Cell-free Protein Synthesis through Solubilisate Exchange in Water/Oil Emulsion Compartments

Adriana V. Pietrini and Pier Luigi Luisi*[a]

This work is aimed at finding conditions under which synthetic compartments used as cell models can fuse with each other and allow reagents contained in the different compartments to react. This goal seems to be best achieved by the use of water in oil emulsions (w/o) with dimensions in the range of 30–60 μm. In particular, cell-free EGFP (enhanced green fluorescent protein) synthesis takes place in Tween 80/Span 80 w/o emulsions, and the extent of the reaction can be monitored directly by fluorescence. The medium is mineral oil, containing 0.5 % v/v aqueous solution. Different premixing configurations of the components (plasmid, amino acids, E. Coli *extract) are used and compared. The in vitro synthesis of EGFP in emulsion droplets proceeds for 1 h, and the yield is 7.5 ng μL^{-1} protein. EGFP synthesis in aqueous solution takes place for at least 5 h. The yield is 10.5 ng μL^{-1} protein after 1 h and 15.8 ng μL^{-1} protein after 5 h. The results with the w/o emulsions show that solubilisate exchange takes place among the different water droplets, but it is not possible to demonstrate clearly that a true fusion takes place.*

Introduction

Over the last few years, the field of cell models has acquired growing importance. The general idea is to utilise closed spherical compartments to host molecular biological reactions, so as to simulate certain aspects of the biochemical behaviour of biological cells. The most classic approach involves liposomes, such as the previously described systems in which liposomes host the polymerase chain reaction,[1] the replication of a RNA template catalysed by Q-beta replicase,[2] the polymerisation of ADP to poly (A)[3,4] and even the expression of polypeptides in ribosome-containing liposomes.[5]

In addition to liposomes, reverse micelles have also been utilised as microcompartments for biological reactions. A large series of enzymatic reactions in reverse micelles has been described in recent years.[6–8] Levashov and co-workers, using Brij 96 in cyclohexane, were able to solubilise a cell extract in reverse micelles and to show the cell-free synthesis of the protein IL-2.[9]

Reverse micelles, or microemulsions, offer the advantage of being thermodynamically stable systems, but their aqueous compartments are generally very small and, in fact, reverse micelles have not been used to investigate intermicellar reactions involving macromolecules solubilised in different compartments. In contrast to microemulsions, which have dimensions ranging between 2 and 10 nm, w/o (water in oil) emulsion compartments offer the advantage of significantly larger size, with radii of the order of 10–50 μm. That such compartments can be used for hosting molecular biology reactions has recently been demonstrated by Tawfik et al.,[10–12] who described the cell-free synthesis of several proteins in w/o emulsion droplets with a variety of surfactant systems. Again, as in the case of Levashov's experiment, detection was possible only after breaking the emulsion at the end of the experiments.

Generally in these experiments, all reagents have to be present initially in the same compartment. As already mentioned, the goal of this work was instead to find conditions under which biological reactions result as the consequence of fusion of different compartments, each containing different reagents at known concentration and activity. This approach would be particularly suitable for studies in the field of the "minimal cell",[2,13] the construction of a semi-artificial, living cell. In fact, the potential reconstitution of the complexity of a cell through fusion of different compartments may permit one to understand what is the critical minimum number of components necessary to elicit cellular life, and by modulation of the concentration of the components it may be possible to achieve a new way of controlling cellular activity. This kind of fusion experiment may also represent a model for symbiogenesis—by which higher molecular complexity can be reached through the integration of two different, simpler cellular compartments.

We will see in this work that fusion is partly possible with w/o emulsions. There are already some indications in the literature that solubilisate exchange between w/o emulsion droplets is possible. Solubilisate-exchanging emulsions have been used, for example, to investigate the conjugation of F+ and F− strains of *E. Coli* resident in separate w/o emulsion droplets.[14] It was also reported that small molecules, including negatively charged dNTP, diffused between emulsion droplets during thermocycling of a polymerase chain reaction in w/o emulsions.[15]

[a] *Dr. A. V. Pietrini, Prof. Dr. P. L. Luisi*
Institute of Polymers, ETH Zentrum
Universitätsstrasse 6, 8092 Zürich (Switzerland)
Fax: (+41) 1-632-10-73
E-mail: luisi@mat.ethz.ch

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Results and Discussion

Setting up the system

Water in oil (w/o) emulsions may appear to have little biological significance, since hydrocarbons (the oil) are not biological solvents. However, this system has the advantage of forced compartmentalisation for hydrophilic compounds, as they are forcibly entrapped in the water pool. W/o emulsions therefore offer well defined localised microenvironments.

As already mentioned, the slant of this paper is in the perspective of the construction of a minimal cell model, through the mixing of different cellular components with each other. Since the term "fusion" is central to this kind of work, we would first like to define this term, also because of a certain degree of confusion present in the literature.

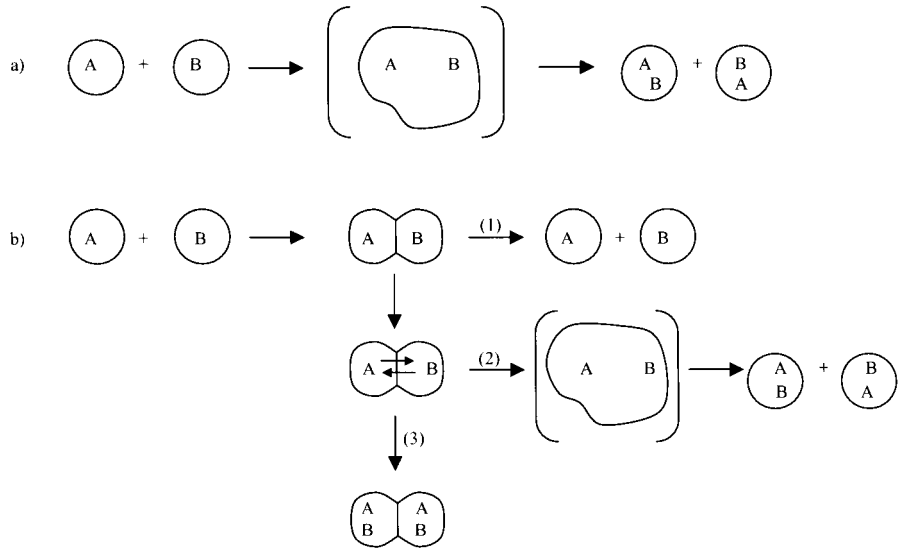
When two compartments come into contact with each other through collisions, quite different events may occur. In the case of reverse micelles (RMs), which are known as very dynamic, mobile systems, there is generally an exchange of the water pool contents upon collision, followed by the formation of reverse micelles of about the same, thermodynamically more stable, size (see Scheme 1 a).[7,17] This rapid and easy exchange of water pool content has been extensively utilised for enzymatic reactions in RMs, as in, for example, the mixing of enzyme-containing RMs with substrate-containing RMs.[18,19] Despite this dynamic behaviour, solubilisate exchange involving macromolecules (e.g., proteins with nucleic acids, or large proteins with large proteins) has not been studied, as it appears that the diffusion of macromolecules from one RM to another RM is not as easy as the diffusion of low molecular weight compounds.

Another kind of event accompanying compartment collision is shown in Scheme 1 b: here, two compartments adhere to each other through one or two layers,[20] and at this point various things may happen. This complex system may collapse back to the original situation without solubilisate exchange, as shown recently in the case of phospholipid liposomes,[21] or there may instead be some solubilisate exchange through the contact region, without a real fusion of the two compartments. Alternatively, there can be a fusion, as in the case of charged phospholipid liposomes.[22] It may be possible to follow fusion through the formation of a different particle size distribution. An increase in size upon fusion, or a change in the size distribution, can generally be easily detected by light scattering.

Reaction after collision of compartments might thus be due either to fusion or to solubilisate exchange without real fusion. At any rate, the term "fusion" is often utilised in the literature without discriminating between these two cases, and often without it being made clear what is going on physically. Of course, it is not always easy to discriminate between all these mechanisms, but it is important in mind to keep all these various possibilities.

In this paper we limit ourselves to the expression of a protein (the enhanced green fluorescence protein, EGFP) through the mixing of different compartments containing the different reagents. We use water in oil (w/o) emulsion droplets with radii in the range of 10–100 μm, generally with mixtures of Span and Tween as surfactants and mineral oil as the oil.

First we describe the utilised emulsion system in detail. It is similar to that described by Tawfik et al.,[10] and the utilised w/o emulsion system is made up of a Span 80 (0.45 % *v/v*)/Tween 80 (0.05 % *v/v*)/aqueous solution (0.5 % *v/v*) in mineral oil. The w/o emulsions are prepared by the direct injection method, the aqueous stock solution being added to a stirred solution of Span 80 (0.45 % *v/v*)/Tween 80 (0.05 % *v/v*) in mineral oil.



Scheme 1. *Schematic presentation of possible mechanisms of solubilisate exchange between compartments. a) Solubilisate exchange is mediated by transient fusion of two droplets and consequent splitting into two emulsion droplets. b) Two droplets adhere to form a common boundary. These adhesive droplets may split into two droplets again without solubilisate exchange (1). Alternatively, they may exchange solubilisates through diffusion through the common boundary, either with (2; the adhesive droplets may fuse before the subsequent splitting) or without (3) subsequent splitting of the adhesive droplets.*

The size distribution of this emulsion system is highly polydisperse. The droplets' sizes, as investigated by light microscopy (see Figure 1), are between 1 and 100 μm, but the vast majority are smaller than 30 μm.

A note should be made concerning the concentrations of reagents in these systems. One could consider two different kinds of concentration: one overall, which refers to the total volume of solution (water plus oil), and a local one referring to the concentration of reagents in the aqueous phase. The concentrations indicated in this work are local ones, as the concentration in water is the relevant one

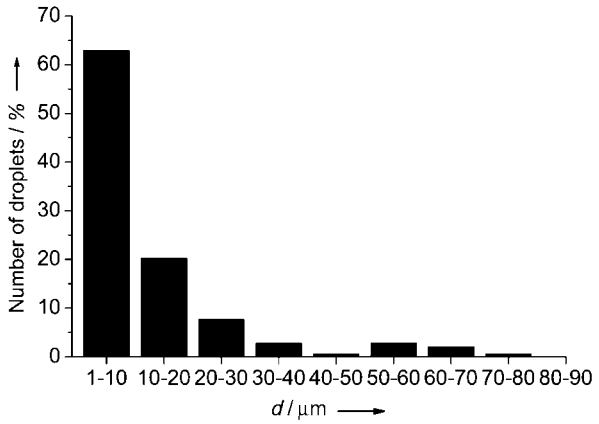


Figure 1. *Diameters of Span 80 (0.45% v/v)/Tween 80 (0.05% v/v)/aqueous solution (0.05% v/v)/mineral oil emulsion droplets visible on a typical micrograph.*

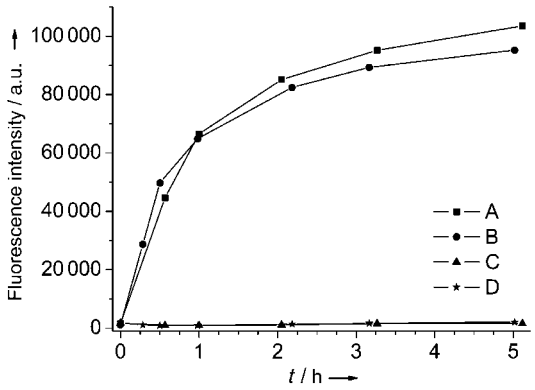


Figure 2. *Cell-free EGFP synthesis in aqueous solution at 37°C; samples are diluted 1:56 v/v with water prior to measurements, excitation at 488 nm, emission max. at 509 nm, $T_m = 25$°C, fluorescence intensity (I) vs. time (t). A) and B) in vitro EGFP synthesis (two independent experiments), C) and D) negative control (two independent experiments).*

for reaction. The concentration in the water phase should generally be the same as in the stock solution used to prepare them.

Protein expression in w/o emulsion droplets

Control system: We now consider a biological reaction, in vitro protein synthesis, taking place because of solubilisate exchange between w/o emulsion droplets. In particular, we consider the expression of the EGFP, utilising a commercially available extract system for cell-free protein expression (*E. Coli* T7 S30 extract system for circular DNA), with addition of the plasmid containing the EGFP gene (pWM T7 EGFP) and the amino acids.

The cell-free EGFP synthesis is first run in water, in order to provide a reference system. A mixture of pWM T7 EGFP, the 20 standard proteinogenic amino acids and extract is used for the in vitro EGFP synthesis, whereas a mixture of extract, 20 amino acids and the plasmid cloning vector pBR322 is chosen for the negative control. The plasmid pBR322 is a cloning vector lacking the T7 promoter and terminator and pBR322 does not carry a gene for cell-free protein synthesis. Therefore, no gene of this vector should be transcribed by T7 RNA polymerase present in the *E. Coli* T7 S30 extract system for circular DNA.[23]

The kinetics of EGFP synthesis in aqueous solution and its negative control are illustrated in Figure 2. The EGFP synthesis is faster during the first hour after sample preparation, but continues at a lower rate for several hours afterwards. The fluorescence intensity of the negative control is 50 times lower than that of the EGFP synthesis sample after five hours' incubation, so the background fluorescence of the samples is negligible. Two sets of independent experiments show that the data for the EGFP synthesis and for the negative control are reproducible; that is, the difference is below 10%.

Please note that the same arbitrary units (a.u.) for fluorescence intensities are used throughout this work to ensure the comparability of the results depicted in different graphs.

It was also investigated whether the EGFP synthesis occurs at a low temperature such as 4°C. In fact, there is no EGFP synthesis within 7 h when samples are prepared and incubated at 4°C (see Figure 3); the fluorescence intensity values for the EGFP synthesis at 4°C and the negative control at 4°C are alike. The emulsion preparation at 4°C for EGFP synthesis in emulsion droplets ensures that EGFP is not synthesised during the emulsification.

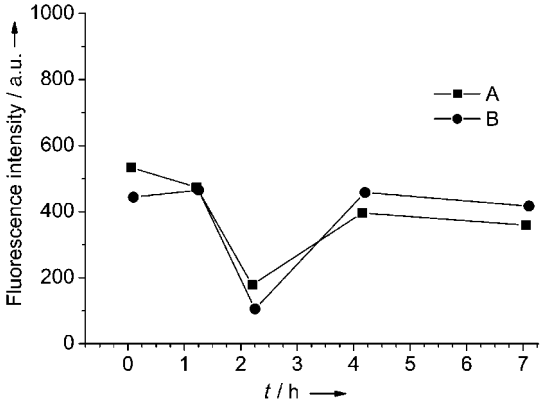


Figure 3. *Cell-free EGFP synthesis does not take place in aqueous solution at 4°C, fluorescence intensity (I) vs. time (t). A) Mixture for cell-free EGFP synthesis with plasmid pWM T7 EGFP, and B) negative control, mixture for cell-free protein synthesis with plasmid pBR322. Samples were diluted 1:200 v/v with water prior to measurements, excitation at 488 nm, emission max. at 509 nm, $T_m = 25$°C.*

The calibration curves in Figure 4 allow us to determine the quantity of protein synthesised by reading the fluorescence intensity in aqueous solution and in emulsion droplets. Extract, 20 amino acids and the plasmid pBR322 at the usual concentrations were mixed with known amounts of commercially available EGFP for the aqueous solution calibration curve, and the fluorescence of these mixtures was then measured. Extract, 20 amino acids and the plasmid pBR322 were added to ensure the same background fluorescence as in the case of the in vitro EGFP synthesis. The plasmid pBR322 was used instead of pWM T7 EGFP to prevent the synthesis of EGFP. The same

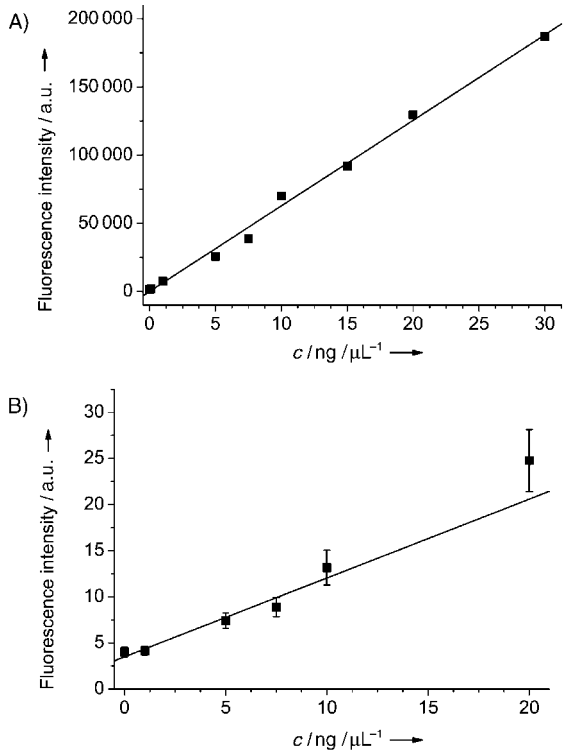


Figure 4. *Calibration curve of ng μL^{-1} EGFP in: A) aqueous solution, samples were diluted 1:56 v/v with water prior to measurement, excitation at 488 nm, emission max. at 509 nm, $T_m = 25$ °C, B) emulsion droplets (local concentration), fluorescence intensity (I) vs. ng μL^{-1} EGFP, on average 10 droplets with diameters of 30–60 μm were evaluated per EGFP concentration.*

aqueous mixture was used for the emulsion droplets calibration curve, but the aqueous mixture was emulsified in the usual organic surfactant solution and micrographs of the emulsion were recorded. The emulsion droplets calibration curve was obtained by electronic evaluation of the fluorescence intensity of the droplets on the micrographs. The amount of EGFP synthesised is presented in Table 1. The linear relationship of fluorescence intensity and EGFP concentration in aqueous solution (obtained from Figure 4A) is Equation (1):

Table 1. *Amounts of EGFP synthesised in emulsion droplets and in aqueous solution.*

In emulsion droplets		In aqueous solution	
t [h]	ng μL^{-1} EGFP	*t* [h]	ng μL^{-1} EGFP
0	0.8 ± 0.9	0	0.2 ± 0.3
0.5	5.2 ± 1.2	0.5	7.5 ± 0.7
1	7.5 ± 1.8	1	10.5 ± 0.5
4	7.4 ± 1.8	5	15.8 ± 1.1

$$\text{ng}\,\mu\text{L}^{-1}\ \text{EGFP} = \frac{\text{fluorescence intensity in a.u.} + 5.0 \pm 2072}{6278 \pm 159} \quad (1)$$

The linear relationship of fluorescence intensity vs. EGFP concentration in emulsion droplets (obtained from Figure 4B) is Equation (2):

$$\text{ng}\,\mu\text{L}^{-1}\ \text{EGFP} = \frac{\text{mean fluorescence intensity in a.u.} + 2.72 \pm 0.74}{1.06 \pm 0.08} \quad (2)$$

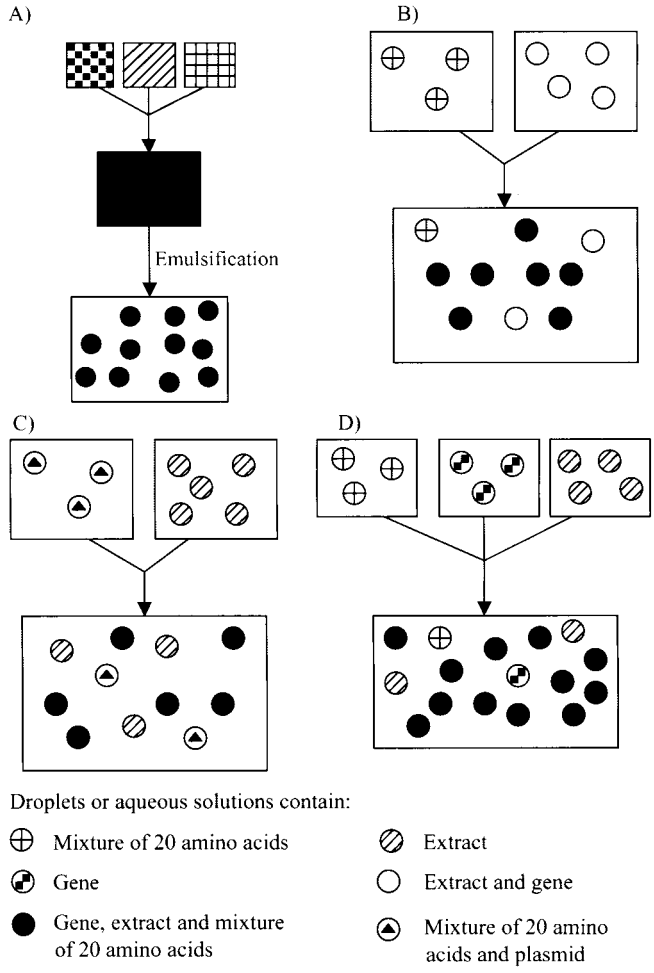
Batch synthesis in w/o emulsions: In this experiment, cell-free EGFP synthesis in emulsion droplets can take place without solubilisate exchange, because all the required compounds for cell-free EGFP synthesis should be present in each emulsion droplet. The data obtained in this way are an important reference system for the next section, dealing with fusion of compartments.

The used plasmid concentration of 80 ng μL^{-1} aqueous solution and the used amino acid concentration of 0.2 mM (local concentration of each amino acid) seem to be rather low. At these concentrations, though, an emulsion droplet with a diameter of 30 μm contains on average 3×10^6 pWM T7 EGFP molecules (3026 bp) and 1×10^7 molecules of each amino acid, and even an emulsion droplet with a diameter of 60 μm contains on average 2×10^7 pWM T7 EGFP molecules and 1×10^8 molecules of each amino acid. There should therefore be plenty of plasmid and amino acids in each emulsion droplet with dimensions equal to or larger than 30 μm in size. The manufacturer has not disclosed the contents of the extract (Premix and S30-extract) and its concentrations, hence it is not possible to make such calculations for the compounds in the extract.

The required aqueous components for cell-free EGFP synthesis are combined in emulsion droplets. This aqueous mixture is then emulsified in an organic surfactant solution. A schematic presentation of the experimental set-up for this "batch" cell-free EGFP synthesis in emulsion droplets is depicted in Scheme 2A.

The time course of the "batch" cell-free EGFP synthesis in emulsion droplets is examined. Typical micrographs are shown in Figure 5A, and the kinetics of three independent experiments are shown in Figure 5B. An identical experiment, in which all was the same except that the EGFP-encoding plasmid pWM T7 EGFP was exchanged for the plasmid-cloning vector pBR322, was chosen as a negative control. Two independent negative control experiments did not display any fluorescence up to 21 h. This was visible by eye in the micrographs and was reconfirmed by computer evaluation of the emulsion droplets on the micrographs. The EGFP synthesis in emulsion droplets continued for 45–60 min, and the fluorescence intensity remained constant from 60 min up to 21 h. EGFP was synthesised in aqueous solution for at least 5 h, although the synthesis was faster during the first hour after sample preparation than later on. A couple of independent experiments showed that the results of the EGFP synthesis in aqueous solution and in emulsion droplets and of the negative controls were reproducible.

Only droplets in the size range 30–60 μm were evaluated electronically because the fluorescence of the synthesised EGFP in emulsion droplets is dependent on the droplet size. The fluorescence of EGFP in the emulsion droplets was too weak for droplets below 30 μm in diameter to be evaluated.



Scheme 2. *Setup for cell-free EGFP synthesis in w/o emulsion droplets. A) "Batch" case. B) Solubilisate exchange between two separate emulsions: two separate emulsions, amino acids dissolved in one emulsion and extract (= Premix and S30-extract) and gene dissolved in another separate emulsion, C) two separate emulsions, amino acids and gene dissolved in one emulsion and extract dissolved in another separate emulsion, D) solubilisate exchange between three separate emulsions, amino acids dissolved in one emulsion, gene dissolved in another separate emulsion, and extract dissolved in another separate emulsion.*

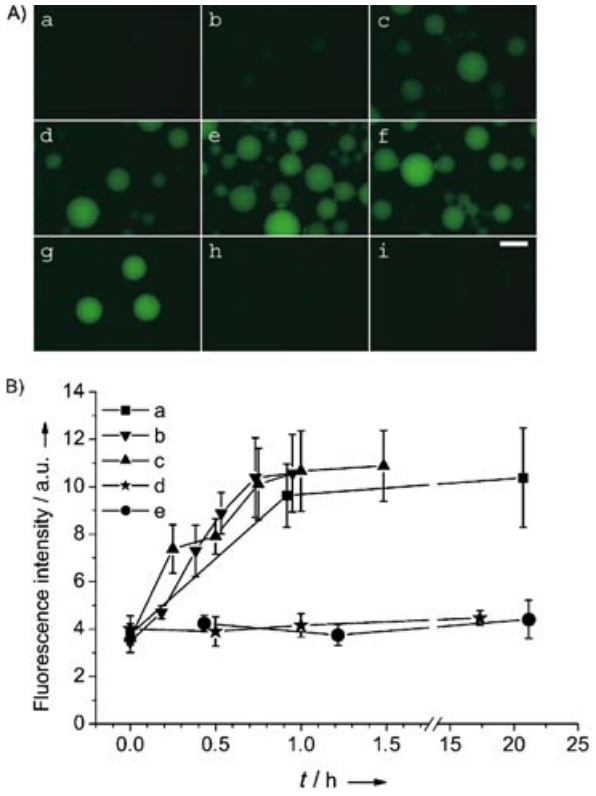


Figure 5. *A) Typical micrographs of the cell-free EGFP synthesis in Span 80 (0.45 % v/v)/Tween 80 (0.05 % v/v)/aqueous solution (0.5 % v/v) in mineral oil emulsion droplets, preparation at 4 °C, incubation at 37 °C: a) 0 min, b) 11 min, c) 23 min, d) 32 min, e) 44 min, f) 57 min, g) 21 h. Negative control: h) 0 min, i) 21 h. The bar represents 50 μm. B) Kinetics of the cell-free EGFP synthesis in emulsion droplets, on average 10 droplets with diameters of 30–60 μm are evaluated per time point, cell-free EGFP synthesis in emulsion droplets (a, b and c are three independent experiments) and negative control (d and e are two independent experiments).*

The calculated EGFP concentrations of the EGFP synthesis in aqueous solution and in emulsion droplets at several time points are presented in Table 1. The most striking differences if the data in Figure 2, Figure 5 and Table 1 are compared are that higher EGFP concentrations are obtained in aqueous solution and that the EGFP synthesis proceeds for a longer time period in water than in the emulsion droplets. The EGFP synthesis in emulsion droplets proceeds for 1 h, with 5ng μL^{-1} EGFP synthesised within 30 min and 7.5 ng μL^{-1} within 1 h. The EGFP synthesis is faster in bulk aqueous solution than in emulsion droplets. In the case of bulk aqueous solution, 7.5 ng μL^{-1} EGFP is synthesised within 30 minutes, 10.5 ng μL^{-1} within 1 hour, and 16 ng μL^{-1} within 5 h. The amount of EGFP synthesised in emulsion droplets is 30% lower than in bulk aqueous solution during the first hour after sample preparation. The detection limit of EGFP in emulsion droplets is below 5 ng μL^{-1}.

Reaction by "fusion" of compartments: Let us now consider the case of the cell-free EGFP synthesis on mixing different compartments with each other. Here the synthesis can only occur if there is fusion, or at least solubilisate exchange. It must be said that under our conditions it is very difficult to discriminate between the two extreme cases. We have not observed a clear increase in size of the droplets—but given the broad dispersion of sizes, this is not proof of the lack of real fusion as indicated in Scheme 1 a. We do have reaction, however, and so we at least have solubilisate exchange. This is why we use the term "fusion" in quotation marks in this section.

The experiments were conducted with three different kinds of components: the extract (that is, Premix and S30-extract), the plasmid and the amino acids. Different mixing combinations are possible, and some of them are schematised in Scheme 2 B–D.

Solubilisate exchange between w/o emulsion droplets was investigated in Span 80 (0.45 % *v/v*)/Tween 80 (0.05 % *v/v*)/aqueous solution (0.5 % *v/v*) in mineral oil emulsion droplets.

The ingredients necessary for the EGFP synthesis in aqueous solution—that is, the extract, 20 amino acids and the plasmid pWM T7 EGFP—were added alone or together with another

ingredient to separate organic surfactant solutions and were emulsified. These two or three separate emulsions were combined, gently mixed and then examined for synthesised EGFP. The synthesis of EGFP—that is, green fluorescence—indicates either that solubilised macromolecules are exchanged between emulsion droplets, or that there is real fusion between emulsion droplets.

The overall concentration of the components in the aqueous phase seems to be a critical parameter for the cell-free protein synthesis. It has been found before that the cell-free EGFP synthesis does not work if the sample is diluted with water prior to the start of the cell-free EGFP synthesis.[24] Conversely, the cell-free EGFP synthesis continues when the reaction mixture is diluted after the start of the reaction (M. Voser, personal communication). The same overall concentrations of required components in the aqueous phase as were used in the "batch" case were therefore chosen.

Solubilisate exchange experiments involving two separate emulsions were conducted. In one experiment, a mixture of 20 amino acids (2 mM each) was emulsified in one organic surfactant solution. The rest of the required ingredients for protein synthesis (90 ng μL^{-1} pWM T7 EGFP, Premix diluted 4:5 *v/v* and S30-extract diluted 1:2 *v/v*) were emulsified in a separate organic surfactant solution (for setup see Scheme 2B). One volume of the former emulsion was combined with nine volumes of the latter emulsion. In another experiment, a mixture of pWM T7 EGFP plasmid (0.4 $\mu g\,\mu L^{-1}$) and 20 amino acids (1 mM each) was emulsified in an organic surfactant solution, and Premix (diluted 1:1 *v/v*) and S30-extract (diluted 3:5 *v/v*) were emulsified in a separate organic surfactant solution (for set-up see Scheme 2C). One volume of the former emulsion was combined with four volumes of the latter emulsion. Some droplets with strong green fluorescence, some with weak green fluorescence, and some with no fluorescence at all were observed in both experiments after a one-hour incubation at 37 °C (see Figure 6).

For a solubilisate-exchange experiment involving three separate emulsions (for set-up see Scheme 2D), the plasmid pWM T7 EGFP (0.8 $\mu g\,\mu L^{-1}$) was emulsified in one organic surfactant solution, a mixture of 20 amino acids (2 mM each) was emulsified in a second organic surfactant solution, and a mixture of Premix (diluted 1:1 *v/v*) and S30-extract (diluted 3:5 *v/v*) was emulsified in a third organic surfactant solution. These three separate emulsions were combined at a volume ratio of 1:1:8 in the order mentioned above. The concentrations in the aqueous part were chosen in such a way that after the combination of the three different emulsions, the overall concentrations in the aqueous part were the same as in the "batch" case. The same experimental set-up was used for the negative control, but the plasmid pWM T7 EGFP was replaced by the plasmid pBR322. Several micrographs are depicted in Figure 7A. The micrographs show that there were some droplets

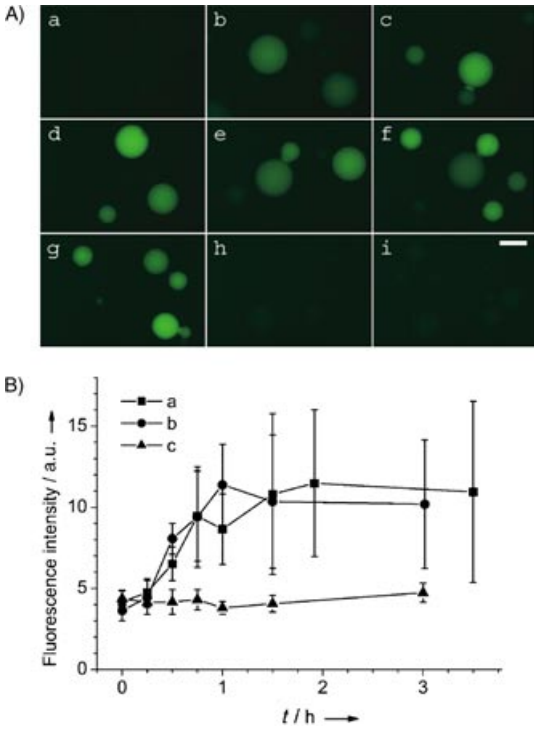


Figure 7. *Solubilisate exchange between Span 80 (0.45% v/v)/Tween 80 (0.05% v/v)/aqueous solution (0.5% v/v) in mineral oil emulsion droplets; one emulsion contained pWM T7 EGFP plasmid, another emulsion contained a mixture of 20 amino acids, and the third emulsion contained a mixture of Premix and S30-extract, combination of these three emulsions at a volume ratio of 1:1:8, preparation at 25 °C, incubation at 37 °C. A) Typical micrographs: a) 0 min, b) 15 min, c) 30 min, d) 45 min, e) 60 min, f) 90 min, g) 3 h. Negative control: h) 0 min, i) 3 h. The bar represents 50 μm. B) Kinetics of solubilisate exchange between w/o emulsion droplets: a) and b) are two independent experiments, c) negative control, an identical experimental set-up but with plasmid pBR322 used instead of the plasmid pWM T7 EGFP. On average, 10 droplets with diameters of 30–60 μm were evaluated per time point.*

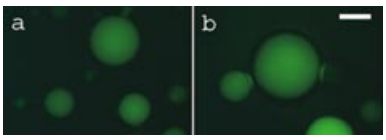


Figure 6. *Solubilisate exchange between droplets of two separate emulsions (Span 80 (0.45% v/v)/Tween 80 (0.05% v/v)/aqueous solution (0.05% v/v) in mineral oil), preparation at 4 °C, incubation for 1 h at 37 °C. a) One emulsion contained a mixture of 20 amino acids and another separate emulsion contained a mixture of pWM T7 EGFP, Premix and S30-extract, with combination of these two emulsions at a volume ratio of 1:9, b) one emulsion contained a mixture of 20 amino acids and pWM T7 EGFP plasmid, another, separate emulsion contained a mixture of Premix and S30-extract, with combination of these two emulsions at a volume ratio of 1:4. The bar represents 50 μm.*

with strong fluorescence, others with weak fluorescence and some others with no fluorescence at all. Hence, in some droplets a large amount of EGFP was synthesised, in others only a small amount, and in some other droplets no or no detectable amount of EGFP was formed. The kinetics of two independent solubilisate exchange experiments are depicted in Figure 7B. The huge error bars in Figure 7B express the same result. The kinetics shown in Figure 7B indicate that EGFP was synthesised up to about one hour.

It is rather surprising that EGFP was synthesised for about the same period of time in a solubilisate-exchange experiment

as it was in the case of the "batch" EGFP synthesis in emulsion droplets, where all the components required for the protein synthesis are first mixed and then dispersed in an organic surfactant solution (see Figure 5B). The process leading to the synthesis of EGFP—that is, solubilisate exchange between emulsion droplets—thus seems to be on a rather short timescale, such as seconds or minutes.

The average fluorescence intensities of EGFP in emulsion droplets in the "batch" case and in the case of droplet solubilisate exchange between three separate emulsions are of the same order. This is an unexpected finding, because in the case of solubilisate exchange some droplets display bright fluorescence, some weak fluorescence and some no fluorescence at all. In the "batch" case, the fluorescence intensity is more uniformly distributed throughout the droplets than in the case of solubilisate exchange between three separate emulsions with its huge error bars. Consequently, the fluorescence intensity in some droplets of the "fusion" case must be larger, namely more EGFP is synthesised in them than in the "batch" case. In turn, this suggests that the concentration statistically established in these droplets is more favourable—perhaps simply higher—than in the rest of the droplets. The finding of the same kinetics for the "batch" case and for the "fusion" case also suggests that, for the solubilisate exchange reaction, the fusion rate and consequent diffusion rate of the reagents is not the rate-limiting step but that the biochemical transformation itself is the rate-limiting step.

The conclusions are that Span 80 (0.45% *v/v*)/Tween 80 (0.05% *v/v*)/aqueous solution (0.5% *v/v*) in mineral oil emulsion droplets exchange solubilised macromolecules, and that the cell-free protein synthesis is a suitable reaction with which to investigate solubilisate exchange between w/o emulsion droplets. Also, the solubilisate exchange between emulsion droplets must be very fast, because there are no significant differences between the time courses of cell-free EGFP synthesis in the "batch" case and in the "fusion" case.

Conclusion

The main result of this investigation is that cell-free EGFP synthesis occurs as the result of solubilisate exchange, or fusion, among droplets in w/o emulsions. The question of the mechanism of solubilisate exchange among the different compartments—namely the question about the definition of the term "fusion" and its detection—remains open and worth further study.

From the biophysical point of view, the data confirm that the emulsion droplets exchange macromolecular solubilisates, and that the synthesis of EGFP takes place upon dispersion of the required aqueous components in two or three separate emulsion compartments that are combined afterwards. Such a synthesis may therefore also work when the required components are dispersed in more than three separate emulsions. An open question is: what is the maximum number of separate emulsions that still allow cell-free protein synthesis after solubilisate exchange between emulsion droplets? This may allow modulation of complex biochemical reactions in various compartments.

The point of mixing many components is indeed interesting for the case of EGFP synthesis, but it is even more so for more complex systems, as in the case of the reconstitution of a prokaryotic cell. Can one achieve the reconstitution of a simple living cell by mixing the various cell components with each other? And how many of them are necessary to have a minimal cell, the simplest cellular system capable of cellular life (i.e., capable of self-maintenance, self-reproduction, and mutation)? Although this is still a far-away target, we consider this work an important step towards the reconstitution of a minimal cell. By inference, this kind of system may also be useful as a simple model of symbiogenesis.

Experimental Section

Materials: Span 80 was purchased from Fluka (Buchs, Switzerland). Tween 80 (SigmaUltra approx. 99%) and mineral oil (for molecular biology, M-5904) were obtained from Sigma (St. Louis, MO, US). Plasmid pBR322 (4361 bp) was purchased from New England BioLabs, Inc. (Beverly, MA, US). *E. Coli* T7 S30 extract system for circular DNA was obtained from Promega Corporation (Madison, WI, US). Recombinant EGFP was obtained from BD Biosciences (Clontech, Basel, Switzerland). The plasmid pWM T7 EGFP (3026 bp) and a mixture of the 20 standard proteinogenic amino acids were bought from BioTecon (Zürich, Switzerland). The construction of the plasmid pWM T7 EGFP is described elsewhere.[16]

Methods: Preparation of Span 80 (0.45% *v/v*)/Tween 80 (0.05% *v/v*)/mineral oil solution: Span 80 (447 mg) was placed in a test tube (10 mL), and mineral oil (9.5 mL, 8.0 g) was added. This mixture was vortexed. Tween 80 (54 mg) was added, and the test tube was vortexed again. This solution was 4.5% *v/v* Span 80/0.5% *v/v* Tween 80 in mineral oil. Less concentrated surfactant mineral oil solutions were obtained by dilution of Span 80 (4.5% *v/v*), Tween 80 (0.5% *v/v*)/mineral oil solution; that is, Span 80 (0.45% *v/v*)/Tween 80 (0.05% *v/v*)/mineral oil was obtained by addition of mineral oil (9 mL) to Span 80 (4.5% *v/v*) /Tween 80 (0.5% *v/v*)/mineral oil solution (1 mL).

The standard procedure for emulsion preparation was as follows: freshly prepared Span 80 (0.45% *v/v*)/Tween 80 (0.05% *v/v*)/mineral oil solution (2 or 3 mL) was stirred at 1500 rpm with a magnetic bar (1.5×0.6 cm) in a glass vial (5 mL, cylindrical with an inner diameter of 1.7 cm). Aqueous solution (10 or 15 μL) was added in aliquots (5 μL) to the stirred surfactant oil solution over 2 min. The stirring was continued for another minute. The aqueous solution consisted of the mixture necessary for the cell-free protein synthesis; that is, each of the 20 amino acids (0.2 mM), plasmid (80 ng μL^{-1}), Premix diluted 2:3 *v/v*, and S30-extract diluted 3:7 *v/v*. The plasmid pWM T7 EGFP was used for the cell-free EGFP synthesis experiments and the plasmid pBR322 was used for the negative control experiments.

The preparation of the mix for cell-free protein synthesis and the emulsification was carried out at 4°C for the "batch" experiment and the "solubilisate-exchange" experiments involving two separate emulsions. The aqueous phase was prepared on ice and was emulsified at ambient temperature for the "solubilisate-exchange" experiments involving three separate emulsions. Emulsions containing the mix for cell-free protein synthesis were incubated at 37°C in aliquots protected from light. Fluorescence micrographs

were collected at various time points with separate aliquots. An inverse light microscope of the Axiovert 135TV type (Carl Zeiss AG, Switzerland) was used to collect micrographs. Live pictures were obtained with a CCD C5810 colour camera (Hamamatsu, Japan) and were recorded with the help of the programme HpdCpx (Hamamatsu, Japan). A LD-Achroplan objective with a 20:1 magnification and a numerical aperture of 0.4 was used. The exposure time was 1.04 s.

For fusion experiments the required components were emulsified in separate surfactant oil solutions and were combined afterwards. Aliquots were incubated with protection from light. Fluorescence micrographs were collected at various time points with separate aliquots.

Reference experiments for the in vitro transcription and translation of pWM T7 EGFP were carried out in aqueous solution. The plasmid pWM T7 EGFP was replaced by pBR322 for the negative control experiments. The required components were mixed at the usual concentrations and the mixture was incubated at 37 °C. An aliquot (7.2 μL) was diluted with water (400 μL) and fluorescence emission was measured at various time points (slits of the fluorimeter were set to 1/1/3/3). Fluorescence spectra were collected on a SPEX Fluorolog F112XE instrument with the help of the dm3000 software package (SPEX Industries, Inc., Edison, N.J., US). Quartz cells (500 μL, Hellma, Germany) with path lengths of 0.5 cm were used.

The amount of synthesised EGFP in aqueous solution and in emulsion droplets was estimated with the help of calibration curves. Samples for the calibration curves were prepared like those for the negative control, but specified amounts of commercially available EGFP were added.

Keywords: biosynthesis · colloids · fusion · green fluorescence protein · microreactors

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