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Linking Genotype and Phenotype in Protein Synthesizing Liposomes with External Supply of Resources

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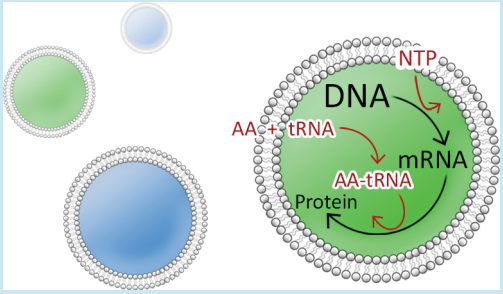
Supporting Information

ABSTRACT: Reconstituting an elementary gene expression system inside self-assembled lipid vesicles to mimic the cellular synthesis machinery is at the core of the development of a minimal cell following a bottom-up synthetic biology approach. The ability to operate the expression of multiple genes in a controlled manner and to generate the output proteins with predictable dynamics in liposomes relies on the link between genotype and phenotype. Here, we established this link in surface-tethered liposomes producing proteins from a linear DNA template using a reconstituted transcription/translation/aminoacylation apparatus fuelled by external supply of feedstock. The amounts of entrapped DNA molecules and synthesized proteins were visualized by fluorescence confocal microscopy in individual vesicles. We showed that there exists no linear correlation between the amount of encapsulated genes and the level of output proteins, which is a consequence of the compositional heterogeneity between liposomes due to the low-copy number of some constituents, as well as interfacing differences with the nutrient-containing environment. In order to decouple gene activity from those sources of variability and, thus, infer the probabilistic occupancy of transcriptionally active genes in protein synthesizing liposomes, we developed a dual gene expression assay consisting of the production of two fluorescent reporter proteins of distinguishable colors from two different DNA templates. The stochastic color-coding of the vesicles was analyzed and compared to the color pattern expected from a Poisson distribution of encapsulated genes. Unexpectedly, we found that the apparent number of transcriptionally active DNA molecules in liposomes corresponds only to ca. 10% of the bulk concentration. We believe that our study provides new insights about the relationship between the genotype and phenotype in protein synthesizing liposomes, which is of primary importance toward the construction of a programmable artificial cell implemented with regulatory gene networks of predictable dynamics.



KEYWORDS: *artificial cell, cell-free translation, lipid vesicle, gene expression, enzyme encapsulation*

Cell-free synthetic biology has emerged as a promising discipline to understand biological functions as well as to design and build up functional molecular assemblies.[1] Ultimately, the goal of such an engineering approach culminates into the construction of an elementary cell having all essential attributes of a living entity.[2−4] The simplest representation of a living cell can be envisioned as a reconstituted synthesis machinery carrying out the expression of a minimal genome inside a synthetic lipid vesicle. In recent years, the production of proteins from a DNA template inside liposomes loaded with a transcription/translation apparatus has been demonstrated by using either cellular extracts usually derived from *Escherichia coli* bacteria[5−8] or a reconstituted gene expression system[9−12] called the PURE system.[13]

A next step toward the creation of a minimal cell consists in the functional assembly of genetic circuits with predictable behaviors. However, two main hurdles need to be overpassed to reach this goal. First, the kinetic parameters of the essential reaction steps have to be determined and, second, the statistical partitioning of every constituent (e.g., the DNA molecules, RNA polymerases and ribosomes) within individual reaction vessels must be known. Indeed, the temporal dynamics of synthesized RNAs and proteins tightly depends on the initial concentrations of entrapped substrates and enzymes, in particular for those present at a low-copy number. Until recently it was commonly believed that the vesicle partitioning of all compounds follows a random (Poisson) distribution. In sharp contrast, protein expression has been measured in unexpectedly small (<400 nm) vesicles, for which the probabilities to contain all reactants permitting transcription and translation were supposedly very low.[12,14] A spontaneous crowding of macromolecular solutes occurring during liposome formation has been suggested and directly visualized by electron microscopy for the case of ribosomes.[15] A remarkable accumulation of ribosomes was observed in a small fraction of vesicles at the expense of most of the liposomes that remain empty, with a ribosome occupancy distribution obeying a power law.[15] To a lesser extent, higher internal concentrations

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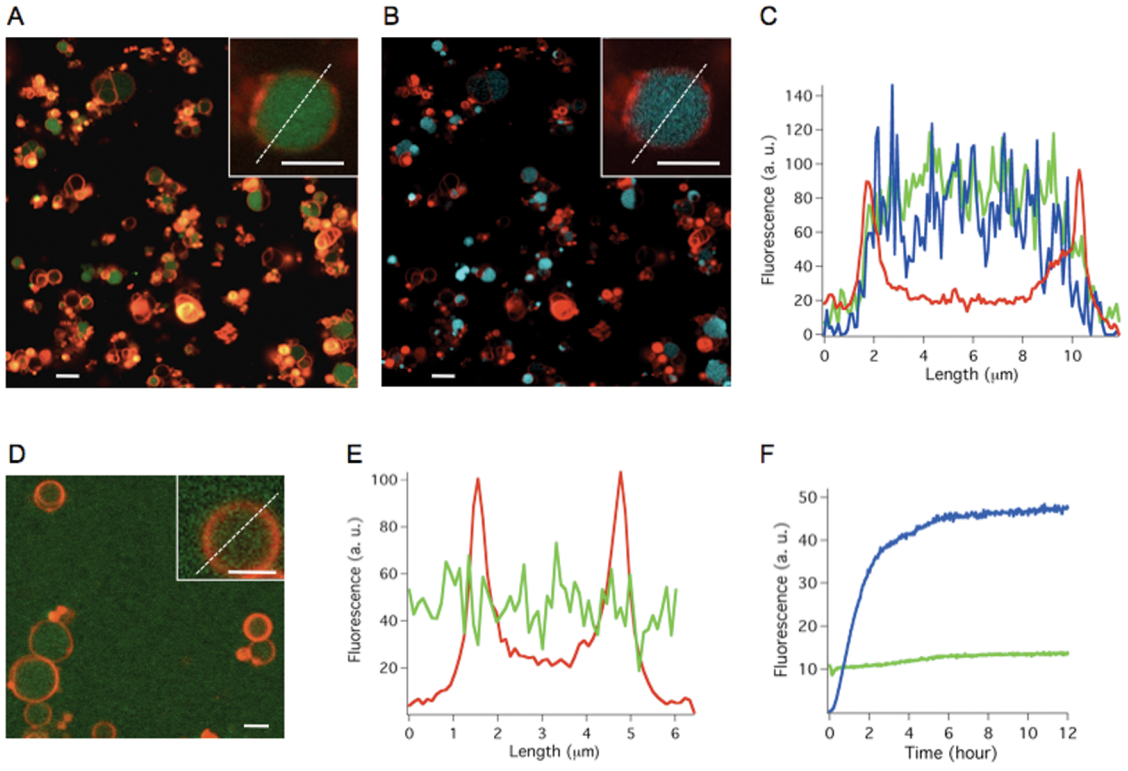

Figure 1. (A, B) Fluorescence confocal images of surface-tethered liposomes (red, TIRTC-labeled lipids) producing CFP proteins (blue) from YOYO-1-labeled DNA (green). Vesicles were imaged after 2 h incubation at 37 °C subsequent to the addition of the feeding solution. Scale bars are 8 μm (insets, 5 μm). (C) Intensity plots of TRITC (red), YOYO-1-DNA (green), and CFP (blue) along the dotted line in (A, B). (D) Fluorescence confocal image of immobilized liposomes and YOYO-1-stained DNA incubated ca. 1 h at 37 °C without dilution with the feeding medium. Scale bar is 4 μm (inset, 2 μm). (E) Intensity plots of TRITC (red) and YOYO-1-DNA (green) along the dotted line in (D). (F) Time course of CFP expression (blue line) from YOYO-1-tagged DNA (green line) in bulk reaction. DNA concentration was 1.4 nM, and expression was carried out at 37 °C.

of small solutes and polymers compared to the bulk solution have also been observed in micrometer-sized vesicles.[16]

This finding raises the questions of whether such an enrichment effect is also present for DNA templates and to what extent it may influence the vesicle-to-vesicle heterogeneity and dynamics of gene expression. In a recent study, the partitioning of fluorescently labeled DNA molecules encapsulated in individual vesicles has been shown to depend on the liposome preparation method, the vesicle size and membrane composition, but no evidence for concentration enhancement has been observed in the tested conditions.[17] Notably, not all encapsulated DNA molecules may be transcriptionally active because of nonspecific binding to other macromolecules or lipids precluding proper activity.

Recently, we established a glass bead-assisted method to produce cell-sized lipid vesicles with a versatile membrane platform enabling to fuel an internal minimalist gene expression machinery with external supply of resources.[12] Liposomes immobilized at high-density on a microscope coverslip were imaged, and the production of the fluorescent reporter protein GFP was analyzed. In analogy to a previous study performed using a different experimental approach,[11] we observed a marked heterogeneity in the levels of synthesized proteins between individual vesicles, which presumably arises, at least partly, from the stochastic entrapment of the many reactant molecules, making every liposome unique in terms of both composition and gene expression dynamics, a property at the basis of the modern view of cell biology.[18] In that context, how can we infer the statistical distribution of *functional* (transcriptionally active) DNA molecules between protein manufacturing vesicles as the variability in the amount of synthesized products also reflects protein compositional differences in the expression system or differences in molecular diffusion across the membrane? We decided to address this question to help us rationally design circuits composed of multiple, physically separated genes with predictable probabilistic abundance in vesicles.

Herein, we report on the simultaneous imaging of both the DNA template and its encoded fluorescent protein, which allowed us to link genotype and phenotype in single liposome microreactors. Moreover, to exclude any expression variability from one vesicle to another due to differences in the protein composition or molecular exchange with the environment, we developed a dual-gene expression assay that consists of the *in vesiculo* synthesis of two distinguishable and independent reporter genes, *cfp* and *emgfp*, controlled by the same promoter. Our reasoning was that the transcriptional activity of a single gene will lead to one-color vesicles, while the presence of at least one copy of each active gene will give two-color liposomes irrespective of the protein composition (except if translation can not occur) and nutrient uptake efficiency since the expression of the two genes will be similarly affected. This approach resembles the one used to decompose the contributions of intrinsic and extrinsic noises in live cells.[19]

Evidences for a spontaneous crowding of some of the PURE system constituents have recently been reported.[14,15] This prompted us to investigate whether a similar effect occurs for DNA molecules when using our methodology for liposome

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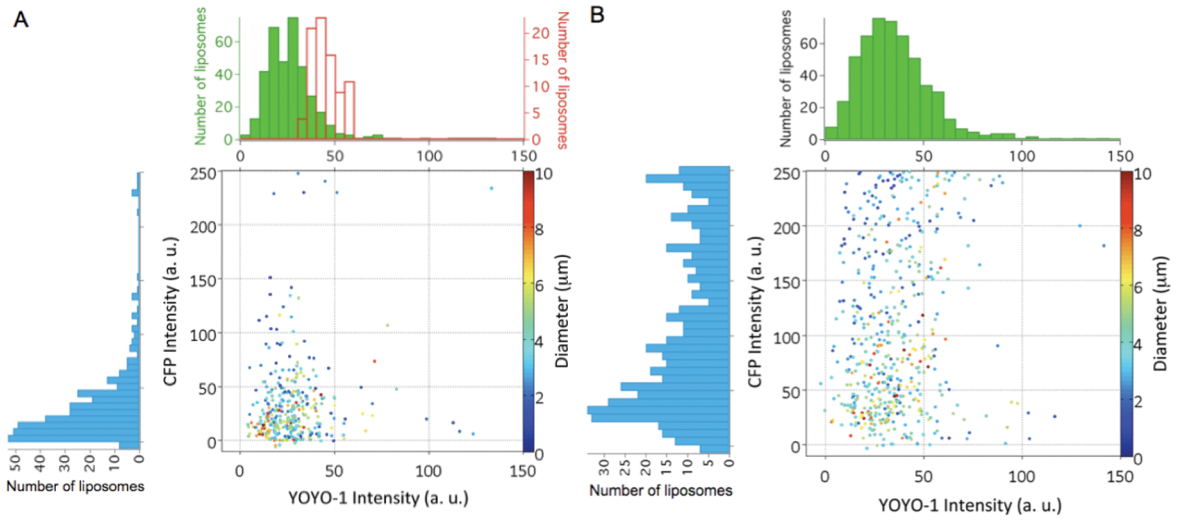

Figure 2. Two-dimensional intensity plots of CFP and YOYO fluorescence inside individual liposomes. DNA was used at a concentration of 1.4 nM. Each point represents the mean fluorescence intensities from one liposome. YOYO-stained DNA template coding for the CFP was used. Surface-immobilized liposomes diluted with the feeding solution were imaged after (A) 2 h or (B) 16 h incubation at 37 °C. The color coding of the points indicates the vesicle diameter (color bar). On each axis, the corresponding intensity histogram is displayed. In (A), the histogram of YOYO-1 intensity inside vesicles without dilution with the feeding medium is appended in red. The total number of analyzed liposomes was 979. The fluorescence plots of a second independent experiment are shown in Figure S2 (Supporting Information).

preparation and immobilization. CFP-coding DNA templates were labeled with the fluorescent dye YOYO-1 and 1.34 nM of DNA was coencapsulated together with the PURE system enzyme mix in liposomes, as described in the Methods section. The vesicles were then immobilized and their fluorescence was imaged (Figure 1D). As shown in Figure S1 (Supporting Information), YOYO-1 fluorescence intensity measured inside and outside liposomes linearly scales with bulk DNA concentration. The narrow distribution of in-liposome YOYO-1 intensities, centered at the intensity value measured in the outside solution (Figure 1E, Figure 2A), indicates that DNA is loaded at identical concentration than that of the bulk solution, and consequently that DNA partitioning follows Poisson statistics. In these conditions, a 2-μm diameter vesicle contains 3.4 DNA molecules on average. The fact that a nonquantized intensity distribution was observed may come from a heterogeneity in DNA loading with YOYO-1 dyes and from the small size of the DNA template (≈ 660 kDa). In other studies, the discrete encapsulation of a few large DNA molecules could be measured by flow cytometry[17] or by fluorescence confocal imaging[20] using a 32-MDa λDNA. Moreover, at least for >1-μm vesicles, DNA fluorescence was found to be evenly distributed in the lumen of vesicles, and no accumulation at the liposome membrane was observed.

In order to initiate transcription and translation inside surface-positioned vesicles, the feeding solution containing the amino acids, nucleotide triphosphates and tRNAs was added in the outside medium, which generates defects in the liposome membrane and, thus, promotes selective exchange of solutes and macromolecules (like tRNAs) between the vesicles and the environment.[12] The effective allocation of resources and entrapment of all constituents of the synthesis machinery are demonstrated by the fluorescence of reporter proteins in the lumen of liposomes (Figures 1B). The amount of encapsulated DNA molecules was also estimated in protein synthesizing vesicles. In contrast to the narrow distribution of DNA fluorescence observed prior to dilution with resources, the CFP-expressing vesicles exhibited a broader distribution of YOYO-1 intensities (Figure 2 and Figure S2, Supporting Information) with a long tail at high values and a ≈0.8-fold average intensity. Changes of YOYO-1 fluorescence properties upon exposure to the feeding solution or upon transcription of dye-loaded DNA have not been observed in bulk reactions (Figure 1F) and, thus, are not the cause of YOYO-1 intensity broadening in liposomes. The results suggest a compositional reorganization of DNA molecules within the first 30 min of incubation, which is accompanied by a larger vesicle-to-vesicle heterogeneity. Diffusion of about 1-kb DNA (≈ 660 kDa) across the lipid bilayer (inward or outward) requires the formation of large defects that could be induced by osmotic pressure.[21] Increased bilayer−bilayer reactivity and fusion upon osmotic stress generation could also be accompanied by leakage of DNA. However, once transcription and translation started, nucleic acids and proteins are engaged into macromolecular complexes that become too large to leak out of the vesicles. Eventually, the osmolarity mismatch between the inside and outside of the liposomes lessens, keeping the buried reagents sequestrated in the lumen. Outward diffusion of DNA occurs down the concentration gradient of total DNA. The net uptake of DNA inside some vesicles, which is reflected by the long tail in YOYO-1 intensity distribution, can be explained by the fact that RNA polymerase-occupied DNA within the vesicle is less prone to cross the lipid bilayer, whereas external "naked", inactive DNA molecules would be able to penetrate into liposomes.

Next, we wondered whether the yield of synthesized proteins was correlated to the amount of encapsulated DNA in liposomes. Bulk experiments have shown that protein production linearly increases with DNA concentration until sharing of the synthesis machineries becomes limiting or until shortage of resources occurs.[22,23] A similar relationship has recently been reported for gene expression compartmentalized in 40 fL microchambers (equivalent to the volume of a vesicle of ≈4.2 μm in diameter).[24] In the latter study, the observed trend is only expected if no other functional constituent than DNA is present at sufficiently low-copy number to introduce

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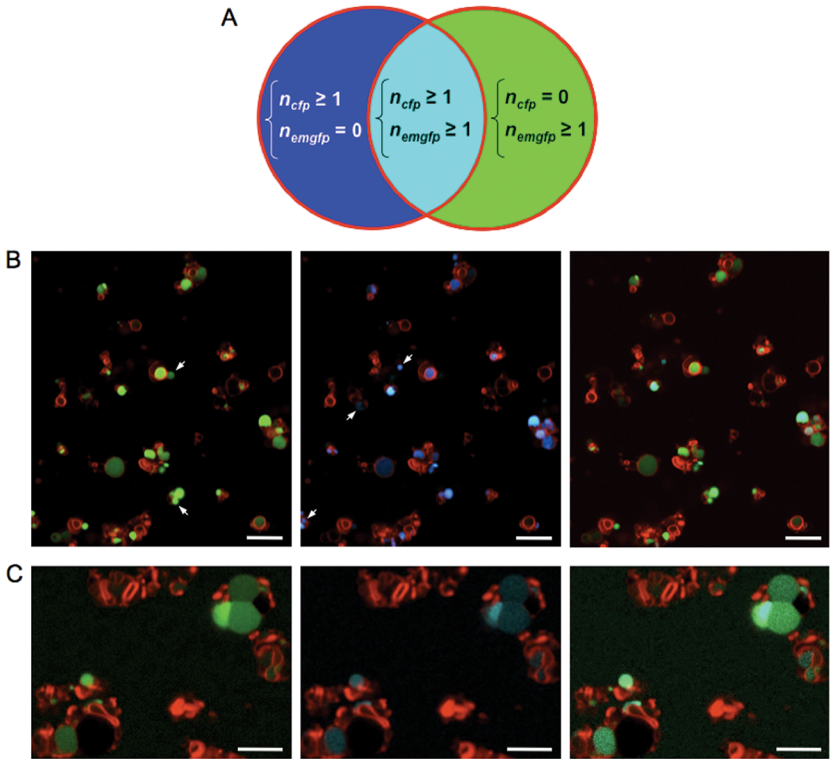

Figure 3. (A) Principle of the two-reporter expression assay. *n* indicates the number of transcriptionally active DNA copies entrapped in a protein synthesizing vesicle. The color coding (phenotype) relies on the expression of either (dark blue or green) or both (light blue) *cfp* and *emgfp* genes (genotype). (B, C) Fluorescence confocal images of surface-immobilized liposomes (red, TRITC-labeled lipids) incubated at 37 °C for 5 h after nutrient supply. The liposomes were produced in the presence of (B) 1.4 nM or (C) 14 nM of each of the two genes in the swelling buffer. (B, C) Left, GFP channel; Middle, CFP channel; Right, overlay. Scale bars are 10 μm. In (B), arrows indicate vesicles expressing either of the two genes.

nonlinearity in the amount of synthesized products with respect to DNA concentration. For instance, one can imagine the situation where a vesicle contains many DNA molecules but lacks RNA polymerases, which prohibits gene expression.

The intensity levels of both fluorescently labeled DNA and CFP proteins synthesized in situ were analyzed in individual surface-tethered vesicles fed during 2 or 16 h (Figure 2 and Figure S2, Supporting Information). While the large fraction of DNA-containing liposomes express proteins, the synthesis yield seems to be uncorrelated to the internal concentration of genes. These results clearly emphasize the nonlinearity of the gene expression process in fed-liposome microreactors, a consequence of the low abundance of some species.

Examining the dependency of CFP gene and output protein contents on the vesicle size does not reveal a striking relationship (Figure 2 and Figure S2, Supporting Information). The efficacy of protein synthesis inside vesicles fed from the external solution relies on two opposing contributions: large liposomes have a higher probability to entrap all reaction species (if one excepts possible crowding effects in particularly small vesicles), while their small surface to volume ratio makes solute exchange (uptake of nutrients and efflux of waste products) less efficient compared to small liposomes. Additionally, the kinetics of transcription and translation reactions may be enhanced in small volume compartments.[5,25,26] Therefore, an optimal size of protein synthesizing vesicles is to be expected,[25] which is not experimentally observed here, indicating that further mechanisms influence gene expression.

Having determined that the amount of DNA molecules, initially partitioned in vesicles following Poisson statistics, is redistributed upon supply of resources, and that no obvious correlation exists between the number of loaded DNA molecules and the yield of internal CFP production, we sought to establish the link between the concentration of DNA in the bulk suspension and the number of transcriptionally active genes in individual protein synthesizing liposomes. We designed a two-reporter assay that hinges on the stochastic color coding of individual vesicles through the expression of either or both *cfp* and *emgfp* genes entrapped in liposomes (Figure 3A). Equimolar amounts of the two linear DNA molecules were used. Here, any inhibitory effects (e.g., the lack of a translational cofactor or of an aminoacyl-tRNA synthetase, or inefficient feeding due to multilamellarity of the vesicle membrane) or promoting effects (e.g., high ribosome concentration) are expected to influence the expression of the *cfp* and *emgfp* genes similarly. In that manner, the displayed color(s) of individual liposomes (the phenotype) merely reflects the presence or absence of each of the two *active* (or apparent) genes (the genotype). For each DNA concentration studied, the probabilities of single-color and double-color vesicles were analyzed (Figure 4A) and compared to the expected values for Poisson statistics using the bulk DNA concentrations (Figure 4B). Liposomes that fail to express fluorescent proteins were not analyzed, while expressing vesicles were pooled in three size categories. To ensure that differences in the folding or maturation times of CFP and emGFP do not bias the measured probabilities, the vesicles were imaged 5 h after triggering gene expression.

As expected, the number of two-color (cyan and green) vesicles increases with higher DNA concentrations and larger diameters (Figure 3B,C, Figure 4A). DNA concentration is not a limiting factor for protein synthesis as long as the probability

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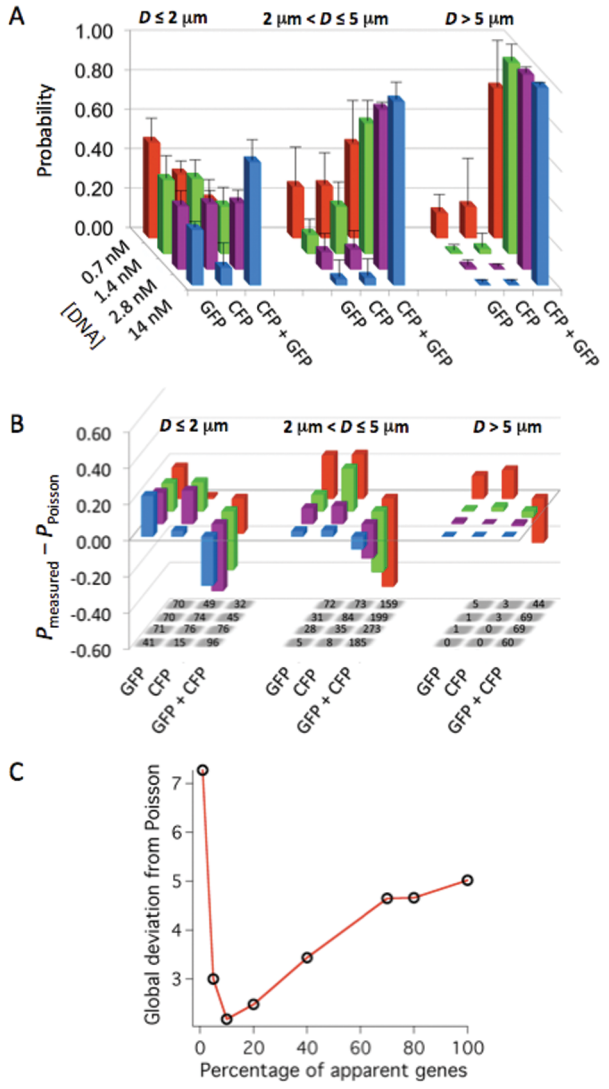

Figure 4. (A) Plot of the measured probabilities of CFP-, GFP- or CFP/GFP-expressing liposomes. Liposomes were incubated for 5 h at 37 °C subsequently to feeding supply. Equimolar amounts of *cfp* and *emgfp* genes were used; DNA concentrations refer to the bulk concentrations of each specific genes. Vesicles were pooled in three size categories: $D \leq 2$ μm, $2 < D \leq 5$ μm and $D > 5$ μm, where D is the liposome diameter. The total number of analyzed liposomes was 2122. Error bars indicate ± SEM; $n \geq 3$. (B) Plot of the deviation of the measured probability ($P_{measured}$) from the expected Poisson probability ($P_{Poisson}$). The numbers depicted in the mirrored shadow are the numbers of analyzed liposomes. Color coding is the same as in (A). (C) Global deviation between the measured and predicted probabilities assuming various percentages of apparent genes. Some of the corresponding probability deviation patterns are shown in Figure S4 (Supporting Information).

of two-color vesicles largely exceeds that of single-color liposomes. In that case, vesicles fail to express because of the lack of a constituent of the translational or aminoacylation machinery, or because inward diffusion of resources is not efficient enough for sustained expression. Only for liposomes smaller than 5 μm and bulk DNA concentrations lower than 2.8 nM does DNA copy number become a limiting factor for gene expression (Figure 4A).

Figure 4B shows that the measured probability pattern substantially deviates from that calculated with Poisson statistics of gene partitioning assuming that all DNA molecules are transcriptionally active. In particular, vesicles with diameters ≤5 μm display a higher probability of being single-colored than predicted. We then sought to predict the partitioning of apparent, transcriptionally active, genes. The minimum deviation between the experimental and theoretical values was obtained if one considers that around 10% of the bulk DNA concentration is entrapped in liposomes and is transcribable (Figure 4C). We showed in Figure 2 the redistribution of intraliposomal DNA molecules upon gene expression trigger. However, this effect alone cannot explain such a large decrease of the average number of apparent genes per vesicle. Although at present we cannot rule out inhibitory interaction between DNA and the membrane of <1-μm vesicles due to the lack of spatial resolution of the fluorescence microscope, this result suggests DNA inactivation due to some PURE system components confined into the liposomes. Interference of some cell-free system constituents with DNA transcription has already been suggested.[22] Nonspecifically bound proteins onto the DNA template can act as roadblocks for the progression of the RNA polymerase with deleterious consequences on the transcription activity. Collisions between the RNA polymerase and DNA-bound proteins can lead to pausing, stalling and even to dissociation of the molecular motor generating incompletely synthesized mRNA.[27,28] In addition, these nonspecific DNA binding proteins compete with the RNA polymerase to dock on the promoter region and thus hinder transcription initiation. While these effects can be marginal in bulk reactions (e.g., only the full length transcript was observed on an RNA gel indicating that premature disassembly of the transcription complex is insignificant; data not shown) they may become highly relevant in the context of a crowded macromolecular environment as invoked for protein synthesizing liposomes[12,14] and contribute to the reduced activity of the encapsulated genes. Even though the detailed mechanism behind DNA redistribution and possible inhibitory effects remain to be clarified, our results demonstrate that the probabilistic occupancy of functionally active genes inside individual vesicles can be assessed using a two-reporter expression assay and that predictable protein output patterns could be reached if one increases bulk DNA concentration to compensate for the low abundance of transcribable genes encapsulated.

The discrepancy sometimes observed between the measured probability values of CFP-only and GFP-only expressing vesicles could certainly be eliminated if more liposomes were analyzed. High-throughput fluorescence detection methods like flow cytometry, if performed in conjunction with liposome preparation methods avoiding vesicle agglutination, would greatly complement single liposome imaging approach.

In this work, we unveiled two important aspects that need to be considered for implementing the expression of multiple genes with predictable output in vesicle-based systems. First, we found no correlation between the amount of synthesized proteins and the intraliposomal DNA concentration. Second, we proved that the relative abundance of two phenotypes in protein synthesizing lipid vesicles reveals the apparent partitioning of two different active DNA templates confined in the reaction vessel, with the unexpected finding that only a small fraction of the bulk DNA molecules is encapsulated in a transcribable manner. This information, hardly tractable from single gene expression, underlines that the bulk DNA concentration needs to be adjusted to higher values to

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compensate for the postfeeding average loss of active genes in order to approach the desired phenotypic pattern.

The elaboration of cell-like functions in protein synthesizing liposomes asks for the coordinated expression of multiple genes. Cell-free regulatory circuits have been developed in test tubes[22,29,30] and lipid vesicles[29,31] laying the promise that harnessing a minimalist gene network, yet capable of coupling and regulating in time the expression of different proteins with specific functions, will soon be under reach. Although in vitro transcription/translation systems relying on cell extracts offer broader combination of transcriptional regulators than currently demonstrated with bacteriophage RNA polymerases, the potential of a PURE system-based platform to implement genetic circuits remains to be fully exploited. Notably, recent advances have been reported that expand the tools for gene expression modulation with the PURE system at the transcription[32,33] or translation[34,35] levels. Further necessary developments include the implementation of specific mRNA and protein degradation systems to enable complex temporal behaviors.

METHODS

Liposome Preparation. Surface-immobilized protein synthesizing lipid vesicles were prepared essentially as previously described,[12] with some modifications. Briefly, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DMPG, Avanti Polar Lipids), *N*-(6-tetramethylrhodamine-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TRITC-DHPE, Invitrogen), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG-biotin, Avanti Polar Lipids), all dissolved in chloroform, were mixed at a molar ratio 79:17:0.5:3.5 and added to 212−300-μm glass beads (Sigma-Aldrich) in a round-bottom glass flask at a mass ratio lipids/beads of 0.002:1. The solvent was rotary-evaporated at about 400 mbar overnight.

Around 10 μL of freshly prepared lipid-coated beads were poured in a reaction tube and immersed in 11.5 μL of swelling solution consisting of 7.5 μL of the PURE system (PURExpress, New England Biolabs) solution B, 2.5 μL of nuclease-free water, 0.5 μL of Superase inhibitor (10 units final, SUPERase.In, Ambion) and 1.0 μL of linear DNA templates to reach the desired final concentrations. Liposomes were formed at 30 °C for 2 h and then subjected to four freeze−thaw cycles.

Next, 1 μL of the liposome-containing solution was carefully harvested and immobilized at the bottom of a poly(dimethylsiloxane) chamber bound onto a microscope coverslip functionalized with BSA-biotin and neutravidin.[12] Lastly, the surface-tethered vesicles were diluted with 2 μL of the PURE system solution A and incubated at 37 °C.

DNA Constructs. Linear DNA templates encoding for the CFP or emGFP protein were prepared by polymerase chain reaction based on pRSET vectors (Invitrogen), as previously described.[12]

DNA Loading with YOYO-1 Dyes. CFP coding DNA was labeled with YOYO-1 iodide (1 mM solution in DMSO, Invitrogen). An equal volume of 10 μM YOYO-1 diluted in nuclease-free water was added to 260 ng/μL of CFP DNA (molar ratio of base pairs to YOYO-1 was 10) and the mixture incubated overnight at 4 °C. CFP-YOYO-1 solution was then diluted 40-fold in 10 mM Tris-HCl, pH 7.4, 100 mM KCl, and dialyzed to 10 ng/μL by using a 30-kDa filter that allows free YOYO-1 elimination. For the fluorescence imaging of DNA in liposomes, 1 μL of CFP-YOYO-1 was added to the swelling mixture.

Spectrofluorometry. The following solution was prepared on ice and transferred in a 15-μL cuvette (Hellma): 7.5 μL of PURExpress solution B, 10 μL of PURExpress solution A, 10 ng of CFP-YOYO-1 DNA template, 0.5 μL of Superase inhibitor and the volume was adjusted to 25 μL with nuclease-free water. The cuvette was mounted in the temperature-controlled holder of a fluorescence spectrophotometer (Cary Eclipse from Varian) set at 37 °C, and the fluorescence was recorded every 2 min (YOYO-1, Exc 490 nm, Em 510 nm; CFP, Exc 440, Em 475).

Fluorescence Imaging. A laser scanning confocal microscope (LSM710, Zeiss) equipped with a ×40 oil immersion objective was used with the following fluorescence settings: TRITC (Exc 543 nm, Em 553−797 nm), emGFP (Exc 488, Em 492−523 nm), CFP (Exc 458 nm, Em 465−483 nm) and YOYO-1 (Exc 488, Em 492−523). The same settings were used in all experiments to enable direct comparison. All measurements were done at room temperature (18 °C).

Image Analysis. The method of liposome preparation yields a rather heterogeneous population of vesicles, including unilamellar as well as multilamellar vesicles. In all conditions tested, we easily obtained tens (sometimes hundreds) of liposomes expressing in the same field of view under the microscope. All the analyzed liposomes present a clear membrane boundary and a hollow (lipid-free) lumen and are considered to be spherical. Filled lipid bodies do not express and were excluded from the analysis. Some vesicles with a well-defined lumen display higher membrane dye intensity reflecting the presence of a few lipid bilayers (although the presence of the different layers cannot be spatially resolved). Those vesicles were also analyzed in Figures 2 and 4A. As a design principle of our dual gene expression assay, the efficacy of molecular exchange across the liposome membrane equally influences the expression of both the *cfp* and *emgfp* genes, which does not bias the probability pattern of one-color and two-color vesicles (Figure 4A).

Fluorescence images were analyzed with the software ImageJ.[36] Single spherical liposomes were localized, and their lumen fluorescence intensity was determined as the averaged intensity inside subtracted from the background (outside) signal. For the dual gene expression assay, only CFP- or/and GFP-expressing liposomes were analyzed, i.e., those vesicles having a fluorescence intensity higher than background in at least one of the two channels. The diameter of the vesicle circular cross-section was considered to be the diameter of the liposome, D. Then, the analyzed liposomes were divided in three different size populations of $D \leq 2$ μm, $2 < D \leq 5$ μm, and $D > 5$ μm.

Statistical Analysis. Assuming that DNA molecules partition in liposomes according to the Poisson probability and that every encapsulated gene expresses its corresponding fluorescent protein, the fraction of vesicles producing only emGFP, CFP, or both genes are as follows:

$$P(\text{GFP}) = P(\text{CFP}) = e^{-\mu} \times (1 - e^{-\mu})$$

$$P(\text{GFP and CFP}) = (1 - e^{-\mu})^2$$

$$\mu = C \times V$$

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where μ is the expected average number of DNA molecules per liposome, C is the DNA concentration in the bulk solution, and V is the liposome volume. In Figure S3 (Supporting Information), the above-mentioned probabilities were plotted as a function of the vesicle diameter for different bulk DNA concentrations. In Figure 4B, the expected probability values were calculated within each of the three size categories by integrating over the corresponding volume range and normalized such that P(GFP) + P(CFP) + P(GFP and CFP) = 1.

Within each vesicle size category, the experimental probability values P(GFP), P(CFP), and P(GFP and CFP) were calculated by dividing the number of either or both of the protein synthesizing liposomes by the total number of protein synthesizing vesicles (Figure 4A).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary discussion and Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Z.N. designed and performed the experiments, analyzed the data, wrote part of the experimental section, and edited the manuscript. C.D. designed the experiments and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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