Cell free expression in emulsions and vesicles

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Nadezda Monina

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Vincent Noireaux

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Abstract

In this study we address the effects that membrane composition has on expression within two common forms of encapsulation: the single layered micelle and the bilayered vesicle. We then proceed to show the effects of oxygen on transcription machinery and the role the membrane plays in optimizing oxygen concentration for protein expression. In each case, the surfactant encapsulation is prepared in mineral oil and encloses an *Escherichia coli* cytoplasmic extract. Measurement of expression efficiency is conducted by both endpoint and kinetic measurement of eGFP (enhanced green fluorescent protein) concentrations via fluorescent microscopy. In the case of micelle encapsulation, results show that micelles of long block copolymer (LBCP) have the highest rate of expression as well as final concentration at 8 hours. Results also show a large discrepancy in eGFP expression between well oxygenated and less oxygenated environments. Similarly, a comparison between lipids suggests longer fatty acid tail length corresponds to more effective inhibition of oxygen diffusion leading to greater expression efficiency. Further expression tests in vesicles show membranes composed of phosphotidylcholine (PC) lipid mixed with LBCP have the most efficient eGFP as well as α hemolysin expression among vesicles of differing composition. With this information comes a newfound knowledge of the effects of synthetic membrane composition, oxygen concentration and the relationship between these two parameters on protein expression.

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Introduction

Gene expression in bacteria. DNA is a biopolymer that stores the sequence information of each protein as genes. One protein is encoded by one gene. DNA is made of two strands twisted in a double helix that is often compared to the blueprint for life. Double strand DNA by itself has very little catalytic activity but instead it is used as a chemically robust template for gene expression which is composed of two steps: transcription and translation. Transcription is a process performed by a protein called RNA polymerase to produce a messenger RNA. There are approximately 2000 RNA polymerases in one *E coli* cell. Messenger RNA is a single strand copy of DNA that is degraded in time. Translation is a process that allows expression of proteins from a messenger RNA. Ribosomes are large macromolecules that read messengers RNA and polymerize a linear chain of amino acids to make proteins. There are approximately 20000 ribosomes in one *E coli* cell. At the end of translation, ribosomes, messenger and the newly synthesized protein disassemble. To be active, the protein folds in a three dimensional structure determined by the amino acid sequence. In bacterial cells, translation and transcription are coupled, ribosomes can translate messenger while it is polymerized by the RNA polymerase from DNA (Fig. 1).

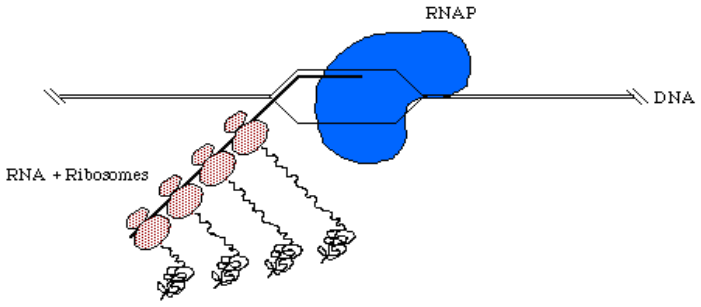

Figure 1. Coupled transcription and translation. During transcription RNA polymerase (RNAP) separates the two DNA strands and forms a short region of base pairing between the coding strand of DNA and the newly synthesized RNA. As the RNA elongates it is displaced as the double stranded DNA rehybridizes. Ribosomes rapidly bind to the resulting single stranded RNA and begin protein synthesis.

Cell-free expression. Cell-free expression systems allow synthesizing large amount of proteins *in vitro*. To express useful amount of proteins in the test tube, these systems use powerful bacteriophage transcription, in most cases the T7 RNA polymerase and its respective promoter, in a cytoplasmic extract from an organism, such as *E. coli*, that provides the translation machinery. They have been prepared for many types of applications such as high-throughput, proteomics or directed evolution. Although not optimized for synthetic biology purposes, these cell-free systems can be modified to study biological information processes. Indeed, in these extracts gene expression is quantitative. Therefore, more parameters can be controlled compared to *in vivo* and there is no background endogenous expression. These properties allow a reductive-constructive approach to biological information. Commercial cell-free systems are not suitable for our study for several reasons. First, the parameters in such systems are fixed. Whereas with our homemade extract we are flexible in adjusting the parameters according to our needs. Second, commercial systems are rather expensive. We have prepared our own system adapted for quantitative studies of biological information processes.

Homemade cell-free system. Our cell-free expression system uses the endogenous *E. coli* RNA polymerase with sigma factor 70 for transcription. The extract is prepared such that it contains the endogenous core RNA polymerase and transcription factor sigma 70.

Cell-free reaction. Cell-free reaction has a typical volume of ten microliters and is composed of the extract and all the other necessary components such as buffer,

nucleotides, amino acids. Plasmids are added to start the reaction.

Encapsulation of in vitro systems. Overview. Within the field of synthetic biology, it has become possible to create novel combinations of cellular components with the ability to exploit specific cellular processes. Some of these synthetic arrangements are built solely for the purpose of producing specific proteins of interest, while others are built for the use of communication, such as signaling cascade, which is important aspect of genetic circuitry [1]. The cell, as a whole, has had the luxury of approximately 3.8 billion years to optimize the efficiency of its assemblages through trials and errors due to the evolutionary mechanism of the "survival of the fittest." For the synthetic biologist, however, interfacing cellular components in an *in vitro* environment while maintaining efficiency or even viability of the new arrangement is often a challenge. This discrepancy in efficiency between *in vivo* and *in vitro* protein expression is due partly to the effects that a small reaction volume has on diffusion, as well as the effect that intracellular packing has on stability of the protein synthesis machinery required for expression [2, 3]. Taking a larger view of *in vitro* cell systems and its goal to create "living" cells using a bottom up approach, it is helpful to note that life is based on the fact that both the informational macromolecule, such as DNA or RNA, and the machinery to substantiate this information, such as ribosomes, are contained in reaction volumes allowing for efficient interaction so as to carry out protein expression [4]. It is for these reasons that encapsulation of *in vitro* systems is of prominent importance for the synthesis of efficient cell-like systems.

The most common forms of encapsulation are the use of micelles and vesicles

which are composed of a spherically organized surfactant monolayer and bilayer, respectively. This popularity is owing partly to the fact that these encapsulations are similar in structure and chemical makeup to prokaryotic cells, as well as that facile encapsulation by mono and bi-lipid layers is enabled by their ability to spontaneously form spherical capsules to reduce edge energy as described by Lipowsky [5]. Nature dictates the size of cell units to be in 1-50μm range for number of reasons: diffusion, membrane mechanics, reactions at low number of molecules. The compartments with the size of order of bacterial cell are advantageous in comparison to larger volumes, for example due to short diffusion time of the molecules. These times can be easily estimated using the theory of diffusion and Stokes Einstein relation. The diffusion coefficient according to the latter one is defined by the following expression

$$D = \frac{k_B T}{6\pi\eta \mathrm{R}},$$

where k_B, T, η, R are Boltzmann constant, temperature, viscosity of the media, and the size of a molecule respectively. Taking the following values for the viscosity of water and the size of eGFP $\eta = 1\, mPa \cdot s,\ R = 4\, nm$ one finds that the diffusion coefficient at room temperature is equal to $D = 5 \cdot 10^{-6}\, cm^2/s.$ As a result for the protein such as eGFP the time it takes to cover the distance of 1 μm is few milliseconds, while the time to cover 1mm is about an hour. Therefore the efficiency of constituents interactions inside small encapsulations is high. Use of these vesicles and micelles as micro reaction “flasks” has led researchers to develop methods to increase the control and efficiency of these encapsulated reactions [3, 6, 7]. The stability of these vesicle reactors have also been the

subject of study leading to the use of different surfactants in vesicle formation as well as the use of the synthetic block copolymer which offers the benefits of stability, decreased water diffusivity and the ability to easily change the chemical structure of the encapsulation [8, 9]. Multiple researchers have acknowledged that the volume of the lipid capsule also affects the efficiency of protein expression for in vitro systems [11, 12] leading to the development of methods allowing further control over the volume of these micro reactors [4, 10 ,13]. In general, higher stability and control of both vesicles and micelles have allowed for more widespread usage as micro-reactors and have been used for high throughput methods such as PCR [14] as well as in methods for studying directed evolution of proteins [15].

In this study, we continue in the direction of exploring aspects which affect the efficiency and control of protein production within lipid encapsulations. While some researchers have looked at the effects of encapsulation of *in vitro* protein expression [4] and others have shown the effects of the lipid composition of membranes on protein expression [16, 17], a comparison of protein expression between membranes composed of synthetic polymers and those composed of biological membranes has yet to be accomplished. It is for this reason that we have undertaken the task of examining the effects of protein expression within both micelles and vesicles composed of some of the more commonly employed surfactants as well as the synthetic block copolymer. To this end, we employ the use of a reporter plasmid in an *Escherichia coli* cytoplasmic extract, encapsulated by the surfactant of choice, to collect expression kinetics as well as final concentrations via fluorescent microscopy. Using these techniques, it is apparent that in

the case of both vesicles and micelles, membranes consisting of synthetic block copolymer increase the rate of expression. Furthermore, we show that these effects hold not only for the expression of eGFP but also that of α-hemolysin. It was shown [7] that α-hemolysin interacts with the natural lipid (PC) membrane of vesicle making pores. That allows nutrients to diffuse freely through the membrane. As a result the period of expression is extended. We would like to study how α-hemolysin affects the expression in vesicles formed with semi synthetic and pure synthetic membranes. Therefore the expression of α-hemolysin is of particular importance for us.

Also of interest in this study is the exploration of the role of oxygen in protein expression. It is common knowledge that oxygen plays a fundamental role in living organisms from the single celled aerobic prokaryote to complex animals. For this wide variety of organisms, oxygen acts as a terminal electron acceptor in the electron transport chain, allowing for the production of the currency of cellular energy, adenosine triphosphate (ATP). Beyond the electron transport chain, many other organic reactions required for life depend on the reduction of oxygen. In fact, the development of an atmosphere containing high oxygen concentration is often noted as a foundational step in the evolution of life today. Here, we show that optimal oxygen concentrations play a fundamental role in allowing for efficient protein expression on the example of two widely used proteins eGFP and Luc. eGFP needs oxygen for both the expression and fluorescence, while Luc does not require oxygen to luminesce. Furthermore, the oxygen concentration required for efficient expression is notably influenced by the effect that membrane composition has on inhibiting oxygen diffusion out of the micelle. These

effects were displayed through expression of a reporter plasmid in membranes of different composition while monitoring intramicellar oxygen content via oxygen sensitive optrodes.

Ultimately, this study adds to the growing list of factors which can affect expression of *in vitro* systems. Essentially, we show that the composition of the micelle or vesicle has profound impact on protein expression. The ability of a membrane to contain oxygen in the reaction solution also offers a new measure of effectiveness of a membrane. In short, the knowledge of these effects helps to achieve a greater efficiency of *in vitro* protein production.

Cell-free expression in emulsions and vesicles

Experimental section

Materials. Surfactants (Span 80, TRITON X-100), phospholipids (PC, DHPC, 24:0PC) and synthetic block copolymers (SBCP, LBCP) were used to prepare water-in-oil emulsions and vesicles. Triton X-100 and Span 80 were purchased from Sigma. Phospholipids: L-α-phosphatidylcholine (PC, Avanti Polar lipids, Alabaster, AL 840051), 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHCP, Avanti Polar lipids, Alabaster, AL 850306), 1,2-dilignoceroyl-sn-glycero-3-phosphocholine (24:0 PC, Avanti Polar lipids, Alabaster, AL 850373). Description and synthesis of block copolymers are given in [18]. We used two block copolymers: 5000 g/mol (poly(ethylene oxide)-polybutadiene with 30 hydrophilic monomers and 70 hydrophobic monomers, called LBCP in this study) and 760 g/mol (poly(ethylene oxide)-polybutadiene, called SBCP in this study). Mineral oil was purchased from Sigma (M5904).

All the plasmids used in the study originate from the plasmid pBEST-Luc (Promega). The green fluorescent protein was expressed with the plasmid pBEST-OR2-OR1-Pr-UTR1-eGFP-Del6-229-T500 described earlier [7]. eGFP-Del6-229 was renamed eGFP in this study. The fusion protein αhemolysin-eGFP, described earlier [7], was expressed through a transcriptional activation cascade with the plasmids pBEST-sigma 28 and pBEST-Ptar-UTR1-AH-eGFP. Sigma factor 28 activates expression of αhemolysin-eGFP with the Ptar promoter.

Experimental protocols. ***Preparation of water-in-oil emulsions.*** SPAN 80 was dissolved in mineral oil at the concentration of 0.23 mM, TRITON X-100 was added into

the cell-free reaction at 0.17 mM. Certain concentrations for SPAN 80 and TRITON X-100 were chosen such that it is still easy to make emulsion and the level of expression in emulsion is good. Phospholipids were dissolved in mineral oil at 5 mg/ml. The solution was incubated at 50°C (at elevated temperature all surfactants are completely dissolved in mineral oil) for one hour, diluted with mineral oil to 2 mg/ml, incubated 30 minutes at 50°C and cooled down to room temperature. Block copolymers were dissolved in mineral oil at 10 mg/ml. The solution was incubated at 50°C for one hour, diluted with mineral oil to 2 mg/ml, incubated 30 minutes at 50°C and cooled down to room temperature. These stocks solution of phospholipids and block copolymers at 5mg/ml and 10mg/ml respectively can be used for a few weeks to prepare emulsions. To prepare emulsion, 1 µl of cell-free reaction was added to 200 µl of mineral oil-surfactant solution. This mixture was immediately vortexed to create the emulsion composed of droplets with a size distribution from 5 to100 micrometers.

Preparation of vesicles. PC, SBCP and LBCP were dissolved in mineral oil at 2, 2, 4 mg/ml respectively, incubated at 50°C for one hour. Mixture of PC and LBCP (mol/mol ratio 4:1) was prepared and heated at 50°C for one hour as well. Vesicles with four types of membranes were prepared: PC, LP, SP and mixture of PC-LBCP. Vesicles were prepared according to Noireaux and Libchaber [7]. Briefly: 1 µl of cell-free reaction was added to 200µl of mineral oil-surfactant solution. The mixture was vortexed immediately to create an emulsion. 50 µl of emulsion were placed on top of the feeding aqueous solution and incubated for a few minutes. PEG 8000, which reduces vesicle formation, was added to the feeding solution after vesicle formation. The biphasic solution was

centrifuged at 11200g for 10 seconds to form vesicles.

Measurements. Samples were deposited between two cover glasses with a 250 μm spacer. Endpoint measurements as well as kinetics were measured at room temperature with a Qimaging Retiga camera mounted on a Olympus IX71 inverted microscope (objectives 20x, 40x, 60x, 100x, FITC fluorescence filter set). Exposure settings: 100 ms using 75% intensity with an EXFO excite 120 mercury vapor arc lamp. Data analysis was done with ImageJ software. Kinetics of expression and endpoint measurements of batch mode reactions were measured with a Wallac Victor III platereader (PerkinElmer). Change in oxygen concentrations in cell-free reactions were measured with Ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride ($Ru(dpp)_3Cl_2$, Sigma) which is a viable oxygen probe. Conversion of fluorescent intensity from emulsions and vesicles into concentration of eGFP was performed in the following way: pure eGFP at concentration 5μM was encapsulated into emulsions and vesicles, measured fluorescence intensity from them allowed us to make the calibration. Measurement were done with a photo multiplier tube (Hamamatsu GaAsp H7421-40) mounted on the same inverted microscope (TRITC fluorescence filter set). Data acquisition was done with a PC counter board and LABVIEW interface (National Instruments).

Image Processing and Data Analysis. Image processing was conducted using ImageJ 1.42q software (Rasband, Wayne, National Institute of Health) to determine the mean grey value and area of each micelle. Endpoint measurements: determining the endpoint expression of eGFP for each lipid emulsion consisted of finding the mean grey value with subtraction of background per micelle volume of 30 different micelles. These values were

then averaged in order to characterize the eGFP expression of the particular emulsion. The background was determined from averaged grey values collected from the photographs of the emulsion excluding the eGFP plasmid. Kinetic measurements: determining expression kinetics of eGFP for each lipid emulsion was accomplished by finding the mean grey value with subtraction of background of 30 micelles for each timepoint. The average value at each timepoint was calculated and plotted against its respective time point in order to characterize the rate of expression over a 8 hour period.

Results and discussion

Measurement of eGFP expression in micelles prepared with different surfactants. Cell-free reaction is a complex solution that has a typical volume of ten microliters and is composed of the cytoplasmic extract, buffer, nucleotides, amino acids, salts. Every component of reaction should be placed on ice in order to keep its activity and functionality. Melted stocks of buffer and amino acids as well as salts are introduced into the extract solution.

Lastly, plasmids coding for eGFP reporter protein are added to initiate the reaction. Oxygen is consumed as an electron donor for the regeneration of ATP, which is the main source of chemical energy for translation. In a cell-free reaction, oxygen is depleted within half a hour, from 0.2 mM to the micromolar level [19]. Thus, oxygen is one of the main basic molecule essential for gene expression.

eGFP is widely used as a reporter protein in fluorescence microscopy and was chosen as a reporter of expression in this study due to its ability to allow visualization of cell-like compartments that are the subjects of our interest.

Various techniques have been used to prepare emulsions, such as high pressure homogenizer, an ultrasound generator or stirring. We have chosen the vortex method of emulsification as it takes only a few second, a crucial advantage in that it is important for emulsification to occur before significant expression takes place.

We tested a range of different surfactants for their ability to form stable emulsions and support protein expression in cell free extract: natural lipids, block copolymers, SPAN and TRITON, both of which are biocompatible, non-denaturing and have

previously been used [20]. All surfactants were dissolved in mineral oil and demonstrated easy emulsion formation (Fig.2.c).

The most common forms of encapsulation are emulsions and vesicles. We would like to address the question of compatibility of membrane composition with the protein expression inside these microreactors. Use of a cell-free system allows us to perform a quantitative study of expression in micelles with different types of membrane.

Cell free extract with a 5nM plasmid concentration was encapsulated by different emulsions. The progress of expression was observed for all samples by means of fluorescence microscopy.

We found that within emulsion formed with each of the various surfactants, as well as emulsion simply prepared with only mineral oil, the level of protein production is lower with respect to batch mode expression (expression in test tube). The batch mode expression with the *E. coli* extract is incubated in a volume of 10µl at room temperature (23°C) for 8 hours with eGFP as reporter protein. After 8 h of incubation 1 µl of extract is encapsulated in micelles and expression is measured. A protein yield of 24 µM is achieved at plasmid concentration 5 nM (Fig.2.a, bar 9).

Some of the tested surfactants used for the emulsion produce rather low level of protein production (Fig.2.a). Protein yield in emulsions formed with PC is 0.4µM which is only 3% of expression in pure synthetic emulsions formed with SBCP, only 4% of expression in semisynthetic emulsions formed with mix of PC and LBCP and only 6% of expression in emulsions formed with SPAN. Apparently, there is an inhibition of protein expression due to presence of TRITON in emulsions, which is below 1% of expression in

batch mode (Fig.2.a,b). However, an interesting result was achieved with LBCP membrane. The protein yield in this case reached the level of 19μM which is 79% of batch mode expression in optimum conditions (Fig.2.a). This fact indicates that this particular synthetic membrane is nontoxic and compatible with biological material present in reaction. Therefore, the use of LBCP as a pure synthetic membrane can be considered as another step towards the artificial cell.

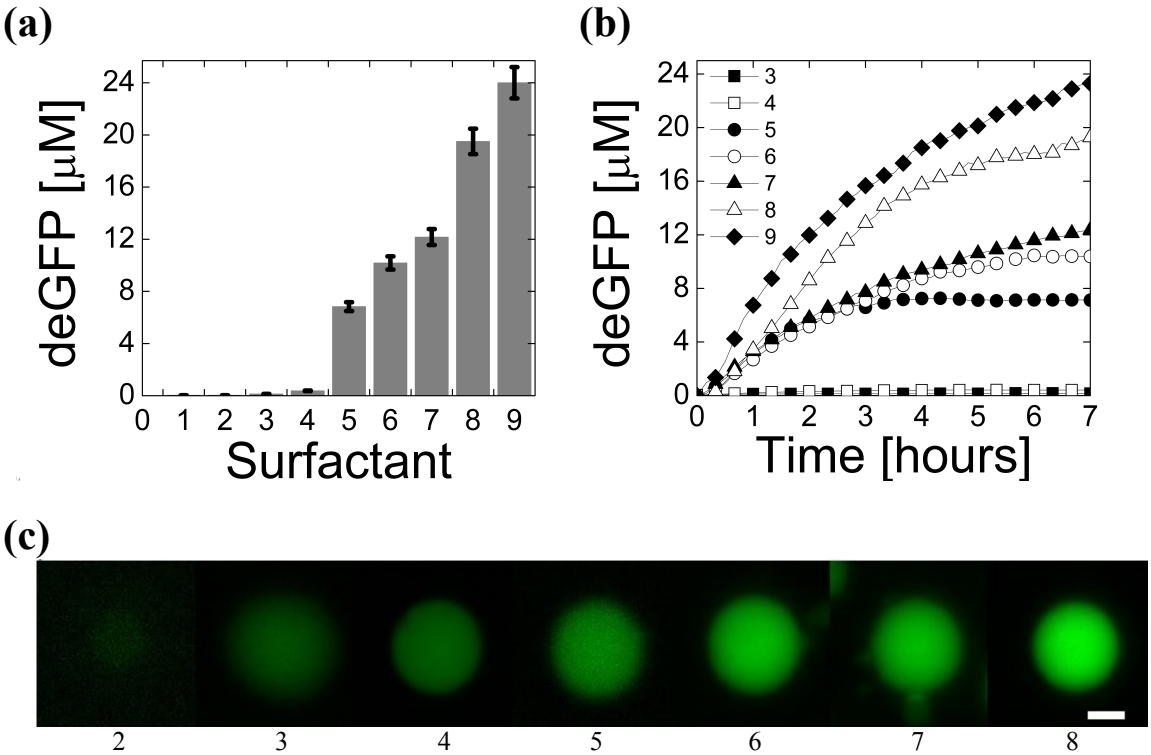

Figure 2. Cell-free expression of eGFP in micelles prepared with different surfactants in mineral oil. (a) End-point measurements after 8 hours of incubation at room temperature (5 nM plasmid pBEST-OR2-OR1-Pr-UTR1-eGFP-T500)): 1 – background signal (no plasmid), 2 – no surfactant, 3 – TRITON X-100, 4 – PC, 5 – SPAN 80, 6 – PC and LBCP (4:1 mol:mol), 7 – SBCP, 8 – LBCP, 9 – batch mode (expression of eGFP in a test tube). (b) Kinetics of eGFP expression in different types of micelles. (c) Fluorescence images of emulsion droplets after 8 hours of expression (scale bar: 10 μm).

From the data one can definitely see that the levels of expression within various surfactants are different, meaning that there is different amount of oxygen which is required for gene expression in these systems. There are two possible hypotheses for that.

One is that the oxygen is sucked out of the micelles in some cases (no surfactant, TRITON, PC, SPAN, SBCP, mix of PC and LBCP) and it is kept inside the emulsions formed with LBCP. The second hypothesis is that in emulsions formed with TRITON, PC or simply mineral oil all oxygen is consumed for expression. While for the emulsions formed with mix of PC and LBCP, SBCP, and LBCP alone there is additionally a diffusion of oxygen inside the micelles. Although, the concentrations of oxygen in mineral oil and reaction are different, the system (mineral oil/reaction) is in the equilibrium in the beginning of expression due to the difference of chemical potentials. Therefore, the first scenario does not make sense since the flow of oxygen out of the micelles is impossible in the equilibrium. Hence, the second scenario seems more attracting, however we do not have any direct evidence in favor of the hypothesis.

Effect of the phospholipid carbon chain length on eGFP expression in micelles prepared with different surfactants. It was proposed that the level of expression as well as fluorescing capability of eGFP is dependent upon oxygen availability. Therefore both expression and fluorescence ultimately depend upon the ability of encapsulation to decrease oxygen diffusion which is in turn dependent on fatty acid tail length in biological membranes. The longer the fatty acid tail length the thicker the membrane itself therefore it is less penetrable for oxygen. As a result the oxygen stored initially in emulsion is not diffusing out.

In order to demonstrate it , emulsions formed with lipids with three different carbon chain length were tested: DHPC, DLPC and 24:0PC.

We assume that an increase in fatty acid tail length will correspond to a decrease

in diffusion of oxygen out of the micelles. The protein yield for emulsions with three surfactants are presented in figure 3.a. Starting from emulsions formed with the shortest tail length: DHPC produced 0.05μM of protein while emulsions formed with DLPC produced 0.4μM of proteins, we conclude with emulsions formed with 24:0PC which gives 2.5μM of proteins. In order to estimate the importance of oxygen for the process of expression the following experiment was carried out: two reactions with eGFP reporter were prepared with 5nM of plasmids. On the top of one of the reactions, 80μl mineral oil was added, preventing oxygen flow into reaction. On the other hand, a reaction sample was also incubated but without mineral oil on the top. The same experiment was performed with reactions containing Luc reporter instead of eGFP. End-point measurements in batch mode were taken after 8 hours. The result revealed that for both reporters, mineral oil on the surface of the reaction drastically changed the level of expression. The level of eGFP production is 9.4 times more in the reaction without mineral oil on the top than in the presence of mineral oil (Fig.3.b). For the Luc this discrepancy is less 1.9 (Fig.3.b) making it apparent that these two reporters have different oxygen sensitivity. Oxygen is crucial for expression of protein itself while eGFP requires molecular oxygen to be oxidized in order to fluoresce [21]. The luminescence of Luc does not depend on oxygen, the difference in the levels of expression in tubes with Luc is due only to the difference in the amount of protein produced in both tubes with and without mineral oil. While for the eGFP the difference of the levels of expression is also caused by amount of oxygen available which is crucial for the eGFP fluorescence. Using these results we show that 9.6μM of eGFP is produced in the tube with mineral oil on the

top and only 1.9μM of them fluoresce.

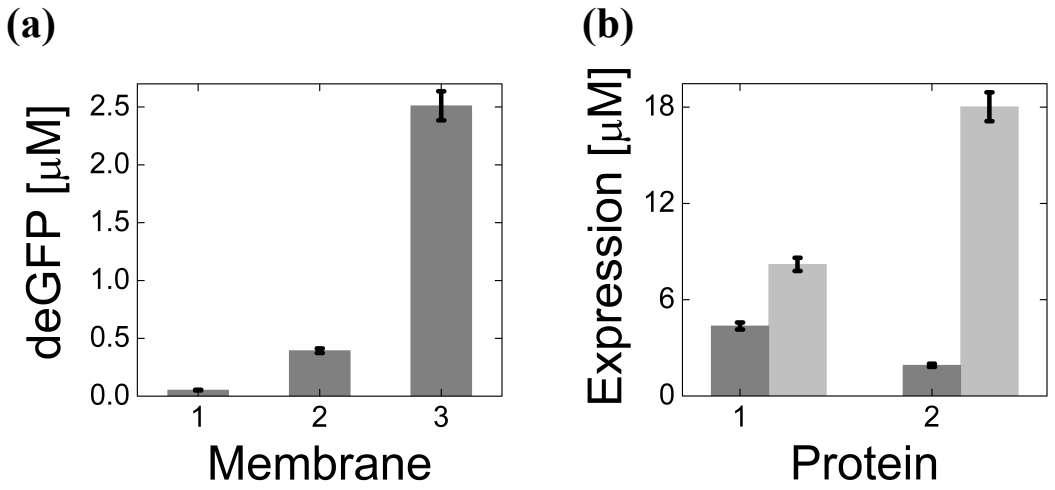

Figure 3. (a) Effect of the phospholipid carbon chain length on the expression of eGFP inside micelles prepared in mineral oil. eGFP concentration was measured after 8 hours of incubation at room temperature (5 nM plasmid pBEST-OR2-OR1-Pr-UTR1-eGFP)): 1 – DHPC, 2 – DLPC, 3 – 24:0 PC. (b) End-point measurements in batch mode: 1 – expression of Luc in a test tube (light grey), expression of Luc in a test tube with mineral oil on top of the reaction (grey), 2 – expression of eGFP in a test tube (light grey), expression of eGFP in a test tube with mineral oil on top of the reaction (grey).

Kinetics of oxygen sensitive $Ru(dpp)_3Cl_2$ in micelles prepared with different surfactants. Formerly, we demonstrated how, oxygen plays an important role in the expression of proteins, particularly in emulsions. In order to investigate the oxygen diffusion out of the emulsion droplets, oxygen sensitive particles were used. Micelles formed with four surfactants with different fatty acid tail length were prepared: no surfactant (in mineral oil), DHPC, 24:0PC, LBCP. Both expression of eGFP and kinetics of oxygen sensitive particles were investigated (Fig.4.c).

Expression in emulsions with no surfactant and in emulsion formed with DHPC are at background level (below 1% of expression in test tube). In contrast, the signal from $Ru(dpp)_3Cl_2$ is increasing, indicating that oxygen diffusion out of the droplets occurs (Fig.4.a,b). For emulsions formed with LBCP and 24:0PC the protein yield is 19μM and 2.8μM respectively, the result for oxygen sensitive particles (Fig.4.b,c) clearly shows that

there is an inhibition of oxygen diffusion out of the emulsion droplets, meaning that a longer fatty acid tail length (24:0PC and LBCP) corresponds to less oxygen leakage out of the micelles (Fig.4.c).

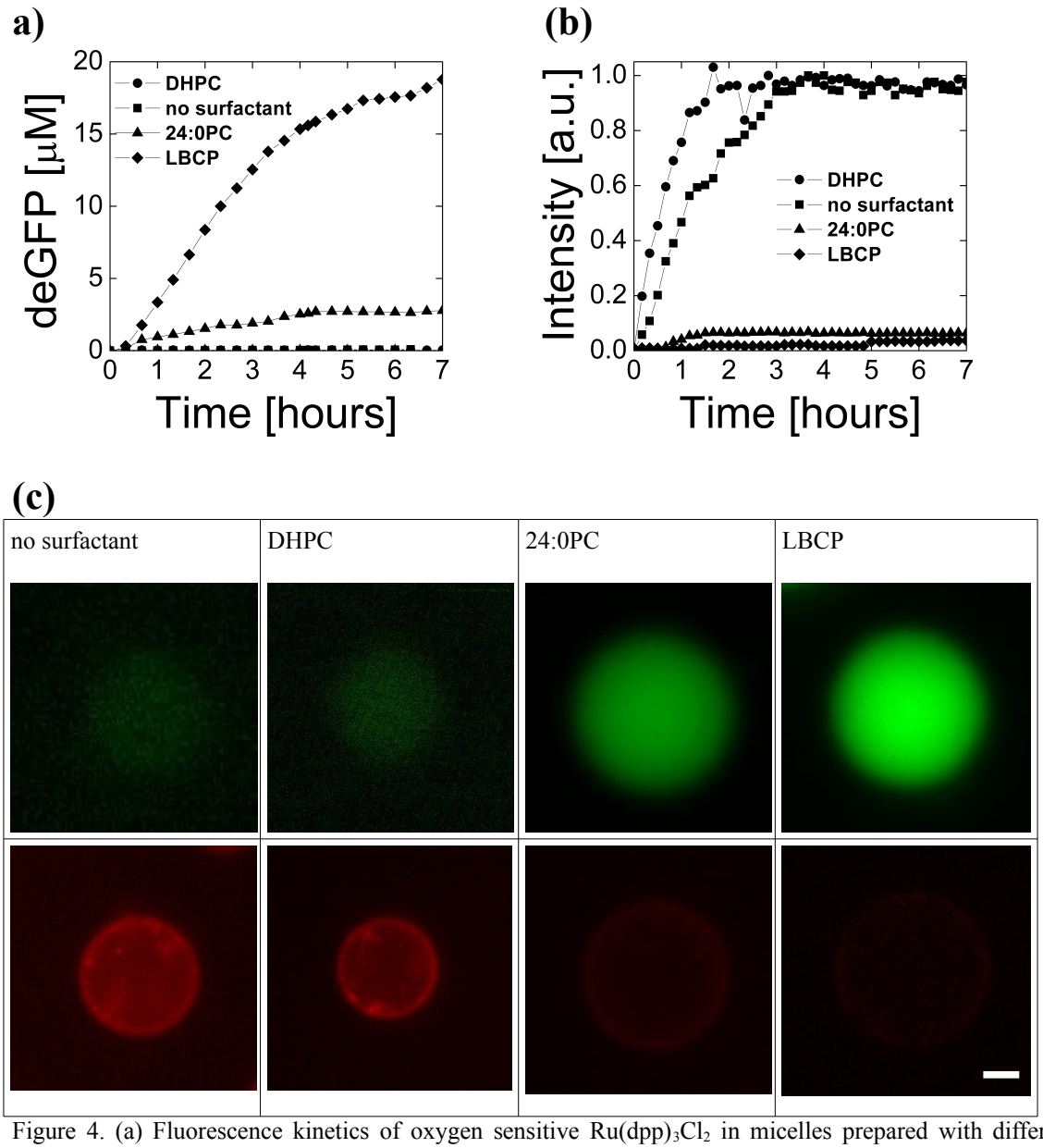

Figure 4. (a) Fluorescence kinetics of oxygen sensitive $Ru(dpp)_3Cl_2$ in micelles prepared with different surfactants in mineral oil. (b) Kinetics of eGFP expression in micelles prepared with different surfactants in mineral oil. (c) Fluorescence images of emulsion droplets after 8 hours of expression (scale bar: 10 µm). Top row: eGFP fluorescence, bottom row: $Ru(dpp)_3Cl_2$ fluorescence.

Measurement of eGFP expression in vesicles. The results for the emulsions with

different surfactants clearly showed that block copolymers are favorable in terms of expression. Therefore, our next step was to encapsulate cell-free expression system into vesicles with semi-synthetic and pure synthetic membranes.

Vesicle formed with four different types of membrane were investigated: PC, SBCP, LBCP, mix of PC and LBCP (Fig.5.c).

Vesicles formed with either PC, SBCP, LBCP or a mix of PC and LBCP show protein expressions of 19 μM, 20 μM, 25 μM and 26 μM respectively corresponding to an increase over batch mode of 25%, 34%, 68% and 75% respectively.

It was demonstrated that the expression in vesicles formed with a mix of PC and LBCP gives the highest protein production (26μM) among all the other membrane types and it produces twice the expression in bulk at room temperature (23°C) (Fig.5.a,b). The stability of the vesicles over time as well as an increased level of expression in vesicles is due to the effects of osmotic pressure which leads to the formation of pores in the membrane allowing nutrients and particularly oxygen to penetrate through the membrane. This formation of pores prolongs the expression process allowing for higher protein yield and a longer duration of expression than batch mode where sources of nutrients are limited.

Cellular extract is a very dense solution that has variety of proteins at 10mg/ml. In a first approximation the osmotic pressure is proportional to the concentration of the proteins inside the vesicles. Approximately, taking all proteins in the extract at 10kDa, we obtain osmotic pressure at about 2000Pa or 0.02atm (this is apparently the lower limit in the size and upper limit in the pressure). The contribution from the newly synthesized

proteins (eGFP) is negligible ~ 5 x 10^{-4} atm or 50 Pa for the protein production at 20μM. In order to balance the osmotic pressure, PEG8000 is added in the feeding solution containing vesicles and mixed by gentle pipetting. This step is crucial in terms of vesicle formation, allowing them to live up to several days. Overall, this period of expression is longer than has been previously accomplished.

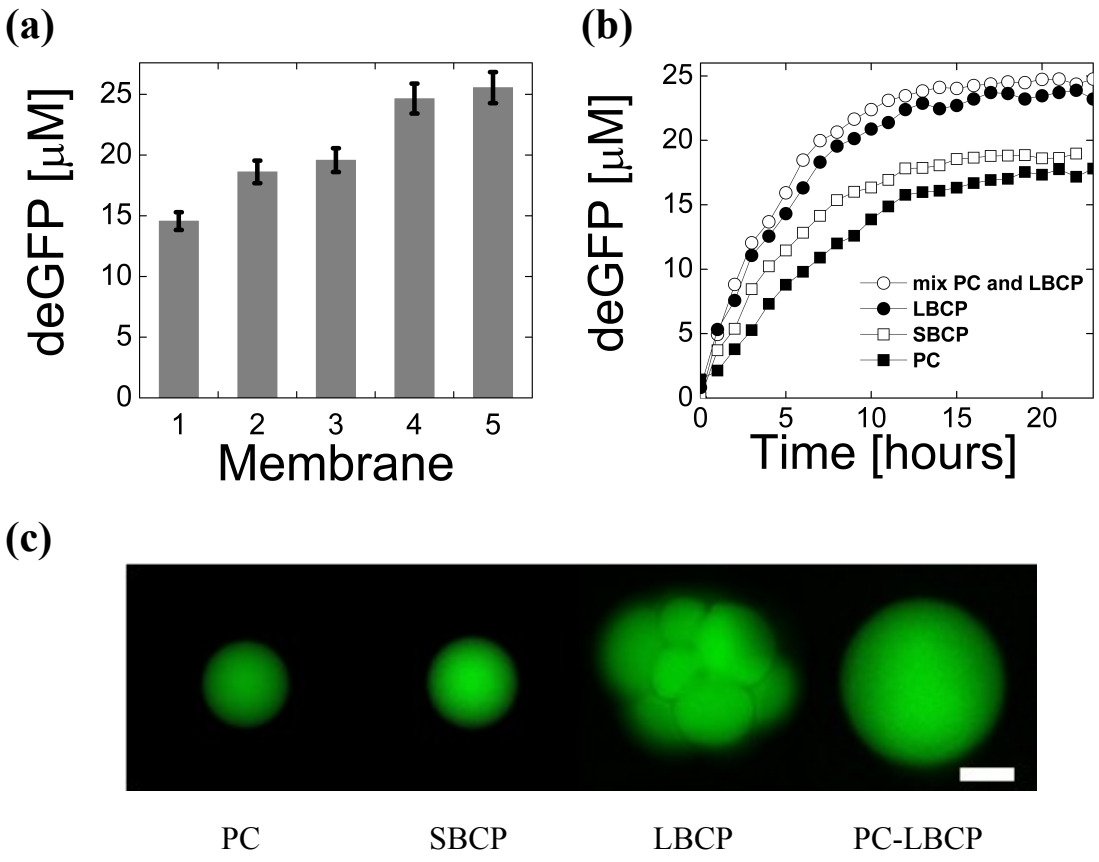

Figure 5. Cell-free expression of eGFP in vesicles with four different types of membranes. (a) End-point measurements after 30 hours incubation at room temperature (5 nM of reporter gene plasmid pBEST-OR2-OR1-Pr-UTR1-eGFP-De6-229-T500): 1 – batch mode (expression of eGFP in a test tube), 2 – PC, 3 – SBCP, 4 – LBCP, 5 – PC and LBCP (4:1 mol:mol). (b) Kinetics of eGFP expression in vesicles with four different types of membranes. Mix PC and LBCP: 4:1 mol:mol. (c) Fluorescence images of vesicles after 30 hours of expression (scale bar: 10 μm).

Measurement of AH-eGFP expression in vesicles. It has been shown by Noieraux and Libchaber [7] that AH-eGFP interacts with vesicle membrane formed with PC makes

pores of 1.4nm such that amino acids and nucleotides could freely diffuse through pores and feed the system. Therefore, expression in the vesicles with such pores at the membrane is longer than in vesicles where just eGFP is expressed. We would like to apply this knowledge to semisynthetic and pure synthetic vesicles. Vesicles formed with four different types of membrane were investigated: PC, SBCP, LBCP, mix of PC and LBCP (Fig.6.c).

eGFP expression in pure synthetic vesicle: SBCP and LBCP stops after 18 hours (Fig.5.b), which is the case for the same type of vesicles with AH-eGFP expressed inside (Fig.6.b). There is a weak interaction of AH-eGFP with the membrane in pure synthetic vesicles formed with SBCP and LBCP (Fig.6.c). However it is not sufficient to make pores at the membrane to feed the system. Expression of AH-eGFP inside vesicles formed with PC gives a protein yield of 3.9μM and stops after about 30 hours when expression of eGFP in the same type of vesicles reaches a plateau after 18 hours (Fig.6.a,b). This clearly demonstrates a strong interaction of AH-eGFP with the PC membrane (Fig.6.c), therefore allowing free passage of nucleotides and amino acids through pores, thus feeding the system. Finally, expression in semisynthetic vesicles (Fig.6.c) stops after 36 hours, demonstrating the longest expression and giving the highest protein production at 7.3μM among all the other membranes in the study.

There is a peculiarity that has been discovered during this study. In spite of the fact that osmotic pressure in the system is relatively high ~ 2000Pa or 0.02atm, there is no need to balance it by adding PEG8000 molecules to the feeding with pure synthetic vesicles formed with SBCP and LBCP as well as semisynthetic vesicles formed with mix

of PC and LBCP, which is not the case for vesicles formed with PC.

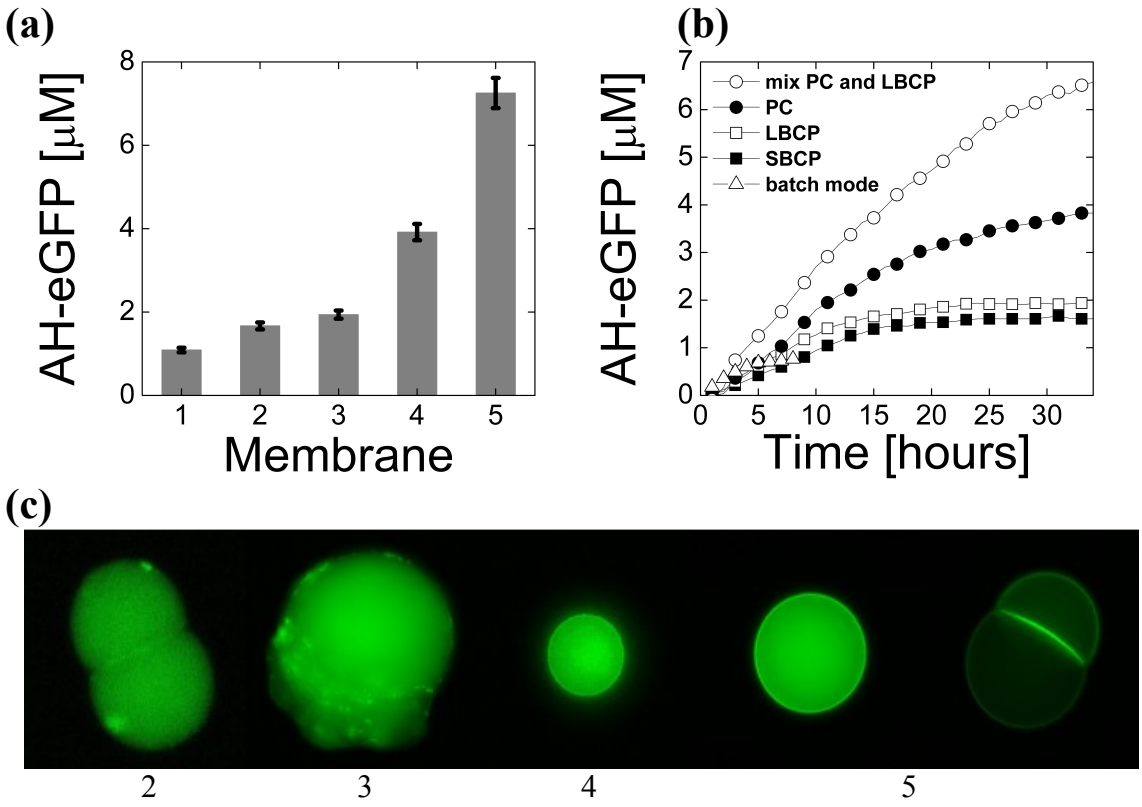

Figure 6. Cell-free expression of the fusion protein α-hemolysin-eGFP in vesicles with four types of membranes. (a) End-point measurements after 40 hours incubation at room temperature (0.5 nM of pBEST-sigma28 and 5 nM of reporter gene plasmid pBEST-Ptar-UTR1-AH-eGFP): 1 – batch mode (expression of α-hemolysin-eGFP in a test tube), 2 – SBCP, 3 – LBCP, 4 – PC, 5 – PC and LBCP (4:1 mol:mol). (b) Kinetics of α-hemolysin-eGFP expression in vesicles with four types of membranes. (c) Fluorescence images of vesicles after 40 hours of expression (scale bar: 10 μm).

Conclusions

- Oxygen in cell-free reaction is vital element for gene expression
- Two forms of encapsulation were performed: micelles and vesicles
- Cell-free expression inside micelles and vesicles was quantitatively studied

Emulsions (micelles):

- Cell-free expression in different types of emulsions is studied. It was found that protein yield inside emulsions formed with pure synthetic membrane LBCP is the highest among all the surfactants that were used in this study. The level of expression in LBCP emulsions is close to one in bulk in optimum conditions. Two possible scenarios of sustaining the level of oxygen in emulsions were proposed. One of them does not agree with assumption about the system being in equilibrium. The second one although more favorable does not have experimental proof. Additional experiments need to be performed to justify this scenario. In order to have strong support of the scenario it must be shown that in emulsions formed with mineral oil (no surfactant) oxygen can not diffuse in the droplet, while for emulsions with LBCP membrane oxygen is replenished.
- The relative contributions of two processes: protein expression and protein fluorescence which are ultimately depend upon oxygen availability were estimated.
- It was shown that protein expression depends on fatty acid tail length in biological membranes. The longer the fatty acid tail the less oxygen diffuses out of the micelles, causing the higher protein yield inside droplets of emulsion.

Vesicles:

- Cell-free expression in different types of vesicles is studied, particularly, in pure synthetic and semi-synthetic vesicles. It was demonstrated that expression in vesicles formed with a mix of PC and LBCP gives the highest eGFP production among all the other membrane types and it produces twice the expression in bulk at room temperature (23^{o}C).
- Strong interactions of expressed AH-eGFP with the natural (PC) and semi-synthetic (mix of PC and LBCP) membranes were observed. Therefore significant prolongation of protein expression was observed. For example, expression of AH-eGFP inside vesicles formed with PC stops after about 30 hours when expression of eGFP in the same type of vesicles reaches a plateau after 18 hours.

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