarose (AG-Co)	metal coordination bond	imidazole
His-ADH*Bs*	cobalt-chelate and disulfide-group-activated agarose	metal coordination bond	imidazole
His-ProA	AG-Co	metal coordination bond	imidazole
Cys-sGFP	Pu-E	covalent	–
	AuNRs	covalent	–
	cobalt-chelate and disulfide-group-activated agarose	thiol exchange	DTT
Lys-sGFP	SiO_2NPs	ion exchange	sodium chloride
	glass slide	ion exchange	sodium chloride
sGFP containing L-AHA	cyclooctyne-activated agarose	copper-free click chemistry	–

As a result, the fluorescence of AP-IgG-Rh was uniformly distributed on the His-ProA agarose beads, pointing an optimal antibody density for antigen recognition as previously reported for the direct immobilization of IgG on porous agarose particles.[16,51] However, the labeled antibody was not quantitatively captured by the beads functionalized with His-ProA; 43% of the offered antibody was immobilized (Figure S16). Hence, we have demonstrated that His-ProA can be synthesized and immobilized in one pot while retaining its capacity to selectively capture conjugated IgGs, giving rise to a two-step process for the fabrication of immobilized immunoconjugates. These results suggest that the protein synthesis conditions inhibit the affinity interaction between the Fc region of the antibody and His-ProA. This strategy may contribute to developing innovative immunological platforms not only for the manufacture of tailor-made biosensors and pull-down systems[52] but also for immunotherapy.[32]

CONCLUSION

We have coordinated protein synthesis and immobilization in one pot by developing a set of plasmids that are ready to use with suspensions of cell-free protein synthesis and solid materials for on-demand fabrication of protein-based biomaterials. This technology is fully portable since it does not require specialized equipment nor infrastructures. Here we have demonstrated that the *in vitro* synthesis of proteins is orthogonal to a variety of solid materials as carriers and a diversity of immobilization chemistries that attach the nascent proteins to the carriers (see the summary in Table 1). We have also exploited this system to produce multimeric proteins and biological conjugates (IgG−proteinA). However, the optimal synthesis of larger proteins with quaternary structure and the *in situ* formation of biological complexes through specific protein−protein interactions will be some of the most important challenges that this technology will have to face in the near future.

The examples reported in this work have encouraged us to expand the CFPS-i technology to continuous-flow manufacturing of protein-based biomaterials, as already proposed for the synthesis of active pharmaceutical ingredients[53,54] and for bionanotechnology solutions. For instance, the integration of the CFPS-i platform into automated and miniaturized systems under good manufacturing practice (GMP) conditions will undoubtedly boost the on-demand manufacturing of protein-based biomaterials at the point of care and the discovery of new biomaterials by prototyping their fabrication and shortening their processing time. In this sense, Schellekens et al.[55] have reviewed some of the clues to make the manufacture of individualized drugs a reality. They suggest that evolving from large-scale pharmaceutical production to small-scale and automated models will allow the manufacture of drugs for ultraorphan and highly rare diseases to be addressed in a cost-efficient manner. Hence, we foresee that our technology will play an important role in personalized medicine when nanotechnology definitively bursts into clinical treatments.

METHODS

Reagents and Materials. The S30/T7 high-yield protein expression system was supplied by Promega Corporation (Fitchburg, WI). Rhodamine B isothiocyanate, dibenzocyclooctyne-amine, anti-rabbit IgG (whole molecule)−alkaline phosphatase antibody produced in goat, 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP), 5,5′-dithiobis(2-nitrobenzoic acid), 1,4-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), cyanogen bromide-activated Sepharose 4B, L-lysine, L-cysteine, L-histidine, and primers were acquired from Sigma-Aldrich (St. Louis, MO). Click-it L-AHA was supplied by Thermo Fisher Scientific. Six-channel μ-Slide VI$^{0.4}$ was purchased from ibidi (Planegg, Germany), and Lifetech ECR8204 epoxymethacrylate beads (Pu-E) were kindly donated by Purolite Ltd. (Llantrisant, U.K.). The colloidal nanofabrication platform from CIC-BiomaGUNE was used to synthesize the AuNRs and SiO_2NPs (see section 6 in the Supporting Information). Agarose-based materials monofunctionalized with cyclooctyne groups and heterofunctionalized with both disulfide and cobalt chelate groups were fabricated using plain agarose beads purchased from ABT Technologies (Madrid, Spain) (see section 1 in the Supporting Information).

Genetic Engineering of a Toolbox of Plasmids. Plasmid pET-28b(+) (Novagen) was used for cloning and protein expression. The genetic construct His-sGFP_pET28b was developed in a previous work.[56] We designed eight primers (Table S1) for cloning of Cys-sGFP, Lys-sGFP, and untagged sGFP using the His-sGFP_pET28b plasmid as a template. First, we amplified by polymerase chain reaction (PCR) the sequence upstream of the sGFP gene using primers 3 and 8 for the construction of Cys-sGFP_pET28b. Second, the downstream sequence was amplified with primers 7 and 4. Finally, we performed an overlapping PCR by using primers 3 and 4 and the previous PCR products as megaprimers. The pET-28b vector was digested with the restriction enzymes NcoI and XhoI. Afterward, the overlapping PCR product and the digested vector were purified and ligated by homologous recombination in *E. coli* strain DH10β (laboratory stock). The same procedure was carried out for the genetic construction of Lys-sGFP_pET28b with primers 3 and 6, primers 5 and 4, and primers 3 and 4. Cloning of sGFP_pET28b was accomplished with primers 2 and 1 for amplification of the untagged sGFP sequence. The resulting PCR product and the vector were further digested with NcoI and XhoI. After dephosphorylation

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and purification of the DNA fragments, we ligated the DNA fragments and transformed them in BL21-Gold (DE3). All of the molecular biology protocols were performed using standard methods.[57]

***In Vitro* Cell-Free Protein Synthesis and Immobilization.** The CFPS system was composed of 40% S30 Premix Plus and 30% T7/S30 extracts. In the case of click reactions, we mixed 40% S30 Premix Plus without amino acids, 10% amino acid mixture minus methionine, 10% amino acid (methionine or L-AHA), and 30% T7/S30 extracts. We added 1 μg of DNA template and 1 mg of carriers to the cell-lysate-based expression system at a final volume of 50 μL, unless otherwise specified. The reactions were performed at 37 °C and 1200 rpm for 12 h in an Eppendorf Thermomixer R, and 1 mM cetyltrimethylammonium bromide was added to CFPS-i reactions with Pu-E and AuNRs to avoid unspecific hydrophobic interactions and particle aggregation, respectively. Once the CFPS was finished, protein-based biomaterials were easily purified from the reaction mixture by rapid and low-cost filtration using mini Biospin chromatography columns.

Determination of His-sGFP Loading on AG-Co. Solutions of *in vivo*-expressed sGFP were incubated with AG-Co for 1 h to achieve protein loadings from 0.5 to 5 mg/g. The sGFP fluorescence from 10 beads of each loading was analyzed using ZEN 2012 software (Zeiss). The results were plotted as a calibration curve to estimate the CFPS-i of His-sGFP on AG-Co.

Enzymatic Activity Assays. The enzymatic assays were carried out in a Varioskan flash multimode reader (Thermo Scientific) using 96-well plates. For both soluble and immobilized enzyme, the activity was measured under orbital shaking integrated into the reader. Additional details are given in section 2 in the Supporting Information.

Fluorescence Microscopy Imaging. After the CFPS-i was completed, 10 μL of the reaction mixture was placed on a channel of a six-channel μ-Slide VI$^{0.4}$. The bright-field transmission and fluorescence (λ_{exc} = 470 nm, λ_{em} = 500–550 nm) images were obtained with a Cell Observer (Axio Observer, Zeiss) microscope. In order to better visualize AuNRs and SiO_2NPs, the samples were washed three times with 25 mM sodium phosphate buffer (pH 7) before their visualization. Images were processed with ZEN 2012 and FIJI/ImageJ software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00383.

Table S1 and Figures S1–S16 (PDF)

Movie S1 (AVI)

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Author Contributions

A.I.B.-M. and F.L.-G. designed the research; A.I.B.-M., I.L., A.S.-I., and F.L.-G. performed the research; A.I.B.-M. and F.L.-G. analyzed the data and wrote and edited the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AG-Co, agarose microbeads activated with cobalt chelate groups; AP-IgG-Rh, immunoglobulin G chemically conjugated with both rhodamine and alkaline phosphatase; AuNRs, gold nanorods; CFPS, cell-free protein synthesis; CFPS-i, cell-free protein synthesis and immobilization; Cys-sGFP, sGFP tagged with poly(6X)Cys; His-ADH*Bs*, alcohol dehydrogenase from *B. stearothermophilus* tagged with poly(6X)His; His-ProA, protein A tagged with poly(6X)His; His-sGFP, sGFP tagged with poly(6X)His; L-AHA, L-azidohomoalanine; Lys-sGFP, sGFP tagged with poly(6X)Lys; Pu-E, methacrylate microbeads activated with epoxide groups; sGFP, superfolded green fluorescent protein; SiO_2NPs, silica nanoparticles

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