Giant Vesicles as Microreactors for Enzymatic mRNA Synthesis

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Giant vesicles have attracted much attention as possible microreactors for the conduction of enzymatic reactions in an artificial, cell-sized compartment. In this context, we demonstrated in the first part of the present work that giant vesicles formed from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine in an alternating electric field can be made more permeable to Ca^{2+} ions or nucleotide triphosphates by addition of ethanol. This methodology is then applied in a second step whereby these giant vesicles are used as microreactors in which mRNA synthesis can occur. The macromolecules (the DNA template and the enzyme T7 RNA polymerase) are microinjected into a selected giant vesicle, while the substrate molecules (nucleotide triphosphates) are added from the external medium. The fact that mRNA synthesis can be detected is a further step towards our aim: the design of a microreactor that can be seen as a model for a protocell.

KEYWORDS:

giant vesicles · liposomes · microinjection technique · microreactors · mRNA synthesis

Introduction

In recent years, giant vesicles (GVs) have attracted the attention of several research groups as biological microreactors.[1–4] Among the various methods for the production of GVs, we have selected the method of electroformation,[5] which allows the GVs to form in such a way that they remain fixed at the location in which they were formed. With these GVs further manipulation becomes easily achievable. In our group we developed a method for the addition of substances into a selected GV by microinjection.[6–8] Other methods for loading giant vesicles use microelectroporation[9] or are a combination of electroporation and microinjection.[10]

The advantages of GVs in comparison with conventional liposomes (small or large unilamellar vesicles (SUVs or LUVs)) are manifold: GVs formed by electroformation allow the observation of individual vesicles over a long period by optical microscopy in real time because they are attached to the electrode and remain at their site of formation, typically for hours. These GVs can be punctured and appropriate substances microinjected with a micropipette without destruction of the vesicle, therefore one can be sure that the injected components are all inside the same compartment. The time course of molecular biology reactions can be followed by observation of appropriate color changes, which accompany the chemical transformation of reactants. Another advantage of GVs lies in the fact that, as a result of their large size (typical diameter = 30 – 100 μm) and curvature radius, GVs formed by electroformation may be better models for biological membranes and, therefore, biological cells, than conventional liposomes with 100 – 1000 times smaller diameters.

An interesting novel aspect of GVs appeared in our previous work[11] in which we reported that certain physical and chemical properties of GVs, in particular permeability, were significantly different from those of conventional liposomes. In fact, in the cited paper we demonstrated that the bilayer of a GV formed by electroformation permits interaction between compartmentalized nucleic acid (DNA or RNA) introduced by microinjection and a specific nuclease (ribonuclease A, deoxyribonuclease I, or deoxyribonuclease II) added externally. The possibility of artefacts such as imperfections of the GV membrane or leakages was ruled out by cross-control experiments, thus we came to the conclusion that GV membranes under certain conditions allow the permeation even of small proteins.

Previous work by other research groups has shown that ethanol or other alcohols may have dramatic effects on the phase behaviour of bilayer systems.[12–14] These studies demonstrated that addition of ethanol changes the phase transition temperature and the phase structure of many lipidic systems and leads to interdigitated bilayer systems. Most of these investigations have been carried out with disaturated-chain phospholipids such as dipalmitoylphosphatidylcholine (DPPC); for monounsaturated phosphatidylcholines the situation is less clear.[15] Several studies also demonstrated that this formation of interdigitated bilayers has the effect of enhanced permeation,[16, 17] primarily when the interdigitated phase coexists with other phases. The leakage of molecules across the bilayers occurs predominantly at these phase boundaries. Most of these permeability studies were also carried out on phospholipids with disaturated acyl chains. In the literature, there are only a few reports that describe the effect of ethanol on the permeability of liposomes composed of mixed-chain phospholipids. One study demonstrated an increased efflux of calcein from LUVs at ethanol concentrations > 0.6 M.[17] Another study showed that the presence of > 1.1 M ethanol may increase the permeability of conventional liposomes composed of egg phosphatidylcholine

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and egg phosphatidic acid towards protons and potassium ions.[18] To the best of our knowledge, only a few studies that deal with the effects of ethanol on giant vesicles have been reported[19] and we found no reports on an increased permeability of GVs towards ions or larger charged molecules in the presence of ethanol.

Permeability is certainly the main problem when studying enzymatic reactions with GVs, as one would like at least some of the reagents to permeate inside from the external medium. This is indeed the main idea behind the present work. Herein, we report evidence of an increased permeability of GVs made from the monounsaturated phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) towards Ca^{2+} ions and nucleotide triphosphates upon the addition of ethanol. This is in contrast to conventional liposomes, where ethanol has no detectable effect on the permeability of the membranes for the appropriate substrate molecules. In the second part of the work, we utilise the transport of externally added nucleotides across the GV membrane to induce the synthesis of RNA by T7 RNA polymerase inside the GV compartment. This can be seen as a further step towards the modeling of precursors of biological cells with vesicles.

Results

Operational methodology

Phospholipid aggregates formed by electroformation show some typical characteristics because many of these apparent vesicles are not closed spherical structures. In particular, those aggregates that are in direct contact with the electrode are often not closed, or not yet closed. Whether an aggregate has a closed spherical structure (and can, therefore, be considered as a real giant vesicle) or belongs to the "open structures" category (structures that we have designated as mushrooms) can often only be decided by performing experiments (see also Ref. [7]). This is demonstrated in Figure 1 in which the hydrophilic fluorescent molecule fluorescein-12-uridine-5′-(tetrahydrogen-triphosphate) (Fl-UTP) is injected externally into aggregates formed in water. 15 minutes after the injection, many of those aggregates which are in direct contact with the electrode are still fluorescent, while the structures that seem to be clearly spherical (see vesicles number 2 and 4 in Figure 1) show no internal fluorescence. Care was taken throughout this work that all presented experiments were carried out with such clearly spherical structures that were about $>20\,\mu m$ distant from the electrode.

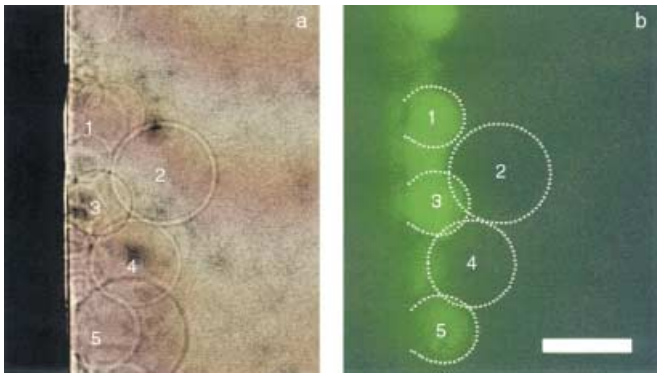

Figure 1. *Distinction between closed spherical vesicles (GVs) and nonclosed spherical aggregates. After the formation of GVs in water for 2 – 4 h, fluorescein-12-UTP (10 mM) was added to the GVs with a femtotip II at a distance of 20 – 50 μm from the membrane of the selected GV. The injection time was 90 × 0.3 s and the injection pressure was 200 kPa. The images were taken 15 min after addition of fluorescein-12-UTP. a) Differential interference contrast (DIC); b) fluorescence mode. Scale bar = 50 μm.*

One of the big advantages of these spherical structures—the giant vesicles—formed by electroformation is that they allow the injection of hydrophilic substances into their aqueous pool. Such injection has shown that smaller molecules such as dye molecules or nucleotides, as well as macromolecules such as enzymes or nucleic acids, are injectable.[6–8] It should be noted here that these kinds of molecules, when injected into the medium surrounding a giant vesicle, have never shown any trace of fluorescence inside the GV. Instead, when these substances were injected into the aqueous pool, they remained inside the GV even after the withdrawal of the micropipette. The "self-healing" capacity of the GV membrane is so high that no clearly observable fluorescent material leaked out during the healing period.

Another important point to recall here is that even if these giant vesicles are closed spherical structures, they are in many cases interconnected by lipid protrusions and tethers to other bilayers.[20] GVs that have no connection to other bilayer systems often lose contact with the electrode after being touched by a micropipette; in most cases such GVs are lost for further experimentation. This behavior is in contrast to that of interconnected GVs, which can often even be punctured several times. Therefore, it becomes possible to inject different aqueous solutions into the same GV by using two or even more punctures. However, GVs that allow multiple puncturing are relatively seldom; in many cases the first puncturing with subsequent injection of liquid into a GV is possible without difficulty but any further puncturing is impossible because the GV starts to move around when touched. The reason for that might be that the mechanical stress on the GV upon the first puncturing loosens the interconnecting lipid layers and makes the GV more mobile (A. Fischer, T. Oberholzer, unpublished observations).

Effect of ethanol on the permeability to Ca^{2+} ions of the GV membrane

Firstly we studied the permeability of the GV membrane to Ca^{2+} ions by making use of the reactions between these ions and Calcium Green-2, which bring about a noteworthy increase of fluorescence. To test whether ethanol could increase the permeability of a GV membrane that consists of POPC, GVs were formed by electroformation in an aqueous solution that contained Calcium Green-2 (2 μM). After turning off the frequency generator, ethylenediaminetetraacetate (EDTA) was added and the system was allowed to equilibrate for more than one hour. EDTA was necessary because the background fluorescence

of the ions present in the water together with Calcium Green-2 was quite high. After this period, a concentrated solution of ethanol was added to the surrounding medium with a pipette (final concentration 1% v/v, corresponding to about 170 mM). The addition of ethanol or other alcohols normally did not change the stability of the GVs; macroscopically, they remained as they had been in the absence of ethanol. Once again, the GVs were allowed to equilibrate but in this instance for a longer period (> 2 hours). Afterwards, a micropipette was loaded with $CaCl_2$ and the solution was injected at a distance of about 50 μm onto the surface of a selected GV. Fluorescence results are shown in Figure 2A. About 50 minutes after addition of calcium chloride, a significant fluorescence increase was detected inside the GV. This means that Ca^{2+} was able to permeate across the GV membrane with the help of ethanol.

Figure 2B presents an equivalent experiment at a concentration of 5.6% ethanol (952 mM). Even at that relatively high concentration, the GVs remained as they were in the absence of ethanol; macroscopically they remained unchanged in size and also attached to each other in the same positions. $CaCl_2$ was injected into the surrounding medium at time zero (Figure 2B, b, c) and the uptake of Ca^{2+} was monitored. Surprisingly, the uptake seemed to be a relatively slow process; only after 12 minutes had a significant amount of Ca^{2+} permeated across the GV membrane and increased the fluorescence intensity inside. The same kind of experiment was also carried out in the absence of ethanol, at 1% (v/v), and at 2.9% (v/v) ethanol (see Figure 2C). Interestingly, the Ca^{2+} uptake did not linearly correlate with the ethanol concentration. At 1% ethanol, there was only a modest uptake of Ca^{2+} (an increase of less than 100 arbitrary units was detected), whereas at 2.9% this increase was almost 600 units and at 5.6% ethanol the uptake reached values above 1500 units.

It should be mentioned here that according to these results, the Ca^{2+} ions could also be partly adsorbed onto the external surface of the GV. The possibility that all the Ca^{2+} ions were only adsorbed onto the external surface could be excluded by several arguments which suggest the substances studied here were mainly entrapped: 1) Ca^{2+} ions, Fl-UTP, and UTP (see below) are all taken up in the same manner. Even if some of the positively charged Ca^{2+} ions are adsorbed onto the membrane surface, it is unlikely that all three are adsorbed in the same way, 2) The uptake of Ca^{2+} ions was clearly ethanol dependent (Figure 2C) and no uptake (or adsorbtion) occurred in the absence of ethanol. Thus, if the ions were only adsorbed, this would have to be an ethanol-dependent process, 3) GVs with externally adsorbed fluorescent material do not appear as homogeneously filled entities (A. Frazzoli, T. Oberholzer, unpublished results and Ref. [21]) as seen here, but as fluorescent rings. It should also be taken into account that the experiments reported herein were carried out at a very low ionic concentration outside the GVs. Therefore, published results on binding of Ca^{2+} ions to the POPC layer are not fully applicable.

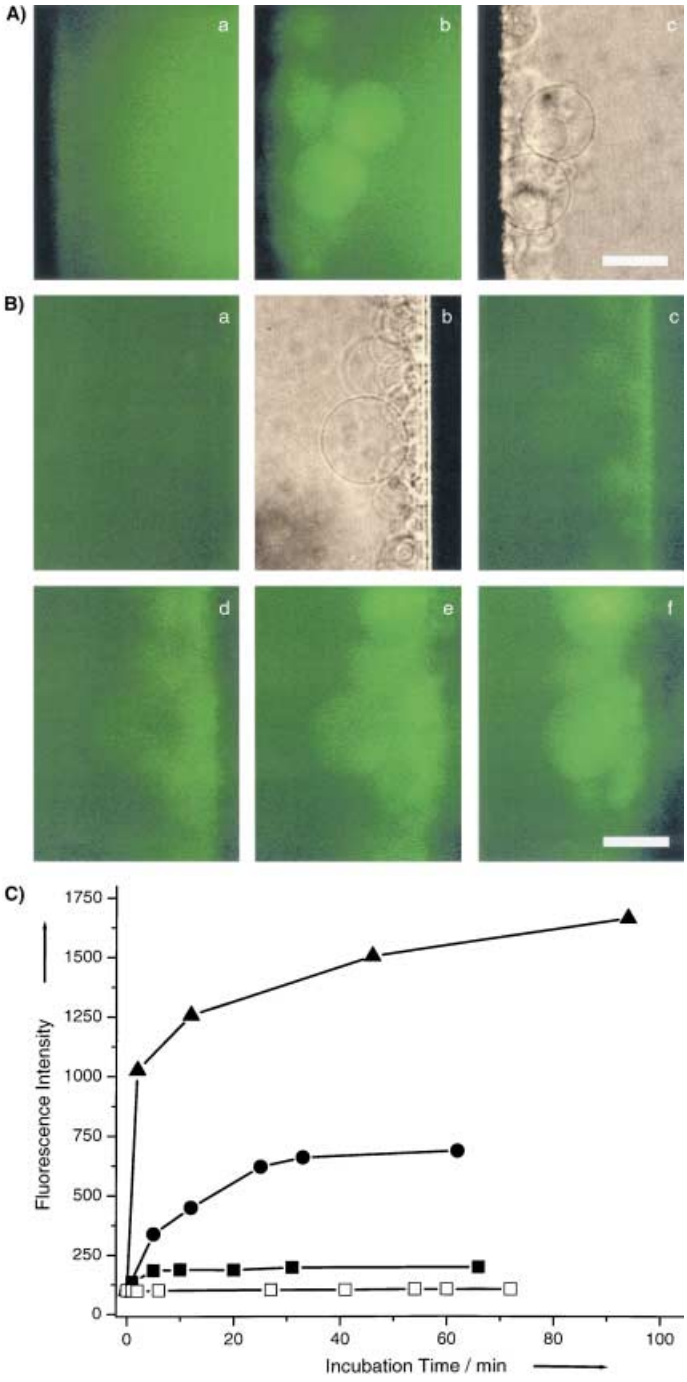

Figure 2. *A) Calcium ions permeate across the membrane of giant vesicles in the presence of 1% (v/v) ethanol. The vesicles were prepared and treated as described in the Experimental Section. $CaCl_2$ (10 mM) was injected into the aqueous solution (6 injections, injection time: 10 s each) in the vicinity of the membrane of the vesicle with a femtotip II at a pressure of 500–600 kPa. a) Before addition of $CaCl_2$; b, c) 50 min after addition of $CaCl_2$. a, b) Fluorescence mode; c) DIC. Scale bar = 50 μm. B) Permeation of calcium ions through the membrane of giant vesicles in the presence of 5.6% ethanol (937 mM). The formation/injection was carried out as in (A). The injection times were 20 × 1 s and 2 × 3 s. The injection pressure was adjusted to 600 kPa. a) Before addition of $CaCl_2$; b, c) 2 min, d) 12 min, e) 46 min, and f) 94 min after addition of $CaCl_2$. a, c–f) Fluorescence mode; b) DIC. Scale bar = 50 μm. C) Time course of the Ca^{2+} uptake at various ethanol concentrations. For details of the method, see the Experimental Section. Filled squares: 1% ethanol (164 mM), circles: 2.9% ethanol (482 mM), triangles: 5.6% ethanol (937 mM); empty squares: identical experiment performed in the absence of ethanol. The fluorescence intensity shown is in arbitrary units.*

We also investigated the Ca^{2+}-loading effect with dimethyl sulfoxide (DMSO) or alcohols other than ethanol (Table 1). At a concentration of 1% (v/v), 1-pentanol (92.5 mM), 1-octanol (63 mM), and 1,2-ethandiol (175 mM), each had a similar effect on the influx of Ca^{2+} ions into the GVs; DMSO (141 mM) and methanol (250 mM), however, increased the Ca^{2+}-ion influx only slightly. In order to test whether the effect of the alcohols was a result of the local concentration of the hydroxy groups, 1,2-ethandiol (1% (v/v, 175 mM) was tested (twice the number of hydroxy groups as in ethanol), but again, no difference with respect to 1% ethanol was observed.

Table 1. *Effect of different alcohols on the permeability of the membrane of a selected GV to Ca^{2+} ions.[a]*

Alcohol	Concentration (v/v)			
	0.1%	1%	3%	6%
ethanol	35	100	550	1500
methanol	effect hardly detectable	low effect	nd[b]	nd[b]
1-pentanol	40	100	nd[b]	nd[b]
1-octanol	30	100	nd[b]	nd[b]
1,2-ethandiol	35	100	nd[b]	nd[b]
DMSO	no effect detectable	effect hardly detectable	nd[b]	nd[b]

[a] The numbers shown are percentage luminosity values. In those cases in which a clear effect could be observed, the value at an alcohol concentration of 1% was arbitrarily set to 100%. Vesicles were formed in the presence of 2 μM Calcium-Green-2, then EDTA was added (final concentration of 40 μM) and the vesicles were allowed to stand for one hour before the appropriate amount of alcohol was added to the medium. After another incubation of two hours, $CaCl_2$ (10 mM) was loaded in a micropipette and added at a distance of about 50 μm to a selected GV. [b] nd = not determined.

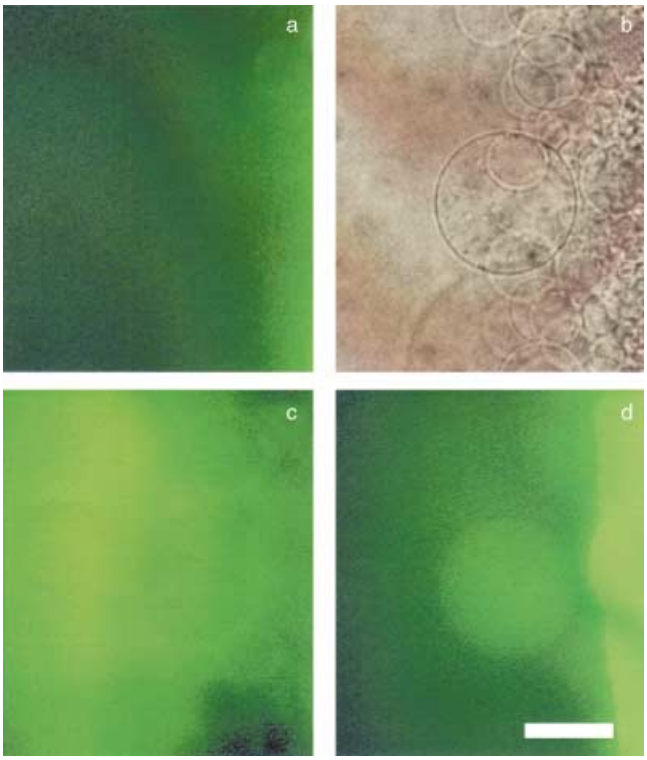

Figure 3. *Permeation of nucleotides across the GV membrane in the presence of ethanol. After the formation of GVs in water, ethanol was added to the external medium (final concentration: 1% (v/v) = 164 mM). Two hours later, fluorescein-12-UTP (10 mM) was added with a femtotip II to the GVs at a distance of 20 – 50 μm from the membrane of the selected GV. The injection time was 90 × 0.3 s and the injection pressure was 200 kPa. a, b) Before addition of fluorescein-12-UTP; c) immediately, and d) 20 minutes after the addition of fluorescein-12-UTP. a, c, d) Fluorescence mode; b) DIC. Scale bar = 50 μm.*

Ethanol-induced permeation of nucleotides across the GV membrane

Having shown that ethanol or structurally similar alcohols increase the permeability of GV membranes towards Ca^{2+} ions, we set up an investigation aimed at clarifying whether biologically important monomers, such as mononucleotide triphosphates, might also permeate across the GV bilayer with the help of ethanol. For this purpose, we used Fl-UTP. The experiment was carried out as previously described: after formation of GVs in water, ethanol (1% (v/v)) was added to the external medium and two hours allowed to make sure that the vesicles were stable then Fl-UTP was added from a micropipette in the vicinity of the selected GV membrane.

An initial series of experiments was carried out without addition of ethanol, so as to determine the background conditions. It should be kept in mind that GVs formed by electroformation, as already mentioned, have quite different permeability properties to those of conventional liposomes.[11] Fl-UTP uptake results are shown in Figure 3. The presented series of images reveals the situation before (a, b), during (c), and after (d) injection of Fl-UTP into the medium surrounding a GV. During the addition of nucleotide, the selected GV seems to be "darker" than the surrounding solution. The increase in fluorescence can only be seen after the external Fl-UTP is sufficiently diluted. Of course this result does not absolutely prove that the Fl-UTP permeated across the GV bilayer; it could also be reasoned that there is an ethanol-induced membrane binding of Fl-UTP. Such a possibility is difficult to exclude. However, it should be taken into account that substances that are incorporated into the GV bilayer (such as fluorescent phospholipids or fluorescent fatty acids) cause a different appearance of the fluorescence. Instead, in such experiments the fluorescence is not homogeneously distributed and the fluorescence appears to be more prominent in the equatorial plane of the observed GV.[20, 22]

One question raised at this point was whether the uptake of Fl-UTP was ethanol-dependent or could also occur in the absence of ethanol. To clarify this, the same kind of experiment was performed under identical conditions without ethanol (see Figure 1). As described above, nonclosed structures that can be loaded with hydrophilic substances also exist. Real spherical vesicles, however, could not take up Fl-UTP in the absence of ethanol.

To clarify whether this ethanol-induced increase in permeability could be observed with all kind of liposomes (SUVs/LUVs/GVs) or whether it was again unique to GVs, we performed the same kind of experiments with conventional extruded LUVs (liposomes produced by extrusion through filters with 100-nm

pores, see Ref. [23]). "Empty" liposomes were incubated in the presence of ethanol and UTP/[^{35}S]UTP for 1 h and the external UTP (together with the ethanol) was removed by spin column chromatography.[24] The isolated liposomes were then analyzed by scintillation counting. The same procedure of external loading of LUVs as was used with nucleotides has also been carried out with detergents. The liposomes that contained nucleotides could be isolated by spin column chromatography.[25] In the case of ethanol, however, no uptake could be detected even at ethanol concentrations of 2 and 10% (v/v) (Figure 4). Note that some of the nucleotides were eluted with the liposomes (in this kind of gel chromatography methodology, fractions 2–6 normally contain >98% of the applied turbid material). The approximately 300–400 counts per minute coeluted with liposomes represent, however, less than 0.05% of the total amount of radioactivity and are also eluted in the absence of ethanol. This effect is always seen when working with radiolabeled substances and is not considered as a specific liposome–substrate interaction. Therefore, these few counts per minute can be ascribed to a nonspecific external UTP–liposome interaction. Again, these results suggest a noteworthy difference between the physical behavior of GVs formed in an electric field and that of conventionally prepared LUVs.

T7 RNA polymerase reaction in giant vesicles

Essentially, we have taken the results of Figure 3 as evidence that all mononucleotide triphosphates, such as ATP, UTP, GTP, and CTP, can permeate across the membrane of a selected GV from the external medium in the presence of ethanol. This provides us with the opportunity to perform RNA or DNA synthesis inside GVs that contain a nucleic acid template and an appropriate polymerase by addition of the monomers to the external medium. We used YO-PRO-1 (4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)propyl]quinolinium iodide) as a fluorescence indicator, a molecule which becomes intensively fluorescent once it binds to nucleic acids.[26] We also used this molecule in our previous studies with GVs.[11] As an enzymatic test reaction, we selected the synthesis of mRNA inside GVs by T7 RNA polymerase. As is well known, this enzyme recognizes a specific promoter sequence on a DNA molecule which works as a start signal for the transcription of RNA.[27] This viral enzyme has the advantage of being commercially available and its structure is relatively simple.[28]

Although the most interesting experiment was the one that involved permeation of mononucleotides from outside the GV, we first carried out experiments in which two mixtures that contained DNA templates, enzymes, and nucleotides were consecutively injected into the selected GV (for details, see the Experimental Section). This was performed in order to determine by a control experiment the expected fluorescence intensity increase and to have an idea about the rate of the process. Figure 5 shows the time course of the change in fluorescence intensity inside the GV and also the corresponding experiment without T7 RNA polymerase. The data points reported in Figure 5B show two independent experiments with a corresponding control experiment in which the enzyme T7 RNA polymerase was omitted. One can see from Figure 5 that under our conditions the reaction proceeds for about 30 min with a fluorescence increase of 80% from the initial value. Most of the newly synthesized product that gave this 80% increase was formed within 5 to 15 minutes after the second injection. It is also evident that this technique has its drawbacks. Once the enzyme solution is injected, the selected GV becomes larger (the enzyme is delivered in a 50% glycerol solution and glycerol normally causes "growth" of these GVs), a phenomenon which can often be seen with this kind of GVs. The consequence is that the injected solution is gradually diluted, and therefore, it is not surprising that the increase of fluorescence (corresponding to the synthesis of RNA by T7 RNA polymerase) occurs for only 30 min.

Let us consider the corresponding experiment in which macromolecules are injected into a selected GV and then ethanol is added to the medium before the nucleotides are injected into the medium in the vicinity of the selected GV. The pictorial view of the fluorescence increase is given in Figure 6A, whilst the corresponding kinetics are shown in Figure 6B. The kinetics are not comparable to those obtained by the injection procedure described above because nucleotides were added at intervals by injection into the surrounding medium (additions are indicated by the asterisks in Figure 6B). Here,

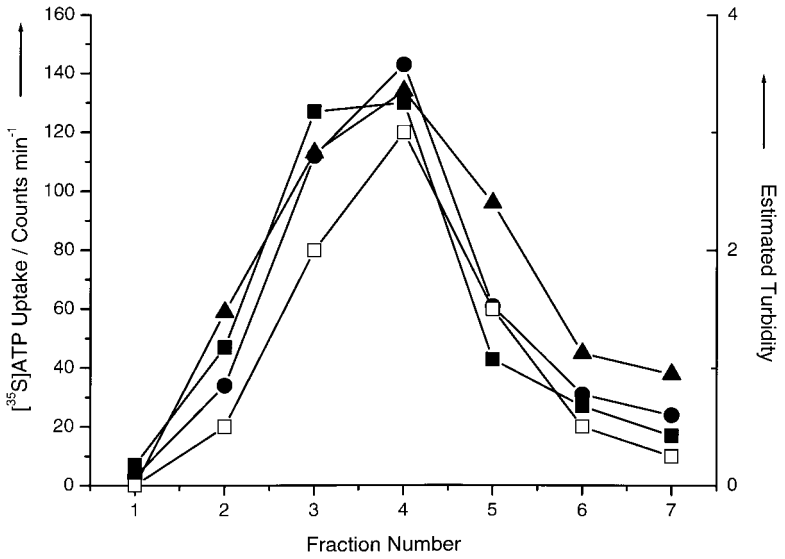

Figure 4. *Ethanol-dependent UTP uptake of conventional liposomes. Conventional LUVs were prepared as described in the Experimental Section. The LUVs were incubated in the absence (filled squares) or presence of ethanol (2% (v/v), circles; 10% (v/v), triangles) for 30 min, before UTP/[^{35}S]UTP was added and the LUVs were incubated for 1 h. Afterwards, the UTP and ethanol molecules were removed by spin column chromatography. The empty squares show the distribution of the turbidity among the individual fractions. After fraction number 7, no significant turbidity could be detected. All three experiments had a similar distribution of light scattering material after the column chromatography therefore only one curve is presented. It should be noted that the count-per-minute values that were eluted together with the liposomes correspond to less than 0.05% of the total applied radioactivity and can be considered as insignificant.*

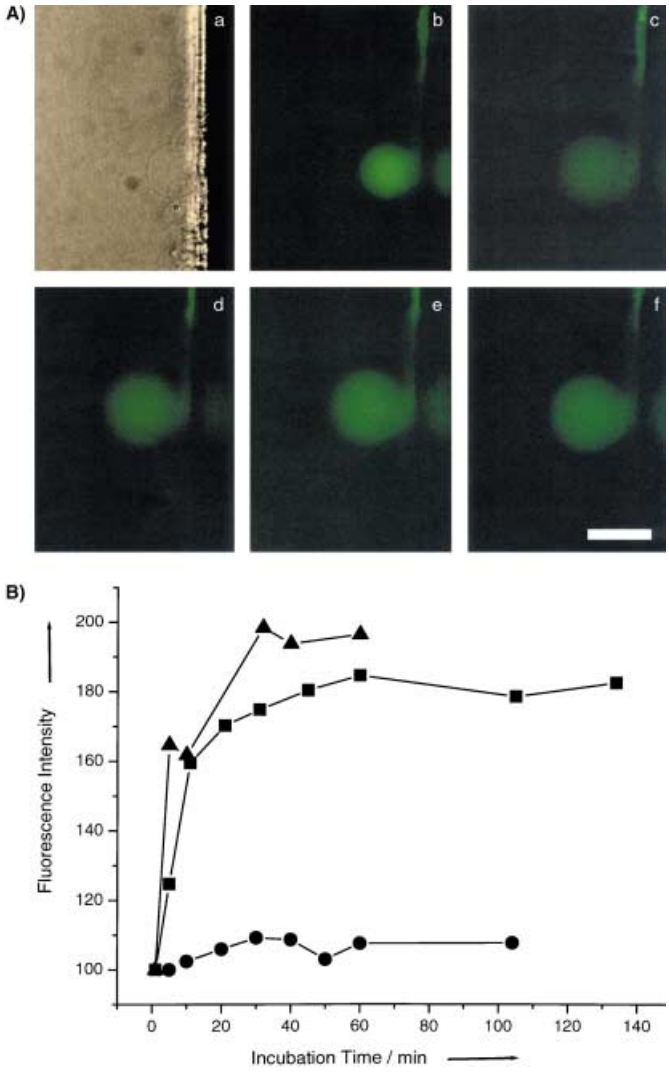

Figure 5. *A) RNA synthesis inside a selected GV, catalyzed by T7 RNA polymerase—double injection method (for details, see the Experimental Section). a, b) 33 min after the injection of the plasmid DNA, c) 1 min, d) 11 min, e) 31 min, and f) 2 hours and 15 minutes after the injection of the solution containing enzyme/nucleotides. a) DIC; b – f) fluorescence mode. Scale bar = 50 μm. B) Kinetics of the RNA synthesis inside giant vesicles. The squares and triangles show two typical experiments with T7 RNA polymerase, the circles show an identical experiment performed without enzyme.*

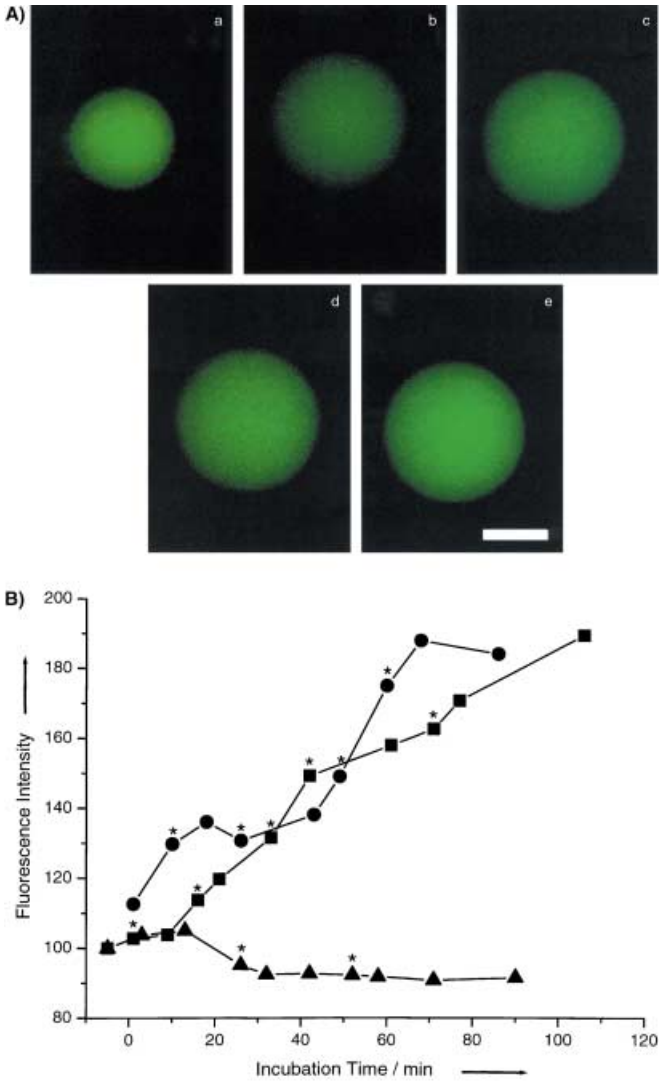

Figure 6. *Modeling of a microreactor by loading a giant vesicle with nucleotides from the outside with occasional addition of nucleotides during the time course of the mRNA synthesis (for details, see the Experimental Section). A) Nucleotides (10 mM each) were injected into the surrounding solution in the vicinity of the GV membrane by using a femtotip II. Fluorescence images: a) 7 min, b) 9 min, c) 42 min, d) 71 minutes, and e) 106 minu after the first addition of nucleotides. Scale bar = 50 μm. B) Quantitative analysis of the increase in fluorescence intensity. Circles/squares show two independent experiments with all reactants; triangles represent an identical experiment in which T7 RNA polymerase was omitted. The asterisks indicate the time points at which nucleotides were externally injected into the surrounding medium.*

there is no depletion of nucleotides and therefore the RNA polymerization by T7 RNA polymerase can proceed for a longer period than in the former experiment. It is important to emphasize that the fluorescence increase cannot be caused by the simple entrance of nucleotides since a nucleotide – YOPRO-1 interaction has never been detected.

An attempt was also made to demonstrate that RNA synthesis can be achieved (and detected) after a single addition of nucleotides (10 punctures for 0.5 s at 200 kPa) at the beginning of the experiment after the injection of a DNA template and T7 RNA polymerase (Figure 7 A). To avoid the increase in size of the selected GV caused by the simultaneous addition of glycerol, 200 μL of the solution containing the T7 RNA polymerase was dialyzed against 50 mL of the appropriate buffer prior to use. The time course of the fluorescence increase can be easily seen in Figure 7 A and the corresponding kinetics are presented in Figure 7 B. From the kinetic data it is obvious that the reaction proceeds for about 30 – 40 min in contrast to the double-injection experiments (Figure 5), where most of the product was synthesized within a few minutes. After 40 min, the fluorescence intensity remained constant.

Discussion

The present paper focuses on the question of whether giant vesicles can be used as microreactors in order to carry out

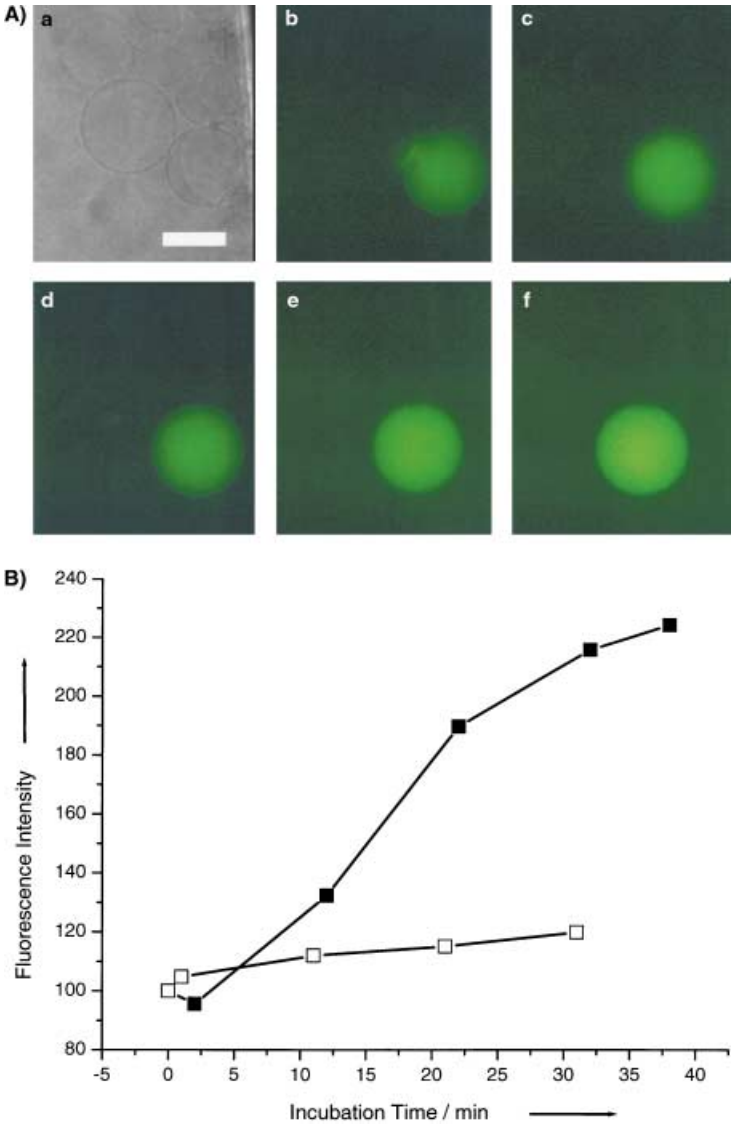

Figure 7. *RNA polymerization inside a GV by T7 RNA polymerase with nucleotide addition from the outside at the beginning of the experiment (for details, see the Experimental Section). A) The injection time for the nucleotide mixture was 30 × 1 s at an injection pressure of 200 kPa. No further nucleotide addition was made after the initiation period. Fluorescence images: a) 2 min, b) 12 min, c) 21 min, d) 32 min, and e) 38 min after the first addition of nucleotides. Scale bar = 50 μm. B) Quantitative analysis of the fluorescence intensity. Filled squares show the kinetics with all the reactants; open squares show the corresponding experiment without T7 RNA polymerase.*

compartmentalized RNA synthesis. With conventional liposomes, similar attempts have already been described.[29–31, 36] The system described herein has many advantages. The first and possibly main reason to use GVs instead of conventional liposomes is that whatever is done in these GVs can be recorded in real time and visualized. Performing biochemical reactions in conventional liposomes always leads to the simple and crucial question, how can one be sure that it really happens inside and not outside the vesicle? This is undoubtedly not a problem when working with our GV system since everything we do is, per se, inside the GV and the results can be easily monitored. Similar arguments are also valid for the reaction time. The reaction begins when the injection has been done or when substrate molecules have been added. Again, this is in contrast to experiments with conventional liposomes, where the starting point is often difficult to determine because the entrapment procedure is relatively complicated and undefined.

In the present work, however, we go one step further. There is not only the question of performing enzymatic reactions inside an artificial compartment, but also the chosen enzymatic reaction is performed in a way that can be considered typical for an origin of life model.[32] Macromolecules are inside a compartment and the substrate molecules are loaded from the external medium. In this way, the GVs work as a bioreactor. Substrate molecules are taken up by the bioreactor with the help of ethanol molecules that increase the permeability of the GV bilayer, then the substrate molecules are incorporated into nucleic acid polymers.

This work is divided into two main parts. In the first part (Sections 1 and 2), we define the conditions under which it is possible for GVs to be loaded from the external medium and demonstrate that a substantial loading is possible with the help of ethanol. In the latter part we apply this new methodology to "feed" liposomes as exemplified by an important biochemical reaction, namely mRNA production by T7 RNA polymerase.

As mentioned above, GVs filled with macromolecules are attractive models for cells; they are large—as large or larger than eukaryotic cells—and a reaction that occurrs in the GV interior can easily be followed if it can be made visible by using fluorescent molecules. This is a minor problem for biochemical reactions with nucleic acids because many suitable fluorescent dyes exist and, therefore, RNA or DNA synthesis can be followed by observation of the increase in fluorescence.[7, 11, 33] For the synthesis of other macromolecules, the fact that appropriate fluorescent analogues must exist can be a serious limitation of these GV compartments.

Experimental Section

Materials: POPC was obtained from Avanti Polar Lipids (Birmingham, AL). YO-PRO-1 iodide and Calcium Green-2 were purchased from Molecular Probes, Inc. (Eugene, OR). ATP, CTP, GTP, UTP, ethanol (puriss. p.a.), glycerol (puriss. p.a.; 86–88%), 1-octanol (puriss.), 1-pentanol (puriss. p.a.), and EDTA were obtained from Fluka (Buchs, Switzerland). Fluorescein-12-UTP tetralithium salt was purchased from Roche Diagnostics (Rotkreuz, Switzerland). All other chemicals were of analytical grade. T7 RNA polymerase (EC 2.7.7.6) was obtained from New England Biolabs (Beverly, MA). The water used was deionized with a Milli-Q system (Millipore, Bedford, MA) and sterilized (> 20 min at 121 °C).

Construction of the plasmid pWMT7–EGFP: The vector pWMT7–EGFP was designed by T. Rechsteiner (BioTecton, Zürich, Switzerland). In brief, the *egfp* gene was excized from the vector pLP-EGFP-C1 (from Clontech) by using the oligonucleotides BT-27 (5′-ATCGCGTCTCCTATGGTGAGCAAG-3′) and BT-28 (5′-

ATCGCGTCTCCCTTATCTAGATCCGG-3′). Two other fragments were produced by the polymerase chain reaction (PCR) by using the vector pET3a (from Novagen); for fragment 1, the following primer oligomers were used: BT-23 = 5′-ATCGCGTCTCGATCGCGGGAGCTGC-3′ and BT-26 = 5′-GGGTACCATGGCGTCTCCCATATGTATATCTCCTTC-3′); for fragment 2 PCR was performed with the oligomers BT-24 (5′-ATCGCGGGGGTCTCACGATCGGTCACAGCTTG-3′) and BT-25 (5′-GGGGTACCCGTCTCCTAAGGATCCGGCTG-3′). All primer oligos contained BsmBI sites in the noncomplementary region. The PCR fragments were digested with BsmBI and ligated. The clones were tested to ensure they contained all fragments. These vectors were the constructs pET3a – EGFP, with a length of 5402 base pairs (bp).

An EcoRV – BamHI fragment (containing the T7 expression box) was excized from pET3a – EGFP and subcloned into the vector pBT100.1 (a derivative of pWM529). The resulting vector, pWMT7, was amplified by PCR with the primers BT-25 and BT-26 (see above). Again, the fragment was digested with BsmBI and ligated with the PCR product (also BsmBI-digested) obtained from pLP – EGFP-C1 with the primers BT-27 and BT-28. The resulting plasmid pWMT7 – EGFP had a length of 3026 bp. Plasmid DNA was purified by the alkali lysis method, precipitated with isopropanol, and treated with Rnase A (10 mg mL^{-1}) for 1 h and then with proteinase K (50 mg mL^{-1}, incubation for 30 min), essentially following the methods described by Sambrook et al.[34]

Preparation of giant vesicles (GVs): Giant vesicles were prepared by the electroformation method as previously described.[11] A POPC solution (2 × 2.5 μL, 0.2 mg mL^{-1}) in diethylether/methanol (9:1) was carefully deposited on each platinum electrode of the investigation chamber and dried under a nitrogen stream for about one minute. Afterwards, the chamber was placed under reduced pressure (< 10 mbar) in a desiccator for at least 15 h. The investigation chamber was then positioned on the stage of an inverted light microscope (Axiovert 135 TV, Carl Zeiss AG), and connected to a frequency generator (Conrad Electronic, Hirschau, Germany) which provided the appropriate ac field (10 Hz, 2 – 3 V, peak-to-peak value; these parameters were controled by an oscilloscope from Velleman, Belgium). The appropriate aqueous solution (1 mL) was added and the giant vesicles were formed in the ac electric field. Typically, after about 2 – 4 h the largest vesicles had reached sizes of 60 – 100 μm in diameter. The alternating electric field was then turned off and further experimentation was started.

Ca^{2+} and fluorescein-12-UTP permeability experiments: The GVs were formed in an aqueous solution containing Calcium Green-2 (2 μM), a fluorescent dye sensitive to calcium ions, to test whether calcium ions can permeate across GV bilayers in the presence of alcohol. EDTA was then added to the external medium at a final concentration of 40 μM to quench some of the background fluorescence outside the GVs. 1 – 2 hours after the EDTA addition, the desired amount of alcohol was added to the external medium and the vesicles were allowed to stand for at least 2 h. A $CaCl_2$ solution (10 mM) was loaded into a micropipette and injected into the external medium in the vicinity of the selected GV (at a distance of approximately 50 μm from the GV) by using a femtotip II (Eppendorf; inner diameter = 0.5 μm). The fluorescence intensity of the selected GVs was recorded at given time points. To quantify of the results, the luminosity values were determined by using the Image-Pro Plus software from MediaCybernetics, MD, USA. The appropriate fluorescent vesicle was selected and the color ranges for red, green, and blue were set to a value of 255 (RGB system). The mean density of luminosity per pixel area was determined and the entire number of pixels was calculated by multiplication of their area. The luminosity at the beginning of the experiment was normalized to 100 and the time course of the fluorescence increase or decrease was followed.

The permeability experiments with fluorescein-12-UTP were carried out in essentially the same way, but with some minor modifications: The vesicles were grown in H_2O and no EDTA was added after turning off the generator. The fluorescein-12-UTP solution which was loaded into the micropipette and injected into the external medium in the vicinity of the selected GV had a concentration of 5 mM.

Permeability studies with conventional liposomes: An aqueous dispersion of conventional liposomes was produced from POPC (40 – 50 mM) by the extrusion technique[23] by using a LiposoFast Basic from Avestin (Avestin Inc., Ottawa, Canada). The diameter of the pores of the polycarbonate filters was 100 nm. These LUVs were diluted to 30 mM POPC with an aqueous solution of ethanol so that the final ethanol concentration was 0, 2, or 10% (v/v). The suspension was mixed and then the LUVs were allowed to stand at room temperature for 30 min. The substrate molecules (0.2 mM UTP/[^{35}S]UTP) were then added and mixed, and the LUVs were again incubated at room temperature for 15 min. Afterwards, the liposomes were loaded onto a BioGel A15m column (BioRad) and a spin-column gel permeation chromatography isolation was performed.[24, 35] This method allows a separation of the liposomes from the substrate molecules that have not been entrapped within about 5 – 10 min. The individual fractions were collected and the [^{35}S]UTP was quantified by liquid scintillation counting by using the scintillation cocktail PICO AQUA (Packard BioScience Company, Meriden, CT, USA).

Enzymatic synthesis of nucleic acid by T7 RNA polymerase inside GVs:

Method of double injection: The vesicles were formed by electroformation in a solution containing sucrose (50 mM), $MgCl_2$ (5 mM), and nucleotides (1 mM each). After their formation, YO-PRO-1 was added to the external medium (final concentration 1 μM) and the GVs were allowed to stand for 30 min. The reaction mixture was added by a double-injection procedure. The first injection mixture contained the plasmid DNA pWMT7 – EGFP (118 μg mL^{-1}) and was injected by puncturing the selected GV and pushing three times at a pressure of 200 kPa for 1 s. The GV was incubated for 2 h before, in a second step, an enzyme solution that contained T7 RNA polymerase (5000 U mL^{-1}), ATP, CTP, GTP, UTP (5 mM each), tris(hydroxymethyl)aminomethane (Tris) – HCl (200 mM, pH 8.0), $MgCl_2$ (40 mM), sucrose (15 mM), 1,4-dithiothreitol (DTT; 10 mM), β-mercaptoethanol (2 mM), and glycerol (5%) was injected into the same GV, which was again punctured and the solution was injected by pushing 5 – 10 times at a pressure of 200 kPa for 1 s). The time course of the enzymatic reaction was followed as described above for the Ca^{2+} – Calcium Green-2 experiments, with the difference that the increase of YO-PRO – nucleic acid fluorescence was determined.

Method of external loading with nucleotides: After formation of the vesicles in YO-PRO-1 (1 μM), ethanol (10 μL) was added to the external aqueous medium and the vesicles were allowed to stand for approximately 2 h. Then a T7 RNA polymerase reaction mixture that contained T7 RNA polymerase (15 000 U mL^{-1}), pWMT7 – EGFP (746 μg mL^{-1}), Tris – HCl (200 mM, pH 8.0), $MgCl_2$ (37.3 mM), EDTA (0.3 mM), NaCl (30 mM), DTT (10 mM), β-mercaptoethanol (6.6 mM), Triton X-100 (0.03%), and glycerol (15%) was injected into the chosen GV (5 – 10 pushes at an injection pressure of 200 kPa for 1 s). Once the fluorescence intensity of the plasmid DNA/YO-PRO-1 complex was constant, a solution containing ATP, CTP, GTP, and UTP (10 mM each) was injected into the surrounding solution in the vicinity of the selected GV with a femtotip II (Eppendorf). The

corresponding control experiment was carried out by using an identical reaction mixture without T7 RNA polymerase.

In some experiments, a solution (100 μL) containing T7 RNA polymerase was dialyzed against 50 mL Tris – HCl (40 mM, pH 8.0), $MgCl_2$ (8 mM), and DTT (5 mM) for 2 h by using a Slide-A-Lyzer with 10 000 Da molecular weight cut-off (from Pierce, Rockford, IL). The dialyzed enzyme solution was kept on ice and used for further experimentation within 2 d.

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