Published in final edited form as:

Biotechnol J. 2013 November ; 8(11): 1341–1346. doi:10.1002/biot.201200388.

Automated formation of multicomponent-encapsulating vesosomes using continuous flow microcentrifugation

Huisoo Jang^{1,2}, Peichi C. Hu³, Sungho Jung^{1,2}, Won Young Kim¹, Sun Min Kim^{2,4}, Noah Malmstadt³, and Tae-Joon Jeon^{1,2}

¹Department of Biological Engineering, Inha University, Incheon, Republic of Korea

²Biohybrid Systems Research Center, Inha University, Incheon, Republic of Korea

³Mork Family Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, CA, USA

⁴Department of Mechanical Engineering, Inha University, Incheon, Republic of Korea

Abstract

Vesosomes – hierarchical assemblies consisting of membrane-bound vesicles of various scales – are potentially powerful models of cellular compartmentalization. Current methods of vesosome fabrication are labor intensive, and offer little control over the size and uniformity of the final product. In this article, we report the development of an automated vesosome formation platform using a microfluidic device and a continuous flow microcentrifuge. In the microfluidic device, water-in-oil droplets containing nanoscale vesicles in the water phase were formed using T-junction geometry, in which a lipid monolayer is formed at the oil/water interface. These water-in-oil droplets were then immediately transferred to the continuous flow microcentrifuge. When a water-in-oil droplet passed through a second lipid monolayer formed in the continuous flow microcentrifuge, a bilayer-encapsulated vesosome was created, which contained all of the contents of the aqueous phase encapsulated within the vesosome. Encapsulation of nanoscale liposomes within the outer vesosome membrane was confirmed by fluorescence microscopy. Laser diffraction analysis showed that the vesosomes we fabricated were uniform (coefficient of variation of 0.029). The yield of the continuous flow microcentrifuge is high, with over 60% of impinging water droplets being converted to vesosomes. Our system provides a fully automatable route for the generation of vesosomes that are able to encapsulate any desired content. The method employed in this work is simple and can be readily applied to a variety of systems, providing a facile platform for fabricating multicomponent carriers and model cells.

Keywords

Giant unilamellar vesicle; Liposome; Vesosome

1 Introduction

A eukaryotic cell is encapsulated by the plasma membrane, which separates the inner and outer environments of the cell [1]. The primary structural scaffolding of the plasma membrane is a phospholipid bilayer. The eukaryotic cell also has several lipid bilayer-encapsulated compartments called organelles, including the endoplasmic reticulum (ER), Golgi apparatus, lysosomes, and peroxisomes. The inner environment and biomolecular contents of each of these organelles differ depending on the specific organelle function. For instance, the primary function of the lysosome is to degrade proteins. Its inner solution has a low pH and contains 70 varieties of proteases. To protect cytoplasmic proteins from uncontrolled proteolytic degradation, a lipid bilayer boundary exists between the interior space and that outside the lysosome. Compartmentalization allows multicompartmentalized cells to regulate the function of each organelle exquisitely, facilitating the broad variety of cellular roles observed in eukaryotic biology.

To mimic properly the biophysical properties of compartmentalized eukaryotic cells, emerging model cell systems must recapitulate this hierarchical compartmentalization, i.e. the compartments-within-compartments nature of the eukaryotic cytosol. Various lipid-bilayer structures have been applied as models of cellular compartmentalization. Liposomes are often used as drug carriers and to mimic real cells [2, 3]. A liposome is surrounded by a phospholipid bilayer that mimics the plasma membrane of real cells. A 10–100 μm liposome is referred to as a giant unilamellar vesicle (GUV). Such structures facilitate direct microscopic observation in real time, and GUVs are widely used for research of lipid membrane dynamics, mechanics, transport, and phase behavior [4]. However, a GUV is composed of a single membrane that just separates two compartments, precluding research and applications of model cells that rely on a multicompartmentalized form.

Vesosomes were introduced in 1997 by Zasadzinski and colleagues [5]. Vesosomes are liposome-encapsulated liposomes, which widens their applicability as model cells. Unlike the GUVs, since the structure is compartmentalized, a vesosome can potentially mimic multicompartment behaviors of real cells. Multiple levels of encapsulation also improve the drug delivery properties of vesosomes relative to single-membrane-bounded liposomes. Vesosomes also show improved stability to lipase attack and in serum [6].

Unfortunately, GUV-scale vesosome fabrication is complicated by the relatively uncontrolled methods available to form GUVs. GUV fabrication approaches include electroformation [7], simple hydration [8] and layer-by-layer emulsification [9, 10]. Recently, GUVs have been fabricated in microfluidic devices [11, 12]. In particular, we have shown the fabrication of asymmetric giant vesicles of uniform size using a microfluidic device [13]. Creating vesosomes usually requires tedious and finely tuned methods and is extremely time-consuming. Automating the whole process of vesosome formation has proven to be difficult. Recently, the interdigitation-fusion method for producing vesosomes was introduced [14]. However, this method is still complicated, requires specialized technical expertise, and is time-consuming. In addition, the size control of vesosomes was accomplished via the addition of ethanol or by varying the composition of cholesterol [15].

These approaches limit the compositions that can be achieved and are relatively uncontrolled and unautomatable.

In this work, we have devised a method to automate vesosome formation using a microfluidic device together with a continuous flow microcentrifuge. This microcentrifuge device was simply fabricated using a speed-controlled motor. We demonstrate that our device can create uniformly sized vesosomes, constructed as liposomes inside GUVs. Using our system, the size of vesosomes is controllable [16], multiple components can be simply encapsulated, and vesosomes can be generated rapidly with a production yield of ~61%. We expect that our system will be applicable to the study of biomimetic cells, multicomponent carriers, etc.

2 Materials and methods

2.1 Materials

Cholesterol was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Asolectin, 10,12-pentacosadiynoic acid (PCDA), [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (HEPES), propylene glycol monomethyl ether acetate (PGMEA), glucose, sucrose, NaCl and squalene were purchased from Sigma-Aldrich (St. Louis, MO, USA). Asolectin is a great source of various fatty acids for membrane study. It contained about 24% saturated fatty acids, 14% mono-unsaturated and 62% poly-unsaturated fatty acids. 1,2-Diphytanoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) was purchased from Avanti Polar Lipids. n-Decane was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). Poly (dimethylsiloxane) (PDMS) prepolymer and curing agent sets were purchased from Dow Corning (Midland, MI, USA). SU-8 2050-negative photoresist was obtained from MicroChem Corp. (Newton, MA, USA).

2.2 Microfluidic device fabrication

A microfluidic device was fabricated using a negative photolithography process. First, a solution of SU-8, the negative photoresist, was coated onto a silicon wafer to a depth of 80 μm by a spin coater. A film mask with the channel pattern was designed using AutoCAD software. A wafer covered masked with this design was exposed to UV light. After washing with a PGMEA solution, only the UV-exposed sections remained as they cross-link with the negative photoresist. Subsequently, a PDMS-curing agent solution (10:1) was poured onto the master wafer with the raised photoresist pattern. To cure the PDMS solution, the mixture was incubated at 70°C for 2 h. The PDMS mixture was detached from the master wafer and was bonded to glass following an oxygen plasma treatment. This bonded structure was stabilized in an oven. Channels in the microfluidic device had a “T-junction” configuration, with two inlet channels and one outlet channel. One inlet was the water inlet (20 μm wide) and the other was the oil inlet (40 μm wide). The outlet ranged from 40 to 80 μm in width. Channel depth for each channel was 80 μm .

2.3 Continuous flow microcentrifuge fabrication

The main components of the centrifugal device are a motor, speed controller, and main body. A central processing unit (CPU) cooling fan that is available as an off-the-shelf

computer component was used as the motor. The main body was fabricated using a disposable petri dish and plastic tubes. The main body was assembled from two petri dish lids. We made an opening in the upper lid and covered it with a conical cover to prevent spillage of the pre-vesosome solution. We glued the speed controller, embedded CPU cooling fan, and main body together.

2.4 Automated vesosome formation

We were able to create vesosomes automatically by inter-locking the continuous flow microcentrifuge and the microfluidic device. First, we made a water-in-oil droplet in the microfluidic device. Water droplets were formed in a continuous oil phase. The oil solution consisted of a 1:1 n-decane:squalene mixture containing 20 mg/mL dissolved lipids. The lipid mixture consisted of 4:1 asolectin:cholesterol with 0.05% NBD-PE lipid for fluorescent images. The aqueous solution consisted of sucrose buffer (0.5 M sucrose, 10 mM HEPES, 100 mM NaCl) containing red fluorescence-labeled polydiacetylene (PDA) vesicles. PDA vesicles were made by a standard hydration method [17] as described below. Each solution was delivered using a syringe pump (Harvard Apparatus Inc., Holliston, MA, USA) into the microfluidic channel. In the microfluidic channel, the aqueous solution flow rate was 20 $\mu\text{L/h}$, while oil solution flow rate was 100 $\mu\text{L/h}$. Water droplets in the oil phase formed at the intersection of two inlet channels. The water droplets in the oil phase were transferred to the rotating continuous flow microcentrifuge automatically. The rotational speed was controlled by a speed controller, which was set to rotate at a rotational force of 9.6 relative centrifugal force (ref). Inside the main body of the continuous flow microcentrifuge the contents were divided by centrifugal force into two layers: an aqueous and an oil phase. The aqueous phase consisted of glucose buffer (0.5 M glucose, 10 mM HEPES, 100 mM NaCl). The oil phase consisted of an oil solution as described above. Prior to use in experiments, the continuous flow microcentrifuge was stabilized for 30 min to form a lipid monolayer along the interface of the two phases. Finally, a two-step process was used for sample collection. The oil layer was removed while the microcentrifuge was rotating. The vesosomes were then collected from the water layer using a pipette.

PDA vesicles encapsulated inside vesosomes were prepared by a standard sonication method, at a concentration of 2 mM. First, PCDA lipid powder was dissolved in chloroform in a vial. The chloroform was evaporated under a flow of argon gas. The vial was filled with deionized water and heated in a bath at 80°C for 40 min. The solution was ultrasonicated using a probe sonicator for 5 min. The solution was filtered using a 0.8- μm syringe filter and then stored at 4°C overnight. The PCDA vesicle solution was irradiated with UV at the wavelength of 254 nm for 2 min to yield blue-colored PCDA vesicles. This blue-colored solution was heated in a water bath 80°C for 5 min, changing the vesicle color to red due to their structural transition [18]. The red-colored vesicles created here are fluorescent since the HOMO-LUMO energy gap increased [18]. We used these fluorescent red-colored vesicles to visualize and confirm the encapsulation of vesicles.

Results and discussion

3.1 Continuous flow microcentrifuge

Previously, we collected the water-in-oil droplets created in the microfluidic device and employed an additional centrifugation step. However, large amounts of water-in-oil droplets fused during the additional centrifugation step. Our present continuous flow microcentrifugal devices provide a centrifugal force that is strong enough to pull droplets through the lipid monolayer created in the continuous flow microcentrifuge, eliminating the additional centrifugation step. In addition, the water-in-oil droplets made in a microfluidic device were immediately transferred into the continuous flow microcentrifuge to generate vesosomes, minimizing the time during which water-in-oil droplets could fuse. To prove the concept of our device design, the continuous flow microcentrifuge was created simply using a commercial CPU cooling fan. The rotational speed is well controlled, giving rise to relatively precise rcf values that can be achieved during the centrifugation. Figure 1 shows a schematic illustration of a continuous flow microcentrifuge and a real device fabricated in our lab. Since the continuous flow microcentrifuge consists of general components that can be acquired in a common laboratory, it is simple to make.

3.2 Water-in-oil droplet formation in a microchannel

Our system comprising T-junction microchannels and continuous flow microcentrifuge devices enables the automated generation of multicompartmentalized vesosomes. At the T-junction, the oil phase containing the lipid molecules and the aqueous phase are brought into contact. Since the streams are immiscible and flowing at different flow rates, the aqueous stream cuts off evenly at the intersection of each inlet channel, while the oil stream flows continuously, creating water droplets interfacing a continuous oil phase. A lipid monolayer is formed along the interface of the aqueous and oil phases due to the amphiphilic nature of the lipids. Since the droplet is hydrophilic, the lipid molecule head groups face the interior of the droplets, making the droplets resemble reverse micelles. PDA vesicles are included in the aqueous stream, so that the reverse micelle-like water droplets contain PDA vesicles. Any other components can be added to the aqueous phase so that they become encapsulated in the vesosomes. The microfluidic device is controlled by two syringe pumps, enabling the continuous generation of water-in-oil droplets. The water-in-oil droplets can be readily collected from the tube connected to the outlet of the device.

3.3 Vesosome formation in a continuous flow microcentrifuge

The continuous flow microcentrifuge device contains both an aqueous and an oil phase containing lipid molecules. When the device rotates, the solutions are laminated into two layers according to their density differences. The oil phase, with a lower density, is located on the inner space of the continuous flow microcentrifuge (nearer the axis of rotation), while the aqueous phase is located in the outer space of the continuous flow microcentrifuge. A lipid monolayer is formed along the interface of the oil and aqueous phases as in the microfluidic device. However, lipid molecules face with their head groups towards the exterior edge of the continuous flow microcentrifuge since the aqueous phase is located here. As water-in-oil droplets created in the microfluidic device pass through the lipid

monolayer interface in the continuous flow microcentrifuges, vesosomes are automatically generated encapsulating the water-in-oil droplets.

When vesosomes are created in the continuous flow microcentrifuge, the internal centrifugal force is an important variable. If the internal centrifugal force is too strong, vesosomes are destroyed during or before vesosome formation. Similarly, if internal centrifugal force is too weak, vesosomes cannot be formed since water-in-oil droplets are not able to penetrate the lipid monolayer in the microcentrifuge. Internal rcf is acquired using following equation:

$$rcf = 1.12 \times R [mm] \times \left(\frac{rpm}{1000} \right)^2$$

where R is the distance from the axis of rotation to the water/oil interface in the centrifuge main body, and rpm is rotations per minute of the CPU cooling fan. By adjusting the rpm of the motor and the radius of the continuous flow microcentrifuge, we were able to optimize the rcf to generate vesosomes in the most efficient manner. From repeated experiments, we confirmed that an rcf of 9.6 is optimal for automatic generation of vesosomes in a continuous flow microcentrifuge. To estimate the production yield, we compared the number of water-in-oil droplets calculated by volumetric flow rate with the number of produced vesosome. We obtained a production yield of ~61.0%. In contrast to the conventional techniques used to create vesosomes, the system reported here is able to create vesosomes automatically by simple manipulation of the flow of aqueous and oil solution containing lipid molecules. The system described in this work is first attempt to create vesosomes automatically, and has, to the best of our knowledge, not been reported elsewhere.

3.4 Uniformity of vesosomes

We are not only able to automate the formation of vesosomes, but also to control the size of vesosomes by adjusting the dimensions of each inlet channel and their flow-through rates[16]. To demonstrate the uniformity of vesosomes, we measured their size distribution using a particle size analyzer from Malvern, which is designed to measure a relatively large particle range from 0.2 to 2000 μm . Since the light scattering intensity differs for each particle size, the size distribution of vesosomes can be simply measured using laser diffraction analysis. As shown in Fig. 2A, the size of vesosomes generated using a continuous flow microcentrifuge peaks at a size of ~20 μm . Although the size distribution of vesosomes appears to be wider than that usually reported in polymer synthesis or nanoparticle synthesis, it compares favorably to the size distribution of vesosomes created using conventional techniques [13, 19, 20]. The coefficient of variation of vesosomes is 0.029, which again compares favorably with previous work [21]. The microscopic image shown in Fig. 2B shows vesosomes consistent with the results of laser diffraction measurement.

3.5 PDA vesicle-encapsulating vesosomes

In order to prove the feasibility of automated generation of multicompartmentalized vesosomes using our system, we encapsulated fluorescent liposomes in vesosomes. Red-

colored PDA vesicles were used as the fluorescent liposomes in this work. To create PDA vesicle-encapsulating vesosomes, the PDA vesicles were simply included in the aqueous solution injected into the microfluidic device. Any other component can be readily encapsulated in vesosomes in the same manner. To visualize the PDA vesicles and GUVs encapsulating the vesicles, images were taken showing the red fluorescence of PDA vesicles and green fluorescence from the leaflets of GUVs. As shown in Fig. 3, we were able to confirm that red-colored inner vesicles were successfully encapsulated in the green-colored outer liposomes, just like peas in a pod. With the system described in this work, multiple encapsulants can be simply encapsulated in GUVs, widening applications of GUVs including multicomponent carrier and model cells.

4 Concluding remarks

In this work we devised a novel method for the automated generation of vesosomes using a microfluidic device and a continuous flow microcentrifuge. Creating vesosomes is typically labor intensive and requires high expertise. However, our system for generating vesosomes is readily reproducible and fully automatable. In addition, multiple encapsulants can be simply introduced inside of GUVs, creating multicompartmental model cell systems. Our system can be potentially applied to a number of research and industrial applications, including multicomponent carriers and model cells. Our system has a high yield and produces unusually uniform vesosomes. Our ongoing research includes the generation of vesosomes in which a simple cellular system is integrated.

Acknowledgments

This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ007995), Rural Development Administration, Republic of Korea and partially by Inha University Grant. The contributions from P.C.H. and N.M. were supported by National Institutes of Health under award 1R01GM093279.

Abbreviations

CPU	central processing unit
GUV	giant unilamellar vesicle
NBD-PE	1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)
PCDA	10,12-pentacosadiynoic acid
PDA	polydi-acetylene
PDMS	polydimethylsiloxane
rcf	relative centrifugal force

References

- [1]. Dacks JB, Peden AA, Field MC. Evolution of specificity in the eukaryotic endomembrane system. *Int. J. Biochem. Cell Biol.* 2009; 41:330–340. [PubMed: 18835459]
- [2]. Bangham AD. Properties and uses of lipid vesicles: An overview. *Ann. N. Y. Acad. Sci.* 1978; 308:2–7. [PubMed: 279288]

- [3]. Kim YR, Jung S, Ryu H, Yoo YE, et al. Synthetic biomimetic membranes and their sensor applications. *Sensors (Basel)*. 2012; 12:9530–9550. [PubMed: 23012557]
- [4]. Walde P, Cosentino K, Engel H, Stano P. Giant vesicles: Preparations and applications. *Chembiochem*. 2010; 11:848–865. [PubMed: 20336703]
- [5]. Walker SA, Kennedy MT, Zasadzinski JA. Encapsulation of bilayer vesicles by self-assembly. *Nature*. 1997; 387:61–64. [PubMed: 9139822]
- [6]. Boyer C, Zasadzinski JA. Multiple lipid compartments slow vesicle contents release in lipases and serum. *ACS Nano*. 2007; 1:176–182. [PubMed: 18797512]
- [7]. Angelova, MI.; Soléau, S.; Méléard, P.; Faucon, F.; Bothorel, P. Preparation of giant vesicles by external AC electric fields. Kinetics and applications. In: Helm, C.; Lösche, M.; Möhwald, H., editors. *Trends in Colloid and Interface Science VI*. Springer; New York: 1992. p. 127-131.
- [8]. Adams DR, Toner M, Langer R. Role of trehalose in prevention of giant vesicle adsorption and encapsulated solute leakage in anhy-drobiotic preservation. *Langmuir*. 2007; 23:13013–13023. [PubMed: 17983248]
- [9]. Pautot S, Frisken BJ, Weitz DA. Production of unilamellar vesicles using an inverted emulsion. *Langmuir*. 2003; 19:2870–2879.
- [10]. Abkarian M, Loiseau E, Massiera G. Continuous droplet interface crossing encapsulation (cDICE) for high throughput monodisperse vesicle design. *Soft Matter*. 2011; 7:4610–4614.
- [11]. Nishimura K, Suzuki H, Toyota T, Yomo T. Size control of giant unilamellar vesicles prepared from inverted emulsion droplets. *J. Colloid Interface Sci*. 2012; 376:119–125. [PubMed: 22444482]
- [12]. Matosevic S. Synthesizing artificial cells from giant unilamellar vesicles: State-of-the art in the development of microfluidic technology. *Bioessays*. 2012; 34:992–1001. [PubMed: 22926929]
- [13]. Hu PC, Li S, Malmstadt N. Microfluidic fabrication of asymmetric giant lipid vesicles. *ACS Appl. Mater. Interfaces*. 2011; 3:1434–440. [PubMed: 21449588]
- [14]. Ahl PL, Perkins WR. Interdigitation – fusion liposomes. *Methods Enzymol*. 2003; 367:80–98. [PubMed: 14611060]
- [15]. de Hoog HPM, Nallani M, Tomczak N. Self-assembled architectures with multiple aqueous compartments. *Soft Matter*. 2012; 8:4552–4561.
- [16]. Xu JH, Li SW, Tan J, Wang YJ, Luo GS. Controllable preparation of monodisperse o/w and w/o emulsions in the same microfluidic device. *Langmuir*. 2006; 22:7943–7946. [PubMed: 16952223]
- [17]. Ji EK, Ahn DJ, Kim JM. The fluorescent polydiacetylene liposome. *Bull. Korean Chem. Soc*. 2003; 24:667–670.
- [18]. Charoenthai N, Pattanatornchai T, Wacharasindhu S, Sukwat-tanasinitt M, Traiphon R. Roles of head group architecture and side chain length on colorimetric response of polydiacetylene vesicles to temperature, ethanol and pH. *J. Colloid Interface Sci*. 2011; 360:565–573. [PubMed: 21601866]
- [19]. Betaneli V, Worch R, Schwille P. Effect of temperature on the formation of liquid phase-separating giant unilamellar vesicles (GUV). *Chem. Phys. Lipids*. 2012; 165:630–637. [PubMed: 22750641]
- [20]. Tamba Y, Terashima H, Yamazaki M. A membrane filtering method for the purification of giant unilamellar vesicles. *Chem. Phys. Lipids*. 2011; 164:351–358. [PubMed: 21524642]
- [21]. Matosevic S, Paegel BM. Stepwise synthesis of giant unilamellar vesicles on a microfluidic assembly line. *J. Am. Chem. Soc*. 2011; 133:2798–2800. [PubMed: 21309555]

Figure 1.

(A) Schematic illustration of the automated vesosome generation system, consisting of a microfluidic device with T channels and a continuous flow microcentrifuge integrated with a speed controlled motor. (B) Photographic images of a continuous flow microcentrifuge made from the lids of petri dishes and a conical tube.

Figure 2.

(A) Size distribution of vesosomes generated from a continuous flow microcentrifuge with the coefficient of variation of 0.029. The size distribution was measured by a laser diffraction method using a particle size analyzer. (B) Microscopic image of vesosomes composed of 4:1 asolectin:cholesterol with 0.05% NBD PE lipids.

Figure 3. Image of vesosomes observed by fluorescence microscopy, indicating the NBD-PE lipid bilayer membrane of vesosome (green). Red dots indicate PDA vesicles encapsulated inside of the vesosome.