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Temperature-Induced Protein Release from Water-in-Oil-in-Water Double Emulsions

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Abstract

A model water-in-oil-in-water ($W_1/O/W_2$) double emulsion was prepared by a two-step emulsification procedure and subsequently subjected to temperature changes that caused the oil phase to freeze and thaw while the two aqueous phases remained liquid. Our previous work on individual double-emulsion globules[1] demonstrated that crystallizing the oil phase (O) preserves stability, while subsequent thawing triggers coalescence of the droplets of the internal aqueous phase (W_1) with the external aqueous phase (W_2), termed external coalescence. Activation of this instability mechanism led to instant release of fluorescently tagged bovine serum albumin (fluorescein isothiocyanate (FITC)-BSA) from the W_1 droplets and into W_2. These results motivated us to apply the proposed temperature-induced globule-breakage mechanism to bulk double emulsions. As expected, no phase separation of the emulsion occurred if stored at temperatures below 18 °C (freezing point of the model oil *n*-hexadecane), whereas oil thawing readily caused instability. Crucial variables were identified during experimentation, and found to greatly influence the behavior of bulk double emulsions following freeze-thaw cycling. Adjustment of these variables accounted for a more efficient release of the encapsulated protein.

Introduction

This study is part of our effort to develop a vaccine cream that will be applied to the skin in the same way as cosmetic formulations. Skin may be a potent immunological induction site since it acts as an immune barrier by its immunocompetent cells.[2] For a long time it was believed that the skin was impenetrable to drugs and bioactive molecules greater than 500 Da;[3] however, vaccine antigens and adjuvants that target the skin only require delivery through the outermost skin layer, the stratum corneum, which is an effective but fragile barrier that can be disrupted by hydration.[4] Therefore, several studies have already reported strong systemic and mucosal immune responses following topical application onto hydrated skin.[5-7] An occlusive dressing (patch) and/or a semiliquid formulation can increase skin hydration and hence penetration.[8,9] The use of lipid-based colloidal carriers[10] and nanoparticles[11] as well as physical penetration-enhancing methods[12] have also proven useful to facilitate delivery of vaccines to the skin. Transcutaneous immunization offers the potential to make vaccine administration easier and cheaper while maintaining efficiency and safety,[13] which would facilitate the implementation of worldwide mass vaccination campaigns and provide the means for a fast response to bioattacks.

A water-in-oil-in-water ($W_1/O/W_2$) double-emulsion system consists of oil (O) globules that contain smaller droplets of an internal aqueous phase (W_1) and are dispersed in an external

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aqueous phase (W_2). $W_1/O/W_2$ double emulsions are traditionally fabricated by a two-step process,[14] but have also been generated in a single-step process that allows precise control of the size and number of inner (W_1) droplets.[15,16] Double emulsions have a compartmentalized structure that can provide high capacity of entrapment, protection of fragile substances, combination of incompatible substances in one product, and controlled release.[17,18] They have been regarded as suitable carriers for vaccines as the emulsification procedure does not affect the protein's molecular weight and antigenicity.[19] In fact, their use has elicited more effective immune responses as compared to other delivery vehicles such as polymeric nanoparticles upon intramuscular antigen administration.[20]

However, the fact that double emulsions are thermodynamically unstable presents a technical challenge to their successful implementation. In general, emulsion stability describes the extent to which the dispersed droplets maintain their uniform distribution over time. According to Florence and Whitehill,[21] instability of $W_1/O/W_2$ double emulsions can occur through four mechanisms: (i) coalescence between W_1 droplets (internal coalescence), (ii) coalescence between the larger oil globules, (iii) coalescence of the W_1 droplets with W_2 (external coalescence), and (iv) transport of water to and from W_2 through the oil phase. Coalescence occurs as the thin film between the contacting interfaces ruptures, while the type and concentration of surfactant adsorbed at the water/oil interfaces determines the nature of such a separating film and, therefore, plays a key role in stability. For instance, a high concentration of surfactant in the oil phase translates into a thick adsorbed layer that produces a repulsive force among W_1 droplets and between W_1 droplets and W_2, while lower surfactant concentrations lead to net attractive forces that favor internal and external coalescence.[22,23] In general, $W_1/O/W_2$ double emulsions require two types of surfactants, an oil-soluble surfactant to stabilize the W_1 droplets and a water-soluble surfactant, in W_2, to stabilize the oil globules.[24]

Besides stability during storage, efficient release of the active substance upon administration is a condition required for successful implementation of a double-emulsion delivery system. The two general mechanisms of solute release from the W_1 droplets of $W_1/O/W_2$ double emulsions are breakdown of the emulsion globules[25] and transport of part of the solute through the oil membrane without breaking the emulsion.[26,27] Depending on the molecule's affinity for the oil phase, solute transfer is triggered by either simple diffusion or surfactant-facilitated transport through reverse micelles, hydrated surfactants, and/or thin lamellae.[22,28,29] Hai and Magdassi[30] compared the release profiles of two different fluorescent markers from W_1 in the absence of globule breakdown, the small-molecule fluorescein sodium salt and the protein fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA). That study reported that the release of FITC-BSA was controlled by diffusion and was much slower than that of fluorescein because of its ability to adsorb at the oil/water interface and its greater molecular weight, which decreases its diffusion coefficient and hinders solubilization within micelle carriers. Thus, it can be inferred that, for applications requiring sudden (rather than prolonged) protein delivery, an external stimulus is required to trigger rupture of the globules, that is, external coalescence. This has been achieved by dilution of the emulsion in a hypo-osmotic solution[31] and submission to a shear stress.[32,33]

We have recently proposed an alternative method which relies on temperature changes to readily destabilize double-emulsion globules.[1] By use of fluorescence capillary videomicroscopy, we demonstrated that the compartmentalized structure of individual $W_1/O/W_2$ double-emulsion globules is not disrupted during or after freezing of the oil membrane separating the two aqueous phases while subsequent thawing causes internal and external coalescence and therefore release of the W_1 contents (FITC-BSA). *n*-Hexadecane was used as the model oil due to its convenient phase transition temperature (~18 °C), which allows crystallization of the oil phase at lower than room temperature while W_1 and W_2 remain liquid.

It is well-known that solutes such as air, water, and surfactant inside or at the surface of oil droplets are expelled during the freezing process due to their lower solubility in the solid phase;[34] hence, we have hypothesized[1] that internal coalescence and external coalescence take place during oil thawing because the oil-soluble surfactant has not yet fully migrated back to the interfaces of the double-emulsion globules, so its effective concentration is actually lower.

In the present work, we further explore the ability of oil phase transitions to promote instant and complete protein release by developing a model temperature-responsive double-emulsion system in the bulk. A major difference between the behavior of a bulk double emulsion and that of an individual double-emulsion globule is that the first consists of many potentially interacting double-emulsion globules instead of just one; consequently, instability may occur not only through internal and/or external coalescence but also through coalescence among neighboring globules. Since only external coalescence results in release of the entrapped protein, the corresponding bulk double-emulsion system must be designed to favor this instability mechanism during oil thawing. Studies on bulk $W_1/O/W_2$ double emulsions conducted by Magadassi[35] and Guery,[36] and their co-workers, have already shown that crystallizing the oil phase can suppress virtually any transport of the aqueous phases, as well as any coalescence, leading to a practically infinite relaxation time. Therefore, the capacity of oil thawing to stimulate instability in bulk $W_1/O/W_2$ double emulsions, leading to instant release, seems desirable for some controlled release applications.

Materials and Methods

Materials

n-Hexadecane (99%) was used as the oil phase for the emulsions, and poly(oxyethylene) sorbitan monooleate (Tween 80, HLB = 15) and sorbitan monooleate (Span 80, HLB = 4) were used as the water-soluble and oil-soluble surfactants, respectively. FITC-BSA, which has excitation and emission wavelengths of 485 and 520 nm, respectively, was chosen as the model protein. Deionized water was generated with a Barnstead E-pure purifier to a resistance of approximately 17 MΩ. A 16.66 mM concentration of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer solution of pH 7.4 was used as the aqueous medium. All reagents were purchased from Aldrich, except for FITC-BSA (purchased from Sigma), and used as received without further purification.

Two-Step Emulsification Procedure

Bulk double emulsions were prepared by a two-step emulsification procedure, first proposed by Matsumoto et al.[37] During the first step, 50 mL of the internal aqueous phase (W_1) was, drop-by-drop, added to 50 mL of the oil phase for about 20 min under strong magnetic stirring (710 rpm). Subsequent high-shear homogenization at 1000 rpm using a Silverson homogenizer (model L4R) with a medium emulsion screen was applied for 5 min to obtain the W_1/O emulsion. During the second step, 80 mL of the freshly prepared W_1/O simple emulsion were, drop-by-drop, added to 20 mL of the external aqueous phase (W_2) for a time period of about 50 min under gentle magnetic stirring (315 rpm); stirring was continued for 5 min more. After several trials, a W_1:O:W_2 volume ratio of 1:1:0.5 was chosen for the double-emulsion systems presented here. Since the continuous phase accounts for only 20% of the emulsion, initial phase separation due to creaming of the double-emulsion globules is greatly prevented, allowing future correlation of phase separation to emulsion instability.

Freeze-Thaw Cycling

After preparation, the double emulsion was split in either 12 × 75 mm borosilicate glass test tubes (3 mL) or 150 × 15 mm polystyrene Petri dishes (6 mL) and then placed in the refrigerator (at ~4 °C) for 1 h to ensure complete crystallization of the oil phase. Control samples were

kept at room temperature (~25 °C). For each freeze-thaw cycle, the emulsions were thawed by transferring them from the refrigerator to a water bath at ~35 °C for 10 min; next, three of the samples were left at room temperature for 50 min more, after which the aqueous bottom, formed upon phase separation, was collected by use of a gastight syringe (model 1001, Hamilton Co.). In the case of the Petri dishes, samples were transferred to test tubes to facilitate collection of the aqueous bottom. Absorbance measurement for the collected bottoms was performed by use of a UV-vis spectrophotometer (model PharmaSpec UV-1700, Shimadzu Scientific Instruments) with the purpose of quantifying the amount of protein released from the emulsion.

Microscopy Observation

After preparation and temperature cycling, an aliquot of the double emulsion was diluted (10-fold) with its corresponding W_2 and slightly stirred for a few seconds. About 5 μL of the diluted emulsion was then placed on a concave glass slide and covered with a coverslip to prevent drying. Samples were observed by use of a Leica DM IRE2 inverted microscope equipped with a fluorescence illuminator and a Leica DC350F camera (Leica Microsystems Imaging Solutions Ltd.). Images were captured onto a computer with an Image-Pro Plus image-analysis system, version 5.0, for Windows 2000 and XP (Media Cybernetics Inc.). All experiments were conducted at least three times to ensure reproducibility of the presented results under the given experimental conditions.

Results and Discussion

On the bases of previous results obtained for individual $W_1/O/W_2$ double-emulsion globules inside glass capillaries,[1] we hypothesize that crystallizing the oil phase preserves the stability of the bulk double emulsion and subsequent thawing instantly triggers release of the FITC-BSA from the W_1 droplets. In other words, the proposed temperature-induced globule-breakage mechanism should macroscopically translate into sharp phase separation that leaves an oil-containing layer on top of a water-containing layer into which the protein has been released. To assess this hypothesis, bulk double emulsions were obtained by two-step emulsification and subsequently subjected to freezing and thawing as described in the Materials and Methods.

Emulsion 1 was prepared with the following composition of the three phases: W_1, 0.25% (w/v) FITC-BSA in buffer solution; O, 0.05 M Span 80 in *n*-hexadecane; W_2, 0.03 M Tween 80 in buffer solution. Figure 1A shows that phase separation did not occur right after preparation, which ruled out initial creaming of the double-emulsion globules due to differences in density. According to Figure 1B,C, the control emulsion was already unstable after 2.5 h of storage at room temperature and continued to phase separate until the bottom corresponded to about 18% of the emulsion. On the other hand, storing emulsion 1 at ~4 °C so that the *n*-hexadecane was crystallized successfully preserved the stability for at least 4 months (Figure 1D); this result agrees with a previous study[35] where a frozen double emulsion remained stable for similar period of time even if subjected to osmotic pressure gradients. Thawing emulsion 1 instantly triggered its phase separation into a “creamy” layer at the top and an “aqueous” layer at the bottom (Figure 1E), similar to those progressively formed in the control emulsion, with no noticeable change after 1 h (Figure 1F) when the bottom was collected. However, absorbance measurements detected only about 4% of the FITC-BSA and thus indicated that most W_1 was still entrapped within the creamy layer. Additional freeze-thaw cycling was conducted as an attempt to trigger additional release, and in fact about 20% of the FITC-BSA was recovered from the aqueous layer after 10 cycles. Only 0.6% of the FITC-BSA was found at the bottom of the control emulsion after 3 days of storage at room temperature (see Figure 1C), which

indicates that oil phase transitions were certainly triggering instant release of encapsulated protein.

Optical microscopy images, taken with bright and fluorescent light, confirmed that the initial sample consisted of well-structured double-emulsion globules and revealed that they were completely filled with many tiny W_1 droplets (Figure 2A,B), which is usually the case for double emulsions prepared by two-step emulsification.[25,38] Experimentation on individual globules[1] demonstrated that relatively small W_1 droplets are not likely to coalesce with W_2 during oil thawing, which hinders protein release. Furthermore, the creamy layer formed after thawing consisted of a water-in-oil (W_1/O) simple emulsion resulting from coalescence among oil globules (Figure 2C,D), which suggests that this instability mechanism was responsible for the observed phase separation. Such a creamy layer remained fairly unchanged for at least 1 week (results not shown), probably because the W_1 droplets were too small to coalescence with each other, and consequently, no additional FITC-BSA was recovered from the aqueous layer. In contrast, conducting several consecutive freeze-thaw cycles instead of just one produced a creamy layer with noticeably larger W_1 droplets (Figure 2E,F), more susceptible to coalesce with W_2, and therefore led to greater release of FITC-BSA. Finally, microscopy observation of the control double emulsion, not subjected to temperature cycling, revealed that its slowly progressing phase separation was also due to formation of a W_1/O emulsion, which had partially taken place after 1 day of storage at room temperature (Figure 2G,H).

Figure 3 summarizes the behavior of emulsion 1 when subjected to freeze-thaw cycling. Freezing the oil phase preserved the double-emulsion stability during storage, and subsequent thawing triggered phase separation. However, the W_1 droplets were very small and therefore fairly stable against external coalescence so that instability occurred primarily through coalescence among oil globules. This instability mechanism further prevented FITC-BSA release by decreasing the total interfacial area available for external coalescence, that is, the number of interior (W_1) droplets in close proximity to the continuous phase (W_2), ultimately forming a W_1/O simple emulsion that phase separated from W_2. Thus, the observed phase separation resulted from elimination of the spaces among globules, which were filled by W_2. Finally, several temperature cycles caused internal coalescence and growth of the W_1 droplets, some of which eventually reached the O/W_2 interface to release their contents.

Accordingly, we proposed two approaches that could improve the delivery efficiency of this temperature-responsive double-emulsion system following freeze-thaw cycling: (1) to increase the concentration of Tween 80 in W_2 to better stabilize the oil globules against coalescence during—and after—thawing and (2) to increase the area of contact between the creamy and the aqueous layers formed after phase separation to facilitate release of the protein.

For the first approach, the concentration of Tween 80 in W_2 was increased from 0.03 to 0.08 M with the intention of further stabilizing the oil globules during freeze-thaw cycling. The procedure of preparation and temperature cycling was the same as for emulsion 1. Again, freezing of the oil phase preserved the stability (results not shown), and subsequent thawing triggered phase separation, although a greater part of emulsion 2 separated at the bottom (Figure 4A). As illustrated in Figure 4B, the volume of the aqueous layer obtained after thawing of emulsion 1 actually corresponds to the original fraction of W_2 (20%) and therefore confirms that phase separation was due to coalescence among oil globules. In contrast, about 45% of emulsion 2 separated at the bottom, which means that at least 25% out of the total 40% of W_1 present in the emulsion must have been released from the creamy layer. Absorbance measurements for the aqueous layers collected from emulsions 1 and 2 revealed a dramatic effect of the Tween 80 concentration on the fraction of the encapsulated FITC-BSA that can be released by oil thawing (Figure 4C). Thus, increasing the concentration of Tween 80 in W_2 from 0.03 to 0.08 M improved the release efficiency from 4% to 40% after the first cycle

and from 10% to 85% after the second cycle. Conducting more than three freeze-thaw cycles did not have a significant additional effect.

Optical microscopy observations (Figure 5A,B) showed that the initial emulsion 2 had a double-emulsion structure similar to that of emulsion 1 (see Figure 2A,B), but in contrast to that system, no appreciable signs of instability were found in the control emulsion 2 even after 3 days of storage at room temperature (Figure 5C,D). In addition, coalescence among oil globules did not take place in emulsion 2 during oil thawing, so the resulting creamy layer consisted of double-emulsion globules, many of which were empty or partially empty (Figure 5E,F). Some W_1/O simple emulsion was found only after the third cycle (Figure 5G,H). These observations demonstrate that the higher concentration of Tween 80 further stabilized the oil globules, preserving the total O/W_2 interfacial area (for at least two freeze-thaw cycles) and thus giving the W_1 droplets a better chance to release the FITC-BSA. We previously proposed[1] that freezing temporarily reduces the oil-soluble surfactant concentration at the interfaces so that double-emulsion globules are more unstable during oil thawing. Thus, although the original W_1 droplets obtained in this bulk system were initially too small for external coalescence to be triggered by oil thawing, we suggest that the W_1 droplets first suffered internal coalescence and grew until they were able to release the protein, all of which occurred very fast. Accordingly, Figure 6 schematizes the behavior of emulsion 2 when subjected to freeze-thaw cycling.

We noticed another difference between emulsions 1 and 2 while monitoring their behavior at room temperature by use of optical microscopy. Whereas a diluted aliquot of emulsion 1 did not experience appreciable changes during 3 h of observation on a covered glass slide (not shown), an interesting phenomenon was observed for emulsion 2 under the same conditions. As illustrated in Figure 7A-F, the initial tiny and fairly undistinguishable W_1 droplets suffered internal coalescence, even generating globules with a single relatively large interior droplet; such larger W_1 droplets eventually coalesced with W_2, leaving empty oil globules that had not yet coalesced with each other. A high concentration of Tween 80 was certainly expected to stabilize the oil globules, but its effect on promoting internal coalescence during microscopy observation at room temperature was rather surprising. This process did not take place in the control emulsion stored at room temperature for days (see Figure 5C,D) but began every time it was diluted (10-fold) with W_2 for microscopy observation, which suggests that this is not a spontaneous process but rather is triggered by dilution. Indeed, emulsion 2 diluted for several hours before being placed onto the glass slide showed similar signs of internal and external coalescence (Figure 7G,H).

The fact that emulsion 1 remained unchanged after dilution with its corresponding W_2 implies that the higher concentration of Tween 80 in emulsion 2 greatly favored internal coalescence upon such a stimulus. The aliquot of emulsion 2 was diluted with W_2, containing 0.08 M Tween 80, and as a result there were 10 times more Tween 80 molecules to cover the same total O/W_2 interfacial area. Given that the amount of Tween 80 in the original, undiluted, double emulsion seemed to be enough to stabilize the globules during room-temperature storage and freeze-thaw cycling, as described above, these surfactant molecules were in great excess in the diluted system. Although the water-soluble surfactant in W_2 is essential to avoid coalescence among double-emulsion globules, it has been reported to have a significant destabilizing effect when present at very high concentrations.[24,39,40] The excess water-soluble surfactant molecules may form micelles and dissolve some of the oil-soluble surfactant, decreasing its effective concentration at the W_1/O interfaces and consequently favoring coalescence of the W_1 droplets;[37] alternatively, this phenomenon has been attributed to displacement of the Span 80 molecules by the excess Tween 80 molecules, causing rupture of the oil film due to a curvature change.[25] This observation agrees with findings by Villa et al.:[23] when W_2 was a highly concentrated solution of Tween 80 (>0.025 M), individual double-emulsion globules

were completely unstable immediately after preparation within glass capillaries. Such individual double-emulsion globules are highly diluted systems since the whole capillary is filled with W_2, so a fairly low concentration of Tween 80 (as compared to formulations for bulk double emulsions) yields too many Tween 80 molecules to cover the interfacial area of a single globule. We propose that a different external stimulus such as dilution with W_2 triggers internal and external coalescence at room temperature in a fashion similar to that of freezing and thawing of the oil phase because both stimuli decrease the effective oil-soluble surfactant concentration at the interfaces. However, dilution at room temperature promotes slow and progressive instability, while freezing-thawing instantly trigger globule bursting.

In the second approach, freshly prepared emulsions 1 and 2 were split into Petri dishes (150 mm i.d) instead of test tubes (12 mm i.d.) before conduction of the freeze-thaw cycles. The area of contact between the creamy and the aqueous layers formed during phase separation was consequently enlarged 150-fold, approximately; because each Petri dish contained 6 mL of emulsion instead of 3 mL (as for the test tubes), the effective increase in the area of contact was about 75-fold. As shown in Figure 8A, spreading emulsion 1 as a relatively thin layer did not have any effect on the fraction of FITC-BSA released after each freeze-thaw cycle. Since temperature cycling of emulsion 1 led to formation of a simple W_1/O emulsion that separated from W_2 (see Figure 3), the total O/W_2 interfacial area—originally created by the dispersed double-emulsion globules—was dramatically reduced to become equivalent to the area of contact between the creamy and aqueous layers. This observation hence illustrates the huge hindering effect of coalescence among oil globules on external coalescence and release of the encapsulated protein. In contrast, much more FITC-BSA was recovered from emulsion 2 after the first freeze-thaw cycle, as compared to the results obtained with test tubes, increasing the amount of protein collected from 40% to 90%, approximately (Figure 8B). We have demonstrated that coalescence among oil globules did not appreciably occur until the third temperature cycle so that the creamy layer still consisted of a dispersed oil phase (see Figure 6); the total interfacial area was therefore preserved along with the space between globules—filled with W_2—into which the FITC-BSA was released. Accordingly, this finding strongly suggests transport limitations during experimentation conducted within test tubes where there was a small area of contact between the creamy and the aqueous layers so that part of the released protein remained entrapped among the globules. In other words, we propose that the 75-fold increase in the area of contact placed many more double-emulsion globules near the aqueous layer, thus facilitating assessment of the released FITC-BSA.

The experimental results presented above correspond to FITC-BSA recovered 1 h after temperature cycling. However, sharp phase separation was always observed immediately after thawing (see Figure 1E), suggesting that the release of protein—due to emulsion instability—occurred instantly. The FITC-BSA release profile over a few hours was obtained for both double-emulsion systems to determine whether a longer time of collection could lead to a higher release efficiency. Thus, fresh emulsions 1 and 2 were prepared, split in Petri dishes, and frozen for 1 h; then all the samples were thawed just once, and their aqueous layers were collected after different times. The temperature cycle was conducted in Petri dishes rather than in test tubes to facilitate quantification of the released protein, as discussed earlier. The release profile for emulsion 1 (not shown) showed that about 4% of FITC-BSA was recovered after 30 min and that this value remained unchanged for longer times of collection (up to 4 h). A constant release profile (not shown) was also obtained for emulsion 2, but corresponding to about 90% of protein recovered. This result confirms that the release of encapsulated protein triggered by oil thawing takes place fast (in less than 30 min) and is fairly independent of time. In other words, a higher release efficiency cannot be achieved simply by waiting for more than 1 h. In fact, no additional FITC-BSA was recovered from the aqueous layers collected after 1 week.

Conclusions

In this study, the globule-breakage mechanism presented during our previous work[1] was applied to obtain temperature-sensitive bulk $W_1/O/W_2$ double emulsions. Freezing the oil membrane (O) separating the two still-liquid aqueous phases preserved the emulsion stability during storage, while subsequent thawing readily triggered instability. Because many double-emulsion globules coexist in a bulk system, a fairly high concentration of the water-soluble surfactant (Tween 80) was required to prevent them from coalescing with each other, so that the main instability mechanism triggered by freezing and thawing of the oil phase was internal coalescence. As a result, the initially tiny W_1 droplets became larger while the total interfacial area remained available for external coalescence, leading to instant release of FITC-BSA. A large area of contact between the creamy and aqueous layers, formed upon phase separation, facilitated collection of the protein at the bottom and therefore seems to be required for highly efficient delivery. Thus, about 90% of the FITC-BSA was instantly released following just one freeze-thaw cycle.

Spreading the double emulsion on a Petri dish, rather than pouring it into a test tube, better simulates the behavior of this delivery system upon topical application. Accordingly, the approach presented here has the potential to provide a stable creamlike formulation that may encapsulate and protect the protein during storage while efficiently releasing it during its administration to the skin. The $W_1/O/W_2$ double emulsion with frozen oil will be spread on the skin, and as the oil melts due to the higher skin temperature, the globules will burst and will release the until-then-encapsulated protein. This globule-breakage mechanism will macroscopically translate into phase separation that leaves a creamy layer on top of an aqueous layer that puts the protein in direct contact with the skin. Additionally, freezing the oil phase of double-emulsion globules has been demonstrated to prevent transport in and out of the W_1 droplets.[35,36] This feature allows us to speculate that potentially denaturating components such as chemical penetration enhancers[9]—whose purpose is to facilitate delivery of the released protein across the stratum corneum—may be stored within the oil (O) and continuous (W_2) phases without modification of the composition of the interior droplets (W_1), chosen to minimize protein instability during storage.[41] Although the presented temperature-responsive double emulsions possess features that make them worth trying for storage and delivery of proteins, and should therefore be further looked into, in this paper we focused exclusively on studying the controlled release of encapsulated protein from such a creamlike delivery vehicle.

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References

(1). Rojas EC, Papadopoulos KD. Langmuir 2007;23:6911–6917. [PubMed: 17521202]

(2). Belyakov IM, Hammond SA, Ahlers JD, Glenn GM, Berzofsky JA. J. Clin. Invest 2004;113:998–1007. [PubMed: 15057306]

(3). Bos JD, Meinardi M. Exp. Dermatol 2000;9:165–169. [PubMed: 10839713]

(4). Warner RR, Stone KJ, Boissy YL. J. Invest. Dermatol 2003;120:275–284. [PubMed: 12542533]

(5). Glenn GM, Taylor DN, Li X, Frankel S, Montemarano A, Alving CR. Nat. Med 2000;6:1403–1406. [PubMed: 11100128]

(6). Guebre-Xabier M, Hammond SA, Epperson DE, Yu J, Ellingsworth L, Glenn GM. J. Virol 2003;77:5218–5225. [PubMed: 12692224]

(7). Beignon JP, Muller BS, Partidos CD. Immunology 2001;102:344–351. [PubMed: 11298834]

(8). Glenn GM, Kenney RT, Hammond SA, Ellingsworth LR. Immunol. Allergy Clin. North Am 2003;23:787–813. [PubMed: 14753392]

(9). Williams AC, Barry BW. Adv. Drug Delivery Rev 2004;56:603–618.

(10). Baca-Estrada ME, Foldvari M, Ewen C, Badea I, Babiuk LA. Vaccine 2000;18:1847–1854. [PubMed: 10699333]

(11). Cui Z, Mumper RJ. J. Controlled Release 2001;75:409–419.

(12). Bramson J, Dayball K, Evelegh C, Wan YH, Page D, Smith A. Gene Ther 2003;10:251–260. [PubMed: 12571633]

(13). Partidos CD, Beignon AS, Mawas F, Belliard G, Briand JP, Muller S. Vaccine 2003;21:776–780. [PubMed: 12531358]

(14). Raynal S, Grossiord J, Seiller M, Clausse D. J. Controlled Release 1993;26:129–140.

(15). Utada AS, Lorenceau E, Link DR, Kaplan PD, Stone HA, Weitz DA. Science 2005;308:537–541. [PubMed: 15845850]

(16). Nisisako T, Okushime S, Torii T. Soft Matter 2005;1:23–27.

(17). Garti N, Lutz R. Interface Sci. Technol 2004;4:557–605.

(18). Aserin, A., editor. Multiple Emulsions: Technology and Applications. Wiley; Hoboken, NJ: 2008.

(19). Bozkir A, Hayta G. J. Drug Targeting 2004;12:157–164.

(20). Bozkir A, Hayta G, Saka OM. Pharmazie 2004;59:723–725. [PubMed: 15497758]

(21). Florence AT, Whitehill D. J. Colloid Interface Sci 1981;79:243–256.

(22). Hou W, Papadopoulos KD. Chem. Eng. Sci 1996;51:5043–5051.

(23). Villa CH, Lawson LB, Li Y, Papadopoulos KD. Langmuir 2003;19:244–249.

(24). Garti, N.; Benichou, A.; Sjoblom, J., editors. Encyclopedic Handbook of Emulsion Technology. Marcel Dekker; New York: 2001. Double Emulsions for Controlled-release Applications—Progress and Trends; p. 377-407.

(25). Pays K, Giermanska-Kahn J, Pouligny B, Bibette J, Leal-Calderon F. Langmuir 2001;17:7758–7769.

(26). Vasiljevic D, Parojcic J, Primorac M, Vuleta G. Int. J. Pharm 2006;209:171–177. [PubMed: 16406403]

(27). Rojas EC, Sahiner N, Lawson LB, John VJ, Papadopoulos KD. J. Colloid Interface Sci 2006;301:617–623. [PubMed: 16781725]

(28). Wen L, Papadopoulos KD. Colloids Surf., A 2000;174:159–167.

(29). Cheng J, Chen JF, Zhao M, Luo Q, Wen LX, Papadopoulos KD. J. Colloid Interface Sci 2007;305:175–182. [PubMed: 17064722]

(30). Hai M, Magdassi S. J. Controlled Release 2004;96:393–402.

(31). Tedajo GM, Bouttier S, Fourniat J, Grossiord JL, Marty JP, Seiller M. Int. J. Pharm 2005;288:63–72. [PubMed: 15607258]

(32). Grossiord JL, Seiller M. S.T.P. Pharma Sci 2001;11:331–339.

(33). Olivieri L, Seiller M, Bromberg L, Besnard M, Duong T, Grossiord JL. J. Controlled Release 2003;88:401–412.

(34). Herhold AB, Ertas D, Levine AJ, King HE. Phys. Rev.E 1999;59:6946–6955.

(35). Magdassi S, Garti N. J. Colloid Interface Sci 1987;120:537–539.

(36). Guery J, Betrand E, Rouzeau C, Levitz P, Weitz DA, Bibette J. Phys. Rev. Lett 2006;96:198301/1–198301/4. [PubMed: 16803145]

(37). Matsumoto S, Kita Y, Yonesawa D. J. Colloid Interface Sci 1976;57:353–361.

(38). Cournarie F, Savelli MP, Rosilio V, Bretez F, Vauthier C, Grossiord JL, Seiller M. Eur. J. Pharm. Biopharm 2004;58:477–482. [PubMed: 15451521]

(39). Jiao J, Burgess D. Pharm. Sci 2003;5:62–73.

(40). Ficheux MF, Bonakdar L, Leal-Calderon F, Bibette J. Langmuir 1998;14:2702–2706.

(41). Chi EY, Krishnan S, Randolph TW, Carpenter JF. Pharm. Res 2003;20:1325–1336. [PubMed: 14567625]

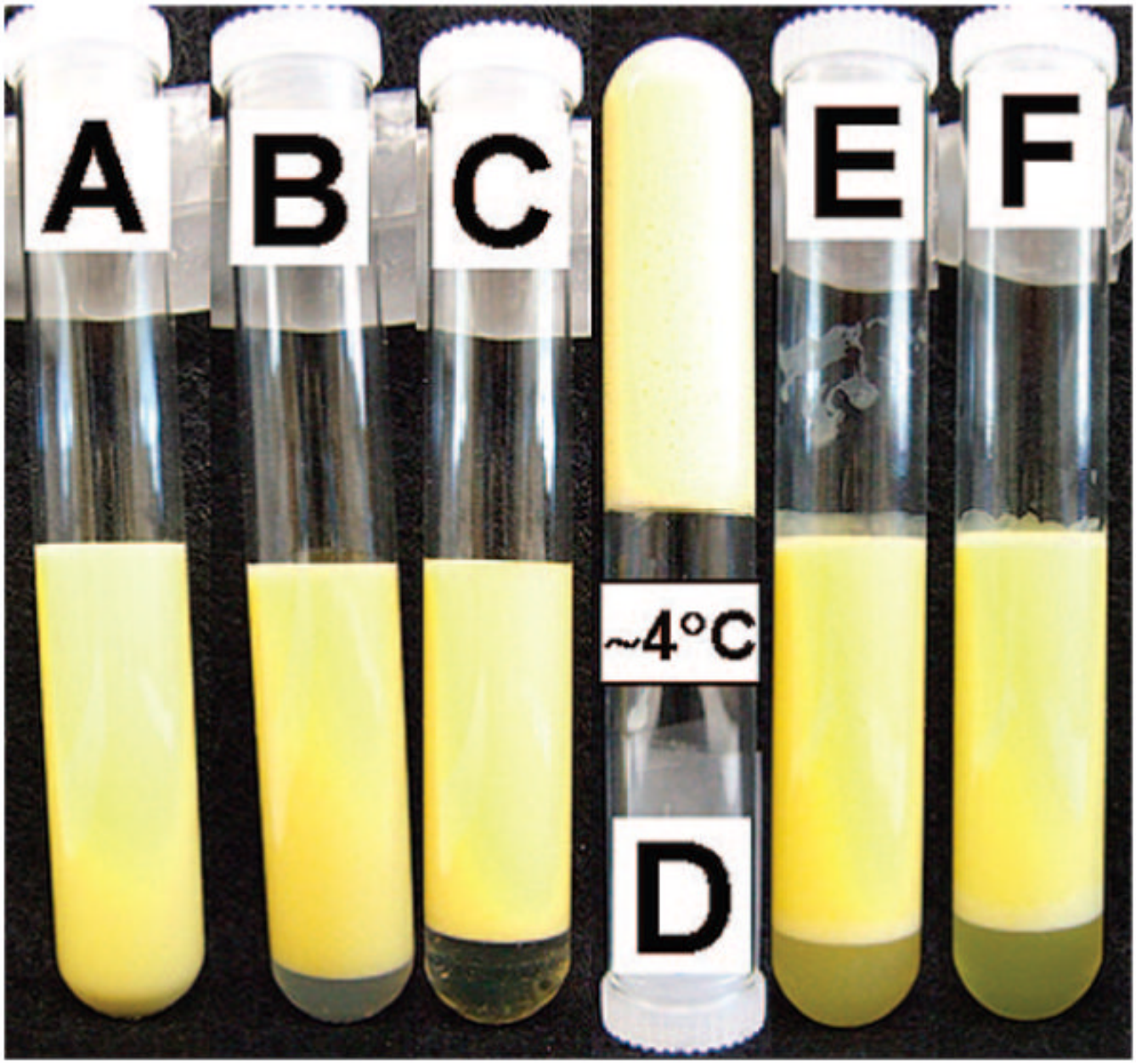

Figure 1.
Macroscopic observation of instability for emulsion 1 during a freeze-thaw cycle: (A) initially; (B) after 2.5 h at room temperature; (C) after 3 days at room temperature; (D) after 4 months of storage at ~4 °C (in the refrigerator); (E) immediately after thawing; (F) 1 h after thawing.

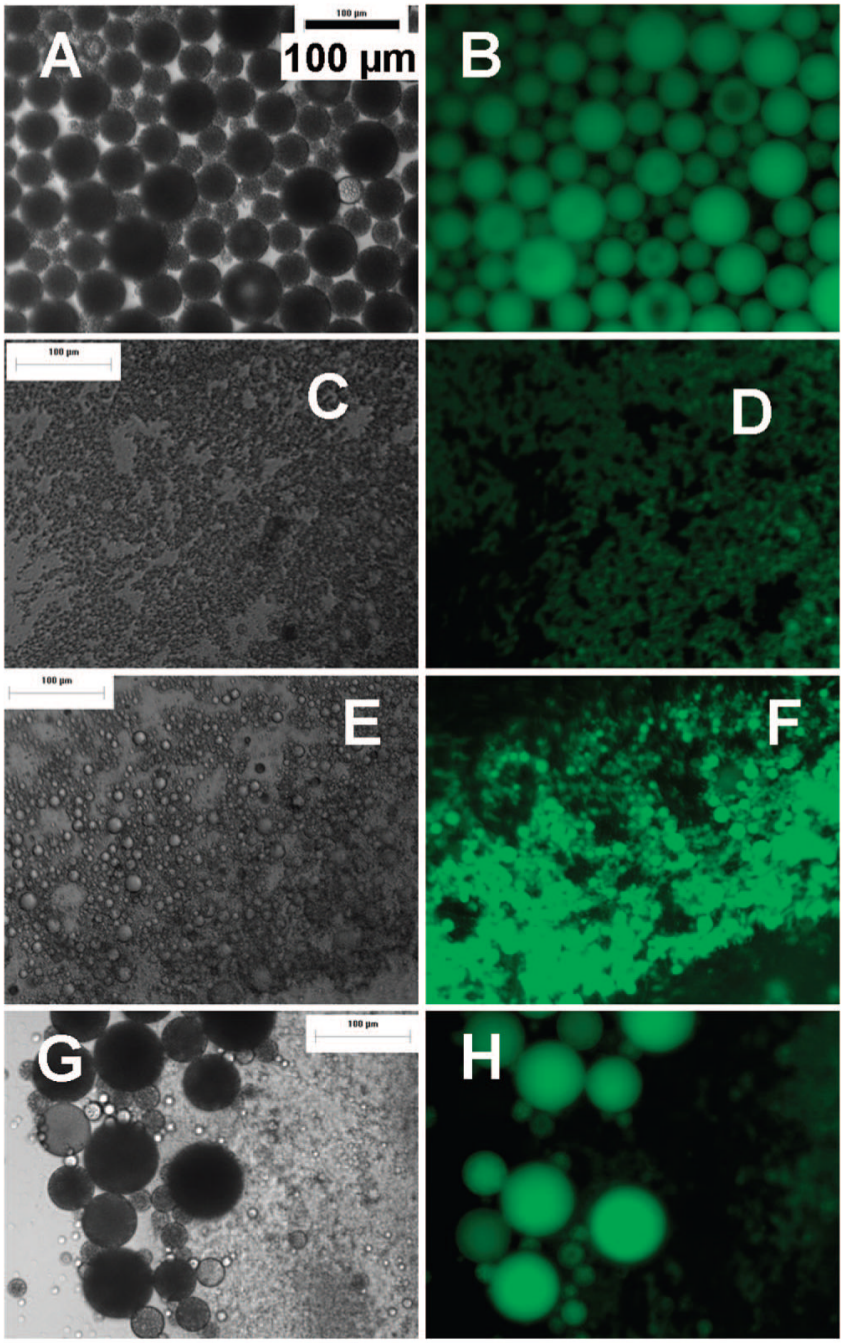

Figure 2.

Microscopy observation of emulsion 1: (A, B) initial sample; (C, D) creamy layer obtained after one freeze-thaw cycle; (E, F) creamy layer obtained after 10 consecutive freeze-thaw cycles; (G, H) creamy layer of the control emulsion, after 1 day of storage at room temperature. The left panels correspond to optical microscopy images, taken with bright light, whereas the right panels are fluorescence microscopy images. The scale bar in panel A also applies to the other images.

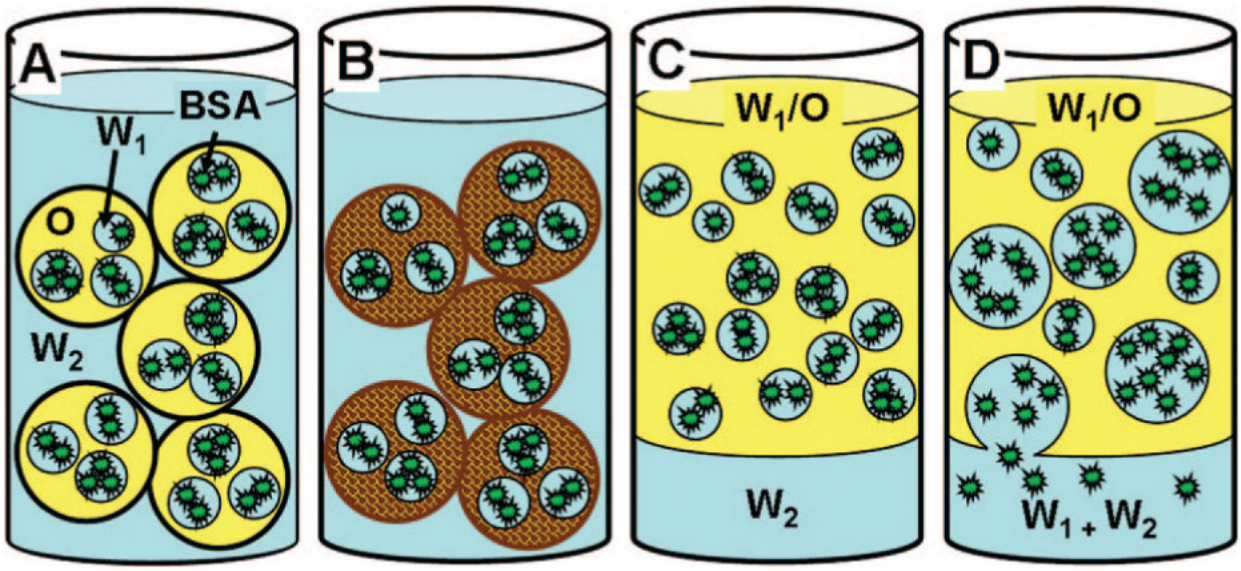

Figure 3.
Scheme describing the behavior for emulsion 1 during freeze-thaw cycling: (A) initially; (B) during storage at low temperature (crystallized oil); (C) after a freeze-thaw cycle; (D) after several freeze-thaw cycles.

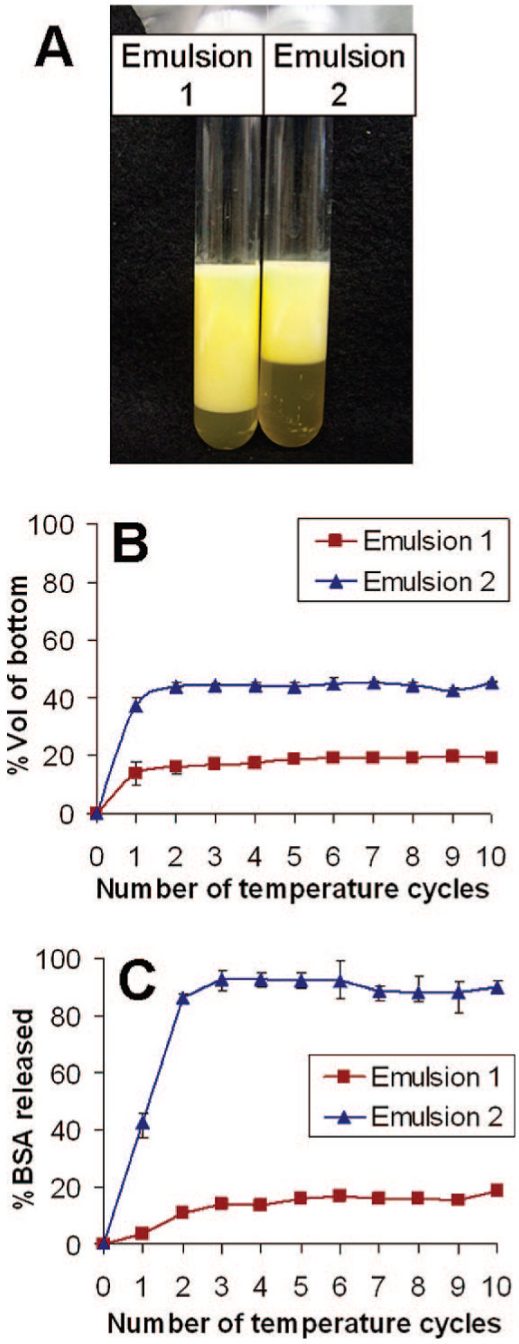

Figure 4.

Effect of the Tween 80 concentration on phase separation and protein release following freeze-thaw cycling: (A) visual comparison of emulsions 1 and 2, 1 h after the third thawing; (B) volume fraction of each double emulsion collected as the bottom aqueous layer; (C) efficiency of FITC-BSA release from each double emulsion. Emulsions 1 and 2 contained 0.03 and 0.08 M Tween 80, respectively.

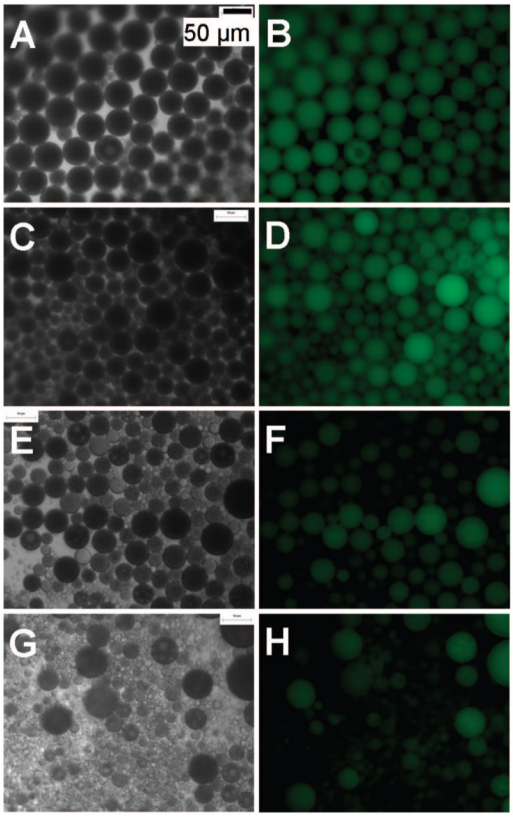

Figure 5.
Microscopy observation of emulsion 2: (A, B) initial emulsion; (C, D) control emulsion after 3 days of storage at room temperature; (E-H) “creamy” layer obtained after (E, F) the first freeze-thaw cycle and (G, H) the third freeze-thaw cycle. The left panels correspond to optical microscopy images, taken with bright light, whereas the right panels are fluorescence microscopy images. The scale bar in panel A also applies to the other images.

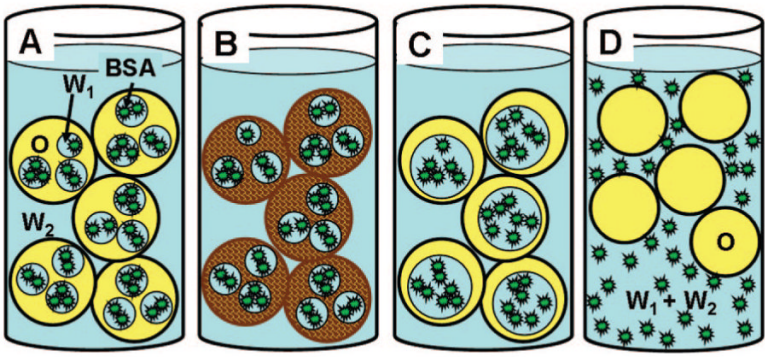

Figure 6.
Scheme describing the behavior for emulsion 2 during freeze-thaw cycling: (A) initially; (B) during storage at low temperature (crystallized oil); (C) after internal coalescence; (D) after external coalescence. Internal coalescence and external coalescence occurred sequentially and were readily triggered by oil thawing.

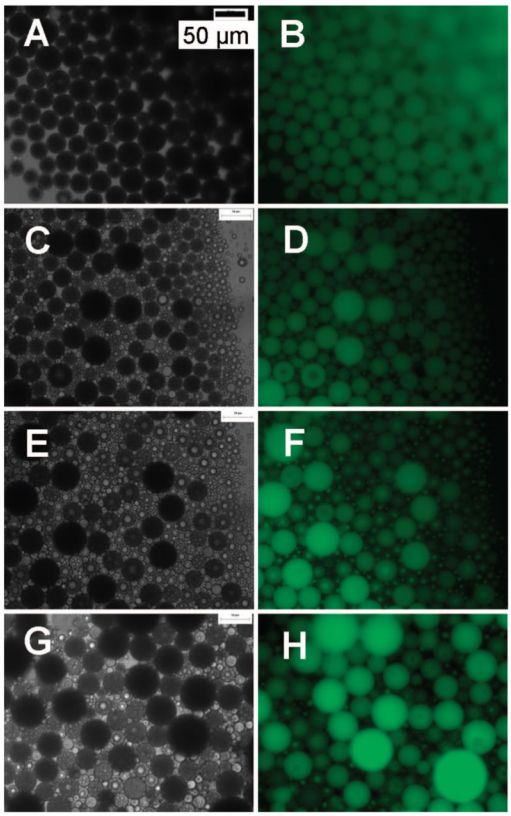

Figure 7.
Microscopy observation of emulsion 2 at room temperature: (A-F) Freshly prepared and diluted emulsion, placed on a covered glass slide for (A, B) about 2 min, (C, D) 1 h, and (E, F) 2 h; (G, H) freshly prepared emulsion diluted for 5 h and then placed on a covered glass slide for about 2 min. The left panels correspond to optical microscopy images, taken with bright light, whereas the right panels are fluorescence microscopy images. The scale bar in panel A also applies to the other images.

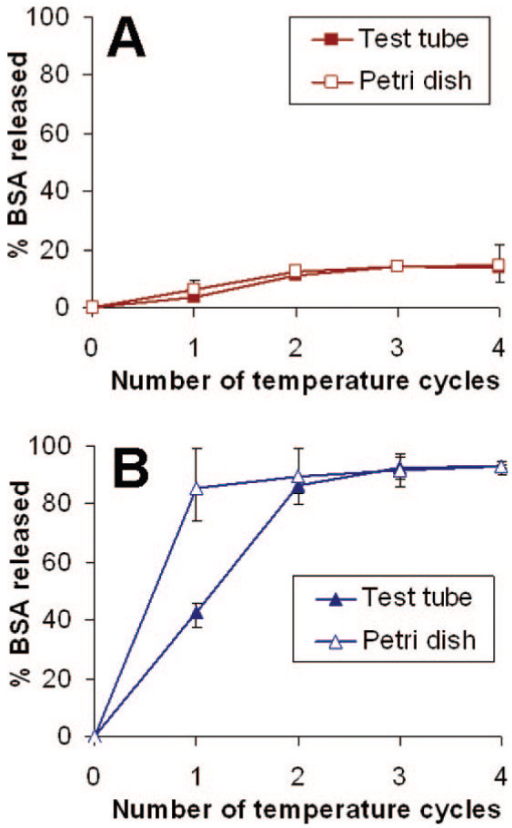

Figure 8.

Effect of the area of contact between the creamy and aqueous layers (formed after phase separation) on FITC-BSA release following freeze-thaw cycling: (A) emulsion 1, containing 0.03 M Tween 80 in W_2; (B) emulsion 2, containing 0.08 M Tween 80 in W_2.