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Liposomes in Double-Emulsion Globules

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Abstract

Tubular liposomes containing a hydrophilic model compound (fluorescein sodium salt, FSS) were entrapped inside the internal aqueous phase (W_1) of water-in-oil-in-water ($W_1/O/W_2$) double-emulsion globules. Our hypothesis was that the oil membrane of double emulsions can function as a layer of protection to liposomes and their contents and thus better control their release. Liposomes were prepared in bulk, and their release was observed microscopically from individual double-emulsion globules. The liposomes containing FSS were released through external coalescence, and the behavior of this system was monitored visually by capillary video microscopy. Double-emulsion globules were stabilized with Tween 80 as the water-soluble surfactant, with Span 80 as the oil-soluble surfactant, while the oil phase (O) was *n*-hexadecane. The lipids in the tubular liposomes consist of 1- α -phosphatidylcholine and Ceramide-VI. Variations of Tween 80 concentration in the external aqueous phase (W_2) and Span 80 concentration in the O phase controlled the release of liposomes from the W_1 phase to the W_2 phase. The major finding of this work is that the sheer presence of liposomes in the W_1 phase is by itself a stabilizing factor for double-emulsion globules.

1. Introduction

Encapsulation of active molecules into micro- or nanodelivery systems^{1–6} allows the properties of such active substances to be protected while also controlling the time and rate of release during administration. There has been a multitude of drug-delivery systems, and double emulsions, liposomes, and hydrogels are just a few to name. All delivery strategies have their advantages as well as drawbacks, and a great deal of work is being done in many laboratories around the world trying to optimize existing delivery vehicles and schemes and also coming up with totally new ones.

Since their first description in 1961, liposomes have drawn attention as one of the most widely used drug-delivery systems, which can be applied to encapsulate not only small drug molecules but also proteins.^{7,8} Recently, the nanoscale size of liposomes was exploited for transdermal drug delivery.⁹ The major limitations in using liposomes for substance delivery lie in the fact

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Supporting Information Available: Experimental description of stability of bulk double emulsions with tubular liposomes in the W_1 phase, and cryo-TEM picture demonstrating the stability of liposomes after 24 h. This material is available free of charge via the Internet at <http://pubs.acs.org>.

that they tend to aggregate, fuse with other liposomes, or leak entrapped substances.¹⁰ Moreover, they are susceptible to factors such as changes in pH or temperature or exposure to serum.^{7,11} Once exposed to unfavorable physicochemical conditions, their unique structures become unstable, and from an application point of view it is necessary to retain the intactness of liposomes before they are administered to a specific target.

One suggested method for improving drug delivery is double encapsulation of the active substance (the use of one carrier to protect another one). For instance, encapsulation of liposomes within a second bilayer,¹² entrapment of liposomes in hydrogel microcapsules,¹³ vesicles in a water-in-oil emulsion system,¹⁴ double-walled microparticles,^{15–17} and our own study of fluorescein sodium salt (FSS) release from nanogels encapsulated in double emulsions.² In all the above-cited studies, an additional protective layer led to better protection and controlled release of the encapsulated molecules. With the aim to prohibit the interaction between liposomes and unfavorable physicochemical environments, we propose a novel double-encapsulation system utilizing a water-in-oil-in-water ($W_1/O/W_2$) double emulsion to encapsulate liposomes.

A liquid $W_1/O/W_2$ double emulsion consists of an external aqueous phase (W_2) in which individual oil globules (O) are dispersed. Within each oil globule, there exists smaller droplets of an internal aqueous phase (W_1). Double emulsions lend themselves to potential applications in drug encapsulation and controlled release,^{18,19} particularly through oral,²⁰ topical,²¹ intramuscular, and intravenous routes.²² For the evaluation of molecular weight and antigenicity of the entrapped antigens, the work by Bozkir and Hayta indicated that the $W_1/O/W_2$ double-emulsion system has potential as a vaccine delivery carrier.²³ In fact, from *in vivo* experiments, it has been proven that the intramuscular administration of double-emulsion formulations stimulates a more effective immune response than conventional vaccination.²⁴ By storing liquid $W_1/O/W_2$ double emulsions at temperatures below the freezing point of the oil phase, Rojas et al. showed that crystallized *n*-hexadecane successfully preserved the stability of emulsions for at least 4 months and may protect proteins during storage. When the emulsions returned to room temperature and the oil phase thawed, a large amount of protein was released spontaneously from the W_1 phase to the W_2 phase.^{25,26}

Our motivation in the development of this double-encapsulation delivery system is to prevent liposomes from interacting with external conditions and to ultimately apply this system in trans-dermal delivery. As a barrier between the body and the outside environment, the skin's *stratum corneum* presents a great hindrance to invading pathogens and toxic molecules. The structure of this barrier must be considered in applications that use the delivery of large molecules through skin as is the case of transcutaneous immunization. However, it can be disrupted by hydration^{27,28} or with the aid of molecules known as penetration enhancers.²⁹ In this Article, we are using tubular liposomes made of α -phosphatidylcholine and Ceramide-VI.³⁰ Ceramide lipids are prevalent in the *stratum corneum*. In the possible application of such a formulation for transdermal drug or vaccine delivery, the water or other penetration enhancers contained in the W_2 aqueous phase of the $W_1/O/W_2$ double emulsions would hydrate/disrupt the *stratum corneum*, thus facilitating the interaction of liposomes with skin.

In our present study, tubular liposomes containing FSS were entrapped in the W_1 phase of $W_1/O/W_2$ double emulsions. Since to the best of our knowledge such a double-encapsulation system has not been reported in the literature, capillary video microscopy³¹ was used to study the behavior of individual $W_1/O/W_2$ double-emulsion globules. We visually monitored the release of tubular liposomes from double-emulsion globules with varying the concentration of surfactants in the oil phase or the W_2 phase.

2. Experimental Section

2.1. Materials

Deionized water obtained from a Barnstead E-pure purifier to a resistivity of approximately 17 M Ω cm was used in the aqueous phases. *n*-Hexadecane (99%), sorbitan monooleate (Span 80), polyoxyethylene sorbitan monooleate (Tween 80), chloroform, methanol, and fluorescein sodium salt (FSS) were purchased from Sigma-Aldrich. For the preparation of liposomes, L- α -phosphatidylcholine was purchased from Avanti Polar Lipids and Ceramide-VI was kindly supplied by Evonik Degussa Corporation (Parsippany, NJ). All the reagents were used as received without further purification.

2.2. Tubular Liposome Synthesis

Recently, Xu et al. reported the preparation of tubular liposomes.³⁰ Ceramide-VI and L- α -phosphatidylcholine (Scheme 1) were combined at a fixed ratio (1:1, w/w) and dissolved in an organic mixture of methanol and chloroform (1:2, v/v). The organic solvents were removed by drying to form a lipid film at 100 mbar pressure by using a rotary evaporator (BUCHI, Switzerland). The lipid film was hydrated in an aqueous phase containing 700 μ M FSS in water at 50 $^{\circ}$ C. The dispersion was then probe-sonicated for 0.5 h at room temperature (22 $\pm$ 1 $^{\circ}$ C). The resulting vesicle suspension was extruded for 11 passes first through 400 nm and then through 100 nm polycarbonate membranes at 55–65 $^{\circ}$ C. The total amount of lipids in the suspension was about 2% (w/v).

2.3. Microcapillary Experimental Setup

Capillary experiments were conducted on an Olympus IMT-2 inverted microscope equipped with a fluorescence illuminator. A high-performance CCD camera (Meyer Instruments, Texas) with a frame rate of 30 frames per second connected to the microscope was used to observe double-emulsion globules. A high-resolution monitor and a S-VHS Hi-Fi VCR (Sony Electronics, California) recorded all the experiments for further analysis by an image-analysis system (Media Cybernetics Inc., Maryland). Two three-dimensional hydraulic micromanipulators (Narishige, Japan) driven by a compressed-nitrogen-powered microinjection system (Narishige, Japan) were mounted on both sides of the microscope to allow the injection of oil globules and aqueous droplets precisely. The details of this equipment were published elsewhere.³²

As shown in Scheme 2, double-emulsion globules were prepared within a thin-walled glass microcapillary. By using a micropipette puller (Narishige PB-7, Japan), the center of the microcapillary (1.5–1.8 mm i.d. $\times$ 100 mm length, Corning) was pulled until the diameter was reduced to 150–200 μ m. This capillary was filled with the W₂ phase and fixed on a specially designed capillary holder, which was in turn mounted on the stage of the microscope. Oil globules and the W₁ droplets were injected into the thinnest part of the capillary from both sides of the microscope by micropipettes. One micropipette with an outer diameter at the tip of approximately 40 μ m was filled with the oil phase. From this micropipette, individual oil globules were formed within the capillary by the microinjection system. A thinner micropipette with an outer diameter at the tip of approximately 15 μ m was used to inject the W₁ droplets within the oil globule. In order for the thinner micropipettes to penetrate the oil globules and for the injected W₁ aqueous droplets to break from the tip of the micropipettes, the outer surface of the micropipette tips was rendered hydrophobic. The hydrophobicity was accomplished by immersing the micropipettes in a solution of siliclad (Gelest Inc., Pennsylvania) for 30 s while nitrogen gas flowed through the micropipettes.

2.4. Cryo-TEM Experiments

The stability of tubular liposomes within the oil phase was tested by cryogenic transmission electron microscopy (cryo-TEM; JEOL 2010, Japan). The inner surface of a rectangular tube (2 mm $\times$ 4 mm i.d. $\times$ 0.5 mm wall, Friedrich & Dimmock Inc.) was rendered hydrophobic using siliclad solution. Then the tube was filled with an oil phase consisting of 0.005 M Span 80 in *n*-hexadecane. Several droplets of aqueous liposome suspension were injected into the oil phase using a 1 mL syringe (Becton Dickinson & Co., New Jersey) with a 25G needle (Becton Dickinson & Co., New Jersey). After 24 h, the liposome droplets were removed using a syringe. A drop of liposome suspension was placed on a Formvar-coated copper TEM grid. The grid was blotted to form a thin film and rapidly vitrified in liquid ethane. The cryo-TEM experiment was conducted at an operating voltage of 120 kV. In addition, the morphology of tubular liposomes after preparation was also determined by cryo-TEM.

3. Results and Discussion

The hypothesized model of a double-encapsulation delivery system illustrates that active molecules are entrapped inside the liposomes, which are in turn confined in the W_1 phase of $W_1/O/W_2$ double emulsions. As shown in Figure 1, the oil phase of the double emulsions functions as a layer of protection to liposomes. When the O phase and W_2 phase have the right amount of surfactants, the double-encapsulation system does not suffer any instability. However, when a stimulus, such as a large amount of water-soluble surfactant, comes into contact with the W_2 phase, the instability of the double emulsions induces the release of liposomes from the W_1 phase to the W_2 phase. Following this, the liposomes would interact with the targeted media to release their contents.

The liposomes used in this study are tubular liposomes that contained 700 μ M FSS in DI-water. According to the cryo-TEM image (Figure 2), the widths of the tubular liposomes are approximately 30–50 nm. When the tubular liposome suspension is encapsulated inside the oil phase, the liposomes remain stable for up to at least 24 h (cryo-TEM image shown in the Supporting Information).

We investigated the release of liposomes from double-emulsion globules as well as the intrinsic effect liposomes may themselves have on the stability of the globules. The latter was accomplished by comparison of liposome-containing- W_1 -phase globules to pure-water- W_1 -phase globules. External coalescence, that is, coalescence between the W_1 and the W_2 aqueous phases, is one of the mechanisms of release of substances from double emulsions, and its occurrence is closely linked with the concentration of surfactants in the oil and aqueous phases.^{33–36} In our experiments, external coalescence was investigated as the mechanism for release of liposomes from the W_1 phase to the W_2 phase. The concentration of Span 80 in the O phase or Tween 80 in the W_2 phase was varied to determine how surfactant concentration may influence liposome release. In experiments performed for each oil globule, three W_1 droplets were formed inside the O phase. Since the size of globules and droplets is one of the factors that influence external coalescence, the ratio of the W_1 droplet diameter to the effective diameter of the oil globule was maintained at 0.4 ± 0.05 . The average external-coalescence time was calculated by the lifetime of all droplets divided by the number of the droplets. All experiments were conducted in triplicate.

3.1. Effect of Oil-Soluble Surfactant Span 80 on the Release of Liposomes

O phases were prepared with 0.005, 0.01, 0.02, and 0.03M Span 80 in *n*-hexadecane. Tween 80 in all W_2 phases was fixed at 0.01 M in water, and the W_1 phase was either liposome suspension or water. Since the critical micelle concentration of Span 80 is approximately 18 μ M in various alkane oils,³⁷ the concentrations used in this study are sufficient to form reverse

micelles within the *n*-hexadecane oil phase. With Span 80 as the only surfactant in the system, that is, no Tween 80 in the W_2 phase, double-emulsion globules entrapping liposomes or water in the W_1 phase were stable at all Span 80 concentrations used in the O phase. This is in agreement with Hou and Papadopoulos's findings that report Span 80 alone can stabilize double-emulsion globules against external coalescence if the concentration of Span 80 is greater than 10^{-3} M.³³ For a double-emulsion globule with a Span 80 concentration of 0.005 M in the oil phase, Figure 3 shows three stable liposome droplets at 5.5 h. Because an uneven presence of salt in the W_1 and W_2 phases may induce water flow from the lower-salt-concentration water phase to the higher-salt-concentration water phase,^{32,38} the existence of FSS in the W_1 phase and its absence in the W_2 phase resulted in spontaneous emulsification of the W_2 phase into the oil phase. As seen in Figure 3b, at 5.5 h, tiny water droplets appeared inside the oil membrane; however, the diameters of the W_1 droplets did not show a measurable change. Therefore, the migration of water from the W_2 to the W_1 phase is not expected to influence the findings of our study because the duration of each experiment is less than 5.5 h.

For 0.01 M Tween 80 present in the W_2 phase, the results of average external-coalescence time for water and liposome suspension in the W_1 phase are summarized in Figure 4. As expected, for water in the W_1 phase, external coalescence slows down as the concentration of Span 80 increases. For double-emulsion globules with the W_1 phase consisting of liposome suspension, external coalescence follows the same trend as globules of pure water in the W_1 phase. For example, when the oil phase contained 0.005 M Span 80 and the W_1 phase was a liposome suspension, average external-coalescence time was 387.67 s/droplet for 0.01 M Tween 80 in the W_2 phase (Figure 5a). When the concentration of Span 80 in the oil phase was increased to 0.03 M, the average external-coalescence time also rose to 1003.67 s/droplet under the same conditions in the W_1 and W_2 phases (Figure 5b). The fluorescent-light images before any of the droplets have coalesced are there to confirm that the internal aqueous phase consisted of a liposome suspension.

The concentration of Span 80 plays a significant role on the stability of double emulsions. As suggested in previous work, the amount of the oil-soluble surfactant may be modeled by the effective thickness of adsorbed layers on the O/ W_2 and W_1 /O interfaces. A higher Span 80 concentration in the oil phase corresponds to thicker adsorbed layers that produce a greater repulsive force to stabilize the double emulsions.³⁹ As the concentration of Span 80 increased, the stability of double emulsions increased, and all the concentrations used were above the CMC.^{33,40,41} One theory recently proposed by Santini et al.⁴² also suggests that the barrier against droplet coalescence becomes stronger due to the increase of the Span 80 concentration, thus enhancing the stability of emulsions.

3.2. Effect of Water-Soluble Surfactant Tween 80 on the Release of Liposomes

The amount of Tween 80 in the W_2 phase was varied in order to observe how Tween 80 may influence the release of water and liposome suspension in the W_1 phase. Tween 80 in the W_2 phase was incorporated at 0.002, 0.005, 0.008, and 0.01 M, while Span 80 in the oil phase for all experiments was 0.02 M. Several previous studies have discussed how external coalescence becomes faster with increasing Tween 80 concentration in the W_2 phase.^{33,34} Also, in the current study, when the concentration of Tween 80 in the W_2 phase increased, external coalescence became faster for both water and liposome suspension in the W_1 phase, as shown in Figure 6. At 0.005 M Tween 80 in the W_2 phase and a liposome suspension in the W_1 phase, the average external-coalescence time was 716.00 s/droplet (Figure 7a). For the same conditions in the W_1 and oil phase, but with Tween 80 concentration increased to 0.01 M in the W_2 phase, average external-coalescence time decreased to 477.33 s/droplet (Figure 7b). Previous studies have provided possible mechanisms for the action of water-soluble

surfactants in the W_2 phase,^{34,40} and the same behavior was confirmed in our current results when the W_1 phase contained liposomes.

3.3. The Effect of Liposomes on External Coalescence

Generally, $W_1/O/W_2$ double emulsions require two types of surfactants, an oil-soluble surfactant and a water-soluble surfactant.³⁶ For instance, a water-soluble surfactant in the W_2 phase is necessary to prevent globule–globule coalescence; however, such surfactant at a high concentration has also been shown to induce external coalescence.^{33–35} Based on the research of Kabalnov and Wennerström,⁴³ Pays et al.³⁴ proposed a mechanism for external coalescence by relating it to the activation energy needed by the double-emulsion system to form a hole in the oil film. Their study showed that the presence of a high amount of water-soluble surfactant in the aqueous phases lowers the energy barrier for forming a hole within the oil film, thereby facilitating coalescence events between the aqueous phases. In our experiments, it should be noted that internal coalescence among the W_1 droplets within double-emulsion globules did not occur at all. This was expected based on the results of the previous studies,^{33,35,44} according to which internal coalescence is an unlikely event, occurring only when there is significantly more water-soluble surfactant in the W_1 phase than in the W_2 phase.

To determine the effect of liposomes on external coalescence, globule behavior with a pure-water W_1 phase was compared to that of the W_1 phase being composed of a liposome suspension. Tween 80 concentration in the W_2 phase was held at 0.01 M while the Span 80 concentration in the oil phase was 0.005 M. Inspection of the coalescence times in Figure 5a and Figure 8 shows that the coalescence of the three W_1 liposome droplets with W_2 , compared to the time needed for pure-water W_1 , is delayed by approximately 19 min. Therefore, we propose that the existence of liposomes in the W_1 phase has a stabilizing effect on the double-emulsion system. This observation agrees with the stabilizing effect of phospholipids from liposomes on the W/O-type emulsions reported by Muderhwa et al.⁴⁵ They demonstrated that liposomes destroyed by emulsification in a bulk experiment donated phospholipid molecules to assist the stabilization effect of the oil-soluble surfactant Arlacel A. O/W liposomal emulsions in which the oil phase is emulsified by phospholipids in liposomes were also developed by this group.⁴⁶

The tubular liposomes in our experiments are prepared with L - α -phosphatidylcholine and Ceramide-VI. The importance of phospholipids as components of all biological systems may also lie in the fact that they form stable adsorption layers in the oil/water interfaces and reduce the interfacial tension of all interfaces in such systems.^{47,48} Phospholipids, being effective emulsifiers and/or stabilizers, are the molecules used to make liposomes, which have been peripherally used to stabilize or costabilize W/O emulsions, O/W emulsions, and $W_1/O/W_2$ double emulsions, showing potential use in vaccine delivery.^{45,46,49} In the liposome suspension reported in our study, there are some free phospho-lipid molecules resulting from preparation and storage. When the liposome suspension is injected into an oil globule, the free L - α -phosphatidylcholine molecules in the W_1 phase will adsorb onto the W_1/O interface and provide more stability by increasing the overall surfactant concentration at the water/oil interfaces of the system. In other words, after the adsorption of L - α -phosphatidylcholine on the W_1/O interface, the activation energy for the rupture of oil film is increased. Therefore, external coalescence for the liposome-containing W_1 droplets with W_2 phase is delayed. For all our experiments, the existence of liposome suspension in the W_1 phase showed a delayed external coalescence of approximately 10–30 min when compared to the case where the W_1 phase consisted only of water.

With the aim to confirm the effect of L - α -phosphatidylcholine on the double emulsions, we did experiments with the W_1 phase containing L - α -phosphatidylcholine instead of liposomes, and the total amount of lipids was about 0.0125%(w/v). The concentration of Span 80 in the oil

phase was 0.02 M while the Tween 80 concentration in the W_2 phase was at 0.005 M. Comparing with the average external-coalescence time of the pure-water W_1 phase (300.22 ± 37.17 s/droplet), the average external-coalescence time for three W_1 droplets containing α -phosphatidylcholine was increased to 702.22 ± 112.05 s/droplet (images not shown).

As reported by McConnell,⁵⁰ unsaturated phospholipids are good fluidizers when compared to saturated ones and spread more rapidly to the air/liquid interface due to their low phase transition. Since the phase-transition temperature of α -phosphatidylcholine is about 19 °C, it is expected to adsorb on the W/O interface rapidly. In addition, the concentrations of Span 80 used in the study are sufficient to form reverse micelles with in the *n*-hexadecane oil phase. Reverse micelles of the oil-soluble surfactant have been reported to facilitate the diffusion of molecules across the oil phase.^{32,51,52} Therefore, it is possible that the reverse micelles of Span 80 incorporate α -phosphatidylcholine molecules to facilitate their adsorption on the interface.

Besides microscopy-in-capillary experiments, the effect of tubular liposomes on the stability of double emulsions was also corroborated by bulk-double-emulsion experiments, where a two-step emulsification procedure was used. Experimental details are available in the Supporting Information.

4. Conclusions

Liposomes containing the model compound fluorescein sodium salt (FSS) were entrapped into the W_1 phase of double-emulsion globules, thus providing a possible double-encapsulation delivery system. Liposomes may thus be protected against the influence of unfavorable physicochemical conditions. The release of liposomes from double-emulsion globules can be controlled from seconds to hours, by varying the water-soluble and/or oil-soluble surfactant concentrations in the W_2 phase or oil phase. The findings also indicate that the mere presence of liposomes in the W_1 phase extends the external-coalescence time in $W_1/O/W_2$ double-emulsion globules due to the adsorption of free α -phosphatidylcholine molecules on the W_1/O interface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Hypothesized model of the double-encapsulation delivery system. (A) The double-encapsulation delivery system does not suffer any instability; (B) by adding stimulus, external coalescence induces the release of liposomes from the W_1 phase to the W_2 phase in a $W_1/O/W_2$ double emulsion.

Figure 2.
Cryo-TEM image of tubular liposomes encapsulating fluorescein sodium salt.

Figure 3. Stability of double-emulsion globules entrapping liposomes. W_1 phase: liposome suspension; O phase: 0.005 M Span80 in *n*-hexadecane; W_2 phase: water. Scale bar in (a) is applicable for both images.

Figure 4.

Average external-coalescence time at 0.01 M Tween 80 in the W_2 phase. W_1 phase: water (■) or liposome suspension (▲); O phase: 0.005, 0.01, 0.02, and 0.03 M Span 80 in *n*-hexadecane; W_2 phase: 0.01 M Tween 80 in water.

Figure 5.

Effect of Span 80 in the oil phase on the release of liposomes. W_1 phase: liposome suspension; O phase: 0.005 M(a) and 0.03 M (b) Span 80 in *n*-hexadecane; W_2 phase: 0.01 M Tween 80 in water. Average external-coalescence time: 387.67 s/droplet (a) and 1003.67 s/droplet (b). Scale bar in (a) is applicable for all images.

Figure 6.

Average external-coalescence time at 0.02 M Span 80 in the O phase. W_1 phase: water (■) or liposome suspension (▲); O phase: 0.02 M Span 80 in *n*-hexadecane; W_2 phase: 0.002, 0.005, 0.008, and 0.01 M Tween 80 in water.

Figure 7.

Effect of Tween 80 in the W_2 phase on the release of liposomes. W_1 phase: liposome suspension; O phase: 0.02 M Span 80 in n -hexadecane; W_2 phase: 0.005 M (a) and 0.01 M (b) Tween 80 in water. Average external-coalescence time: 716.00 s/droplet (a) and 477.33 s/droplet (b). Scale bar in (a) is applicable for all images.

Figure 8.

Time elapsed images of a $W_1/O/W_2$ double-emulsion globule prepared with W_1 phase: water; O phase: 0.005 M Span 80 in *n*-hexadecane; W_2 phase: 0.01 M Tween 80 in water. Average external-coalescence time: 5.6 s/droplet. Scale bar is applicable for all images.

Scheme 1.
Chemical Structures of L-α-Phosphatidylcholine (A) and Ceramide-VI (B)

Scheme 2.
 $W_1/O/W_2$ System inside a Capillary