Long-lived protein expression in hydrogel particles: towards artificial cells†

Xiaoyu Zhou, Han Wu, Miao Cui, Sze Nga Lai and Bo Zheng*†

Herein we report a new type of cell-mimic particle capable of long-lived protein expression. We constructed the cell-mimic particles by immobilizing the proteinaceous factors of the cell-free transcription and translation system on the polymer backbone of hydrogel particles and encapsulating the plasmid template and ribosome inside the hydrogel. With the continuous supply of nutrients and energy, the protein expression in the cell-mimic particles remained stable for at least 11 days. We achieved the regulation of protein expression in the cell-mimic particles by the usage of lac operon. The cell-mimic particles quickly responded to the concentration change of isopropyl β-D-1-thiogalactopyranoside (IPTG) in the feeding buffer to regulate the mCherry expression level. We also constructed an in vitro genetic oscillator in the cell-mimic particles. Protein LacI provided a negative feedback to the expression of both LacI and eGFP, and the expression level change of eGFP presented an oscillation. We expect the cell-mimic particles to be a useful platform for gene circuit engineering, metabolic engineering, and biosensors.

Introduction

Artificial cells are an essential tool in the research of the origin of life,1–3 synthetic biology,4–7 drug delivery,8–11 gene therapy,12,13 etc. Artificial cells are generally capable of some functions or behaviors of natural cells, such as protein expression,14–17 communication,18–21 and self-replication.22,23 These artificial cells are mostly liposomes,21,24 polymersomes,25,26 hydrogels,27,28 or simply aqueous droplets29 encapsulating the cell-free expression system. Cell-free expression systems are usually cell extracts from Escherichia coli (E. coli), wheat embryo and rabbit reticulocytes29,31 supplemented with energy components and free amino acids.32–34 In addition, a purified cell-free transcription and translation system, termed the protein synthesis using recombinant elements (PURE) system was developed by Ueda and co-workers.35 The PURE system was reconstituted from the essential elements of the E. coli transcription and translation system.

Cell-free protein expression in an artificial cell in the batch format mostly terminated within a few hours due to the depletion of energy and nutrients and the accumulation of waste products.33,35 To solve this problem, many efforts were devoted to building artificial cells capable of long-lived protein expression through continuous energy and nutrient uptake and waste elimination. Dialysis membranes encapsulating the cell-free protein expression system inside were introduced, and the protein expression in the artificial cells could last tens of hours with higher protein yield.36 However, dialysis membranes presented a molecular weight cut-off, making it difficult for macromolecules including protein products to pass through the pores. Recently, Maerkl and co-workers built a micro-reactor that supported regulated protein expression up to 30 hours.37 Mass exchange and mixing were actively controlled by the integrated pneumatic valves. Noireaux et al. reported vesicle-based artificial cells which expressed and anchored the pore proteins to the vesicle membrane.37 Small molecules such as the energy source and nutrients can diffuse through the nanopores in the membrane, thus facilitating continuous protein expression up to 4 days. However, vesicle-based artificial cells required a delicate balance of osmotic pressure to prevent vesicle bursting. In contrast to the microencapsulation of the cell free expression system, micro-compartmentation of DNA templates, e.g., DNA gel38 and DNA brushes,39,40 allowed continuous protein expression when the cell free expression system was supplied together with energy and nutrients. DNA brush based artificial cells provide a useful platform for studying synthetic networks.41 However, DNA brushes present characteristics more of a biochips than an artificial cell, and functions such as multi-compartments, motility and chemotaxis42 are difficult to achieve.

Herein, we aimed to construct hydrogel-based cell-mimic particles capable of long-lived protein expression. Instead of physical entrapment of the transcription and translation system in polymeric particles as in previous studies40–42 to achieve cell-free protein expression, we immobilized the proteinaceous factors of the PURE system on the polymer backbone of
hydrogel particles through chelating bonds. We achieved selective nutrient, waste, and energy exchange by the diffusion process between the surrounding buffer and the cell-mimic particles. The resulting cell-mimetic particles were an open system that supported not only long-lived protein expression, but also expression regulation and genetic oscillators.

Results and discussion

We first prepared a functionalized polyacrylamide (PA) hydrogel that presented a nickel(n)–nitrilotriacetic acid (Ni$^{2+}$–NTA) moiety on the polymer backbone (Fig. 1a). First, acrylic acid N-hydroxysuccinimide ester was copolymerized with the hydrogel precursors. Then $N_{x}N_{x}$-bis(carboxymethyl)-$\gamma$-lysine hydrate (AB-NTA) was incorporated into the polymer backbone through the reaction with the N-hydroxysuccinimide (NHS) group. After the formation of the Ni$^{2+}$–NTA complex, the resulting functionalized PA hydrogel can be used for the immobilization of His-tagged proteins.46 We evaluated the immobilization efficiency of the functionalized hydrogel by using the His-tagged eGFP. The PA hydrogel particles were treated with the solution of His-tagged eGFP, followed by washing with water several times. A control experiment was performed by treating the unfunctionalized PA hydrogel with the same solution of His-tagged eGFP. The His-tagged eGFP remained in the functionalized hydrogel, probably due to both the entanglement of the DNA strands with the hydrogel polymer backbone and the reaction of the His tag and the Ni$^{2+}$–NTA complex. The expression of non-His-tagged mCherry was performed in the cell-mimetic particles to verify the capability of protein expression. A red fluorescence was observed in the cell-mimetic particles when the plasmid containing the non-His-tagged mCherry coding gene was encapsulated into the cell-mimetic particles (Fig. 2a and b). The SDS-PAGE results (Fig. 2c) confirmed that non-His-tagged mCherry was expressed inside the cell-mimetic particles and then diffused out to the surroundings. By contrast, no proteinaceous factors or ribosome bands could be found in the surroundings, indicating that the proteinaceous factors and ribosome of the PURE system remained inside the cell-mimetic particles. The non-His-tagged mCherry observed in the surroundings indicated that the protein product freely diffused out of the cell-mimetic particles, providing a potential means of communication with nearby cell-mimetic particles. The results proved that the cell-mimetic particle was (i) a self-maintaining system that kept the proteinaceous factors, ribosome and plasmid template inside the cell-mimetic particle; and (ii) an open system that allowed exchange of nutrients, energy, and expression products between the cell-mimetic particle and the surroundings.

The yield of non-His-tagged eGFP in the solution of the predried PA hydrogel particles was (i) a self-maintaining system that kept the proteinaceous factors, ribosome and plasmid template inside the cell-mimetic particle; and (ii) an open system that allowed exchange of nutrients, energy, and expression products between the cell-mimetic particle and the surroundings.

The expression yield of non-His-tagged eGFP was measured to evaluate the efficiency of the cell-mimetic particles. A group of ten cell-mimetic particles in 5 μL feeding buffer reached the expression equilibrium after 4 hours, with the final non-His-tagged eGFP concentration in the surrounding buffer at 197 μg mL$^{-1}$. In the control experiment, we prepared 5 μL feeding buffer with the same composition. The buffer solution also contained the same amount of proteinaceous factors and ribosome of the PURE system as in the combined ten cell-mimetic particles. The yield of non-His-tagged eGFP in the solution of

Fig. 1 (a) The schematic of the synthesis of the functionalized polyacrylamide (PA) hydrogel; (b) confocal images of the functionalized PA hydrogel containing the His-tagged eGFP; (c) and (d) confocal images of the same functionalized PA hydrogel as in (b) after washing with water and with imidazole, respectively; (e) and (f) confocal images of the same unfunctionalized PA hydrogel containing the His-tagged eGFP before and after washing with water, respectively. Scale bars in the images are 500 μm.
The control experiment reached equilibrium after 2 hours, with the final non-His-tagged eGFP concentration of 87 μg mL⁻¹. By comparison, the in vivo expression in E. coli yielded eGFP with the concentration on the scale of tens of μg mL⁻¹. The results showed that the protein yield and the expression efficiency were higher in the cell-mimic particles than in the aqueous solution and in the E. coli cells. The high expression efficiency of the cell-mimic particles was an unexpected observation. In E. coli, on which the PURE system is based, the transcription and translation processes are coupled, which require a close proximity of ribosome, RNA polymerase, and other related factors. The efficient expression of non-His-tagged eGFP in the cell-mimic particles suggested that the polymer backbone was flexible enough to support the diffusive movement of the immobilized factors and facilitate the coupled transcription and translation processes. The better performance of the cell-mimic particles in protein expression is probably because the proteinaceous factors were confined inside the cell-mimic particles with a much higher local concentration. We expect that smaller or thinner cell-mimic particles with higher surface-area-to-volume ratio will have higher expression yield, as the exchange of nutrients, energy, and expression products by diffusion will take less time.

Next, we tested the capability of long-lived protein expression of the cell-mimic particles. The cell-mimic particles were placed in a microchamber which was connected to the main channel through a side channel (Fig. 3a). Due to the sub-millimeter dimension of the microchannel and the low flow rate, the exchange of nutrients, energy, and expression products between the cell-mimic particles and the solution in the main channel was achieved by diffusion through the side channel. The openness of the cell-mimic particles was crucial to the long-lived protein expression in the cell-mimic particles for the efficient energy and nutrient supply and waste discharge. During the experiment, the nutrients and the energy agents continuously diffused into the cell-mimic particles to support the protein expression, while the protein products and by-products diffused out of the cell-mimic particles. When continuously feeding the cell-mimic particles, we observed stable expression of non-His-tagged mCherry for at least 11 days (Fig. 3b). In the control experiment, the temperature was decreased to room temperature to pause the expression. The associated decrease of the fluorescence signal indicated the loss of non-His-tagged mCherry in the cell-mimic particles by diffusion (Fig. S2†). Once the temperature returned to 37 °C, the expression resumed and the fluorescence signal in the cell-mimic particles was recovered (Fig. 3b). We expect the protein expression to continue until the immobilized proteinaceous factors of the PURE system and the ribosomes degrade. The long-lived protein expression of the cell-mimic particles facilitated more complex functions of the cell-mimic particles such as protein expression regulation and gene networks.

The cell-mimic particles were then used to achieve the regulation of the gene expression. E. coli RNA polymerase was added to the PURE system to initiate the transcription and translation of the protein LacI, which would impede the transcription of the non-His-tagged mCherry gene by binding with the Lac operator linked to the mCherry gene. When isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into the feeding buffer, LacI would bind to IPTG, releasing the Lac operator to activate the mCherry expression (Fig. 4a). After the addition of IPTG into the feeding buffer, the mCherry expression level would be enhanced. The mCherry expression level increased with the increase of the IPTG concentration until the IPTG concentration reached 10 mM (Fig. 4b). When IPTG was removed from the feeding buffer, the mCherry expression returned to the initial low level as the LacI protein would bind to...
removal rate of the proteins.

the period of the same oscillator in natural cells.

lation, with a period of about 2 minutes (Fig. 5b). The period of

promoters became free of bound LacI and the cycle began anew.

the Lac operator again. The cell-mimic particles’ response to

IPTG showed their potential to be a biosensor platform in

addition to being a useful cell model for the research of gene

networks and cell–cell communication.

We further demonstrated a negative feedback gene oscillator

in the cell-mimic particles. With the transcription action of T7

RNA polymerase, protein LacI and non-His-tagged eGFP were

expressed simultaneously in the cell-mimic particles. The

accumulation of protein LacI would inhibit the expression of
eGFP and LacI as a negative feedback, leading to the decreased
expression level of LacI and eGFP (Fig. 5a). Once the accumu-
lated LacI diffused out of the cell-mimic particles, the

promoters became free of bound LacI and the cycle began anew.

As expected, the fluorescence signal of eGFP showed an oscil-
lation, with a period of about 2 minutes (Fig. 5b). The period of

the oscillator in the cell-mimic particles was much shorter than
the period of the same oscillator in natural cells. The differ-
ence is likely because the oscillator period was related to the
removal rate of the proteins. In natural cells, the protein
products were degraded by catabolic enzymes, while in the cell-
mimic particles, the decrease of the protein level was dependent

on the diffusion process, which was much quicker than the
degradation process. With the demonstration of the gene
oscillator, we expect that cell-mimic particles possess great
potential as a platform to test the dynamic behavior of the gene

network.

Conclusions

In conclusion, we built a new type of self-maintaining and self-
regulating cell-mimic particle. The prominent features of the

cell-mimic particles are the long-lived and efficient protein
expression in the particles. The features were achieved through
the immobilization of the cell-free transcription and translation
system on the polymer backbone of the cell-mimic particles,
which rendered the particles capable of continuous uptake of

nutrients and energy from the surroundings and the diffusion of
the protein expression products out of the particles.

With the long-lived and efficient protein expression, the cell-
mimic particles supported the gene regulation and gene circuit
engineering. We envision that the cell-mimic particles will also
be a useful platform for biosensors such as toehold-based

sensors for viruses and for the research of cell–cell commu-
nication such as biofilm disruption. In addition, with a
coating of a lipid bilayer, the cell-mimic particles provide
a potential starting point to the construction of artificial cells
and drug delivery vehicles.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We acknowledge the financial support by the Research Grants Council of Hong Kong (GRF 14303917), The Chinese University of Hong Kong (Direct Grant 4053158), and the Hong Kong Ph.D. Fellowship (H.-W.) from the Research Grants Council of Hong Kong.

Notes and references

387–390.
3 P. L. Luisi, F. Ferri and P. Stano, Naturwissenschaften, 2006,
93, 1–13.
4 S. Rasmussen, L. Chen, D. Deamer, D. C. Krakauer,
N. H. Packard, P. F. Stadler and M. A. Bedau, Science, 2004,
303, 963–965.
5 C. Larigouze, J. I. Glass, N. Alperovich, R. Pieper, P. P. Parmar,
C. A. Hutchison III, H. O. Smith and J. C. Venter, Science,
6 A. Pohorille and D. Deamer, Trends Biotechnol., 2002, 20,
123–128.
7 C. Chiarabelli, P. Stano and P. L. Luisi, Curr. Opin.


