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# A Freeze-Concentration and Polyampholyte-Modified Liposome-Based Antigen Delivery System for Effective Immunotherapy

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Immunotherapy is an exciting new approach in cancer treatment. Here, we describe the development of a novel freeze-concentration method that could be applicable in immunotherapy. The method involves freezing cells in the presence of pH-sensitive, polyampholyte-modified liposomes with encapsulated ovalbumin (OVA) as the antigen. In RAW 264.7 cells, compared to the non-frozen condition, freeze-concentration of polyampholyte-modified liposomes encapsulating OVA resulted in efficient OVA uptake and also allowed for its delivery to the cytosol. Efficient delivery of OVA to the cytosol was shown to be partly due to the pH-dependence of the polyampholyte-modified liposomes. Cytosolic OVA delivery also resulted in significant up-regulation of the major histocompatibility complex class I pathway through a process known as cross-stimulation, as well as an increase in the release of IL-1β, IL-6, and TNF-α. Our results demonstrate that the combination of a simple freeze-concentration method and polyampholyte-modified liposomes might be useful in future immunotherapy applications.

## 1. Introduction

In recent years, enormous research efforts have been focused on the development of novel strategies for the treatment of serious diseases such as cancer.[1] Immunotherapy is one of these novel approaches that uses the body's own immune system to directly attack and destroy cancer cells.[2] Thus, the activation of the immune system in cancer therapy has become a very important topic amongst cancer researchers.[3] Antigen presenting cells (APCs) such as dendritic cells, macrophages, and B-cells, are essential in the activation of immune responses and therefore play an important role in immunotherapy. Antigen presentation can occur through both major histocompatibility complex (MHC) class I and MHC class II routes.[4] Generally, following internalization of an exogenous antigenic protein by APCs, the protein molecule is degraded to peptide fragments and these fragments are then presented at the cell surface by MHC class II molecules with the resulting induction of humoral immunity.[5] In contrast,

endogenous protein molecules are degraded by cytosolic proteasomes present in the cytosol of APCs. Peptide fragments generated as a result of proteolysis are then presented by MHC class I molecules. An important function of the MHC class I molecule in cancer immunotherapy is to display antigenic proteins to cytotoxic T cells (CTLs).[6] After recognition of the antigen by CTLs, the target cell, which may be infected with a virus or be cancerous, is directly killed by the CTL. In some cases, exogenous antigen can be transferred to the cytosol resulting in the induction of MHC class I-presentation, a process known as cross-presentation.[7] Previously, Hanlon et al. reported the cross-presentation through MHC class I instead of MHC class II using protein-loaded poly (lactic-co-glycolic acid) (or PLGA) nanoparticles that can escape from the endosomal compartment.[8] Similarly, Akagi et al. have described the use of γ-poly (glutamic acid)-based nanoparticles with entrapped ovalbumin (OVA) that allow for its delivery to the cytosol of cells and its subsequent cross-presentation.[9]

Many researchers in this area have recently focused on the development of carriers, which provide additional adjuvant activity for the induction of immune response. Nanocarriers such as nanoparticles[10], micelles[11], and nanogels[12] have been developed for the cytoplasmic delivery of antigens such as proteins, peptides, or genes. However, many of these are toxic[13] and are unstable.[14] Lipid-based delivery systems, such as liposomes, have been extensively used as carriers because of their biocompatibility, non-toxicity, and ability to undergo membrane fusion.[15] pH-sensitive liposomes modified by polymers have also recently been shown to be an effective approach for the efficient delivery of antigen molecules to APCs such as dendritic cells. Recently, Kono et al. have developed an efficient pH-sensitive liposome by modification with pH-sensitive polyglycidol derivatives.[16] They demonstrated pH sensitivity of the liposomes through their ability to deliver proteins to the cytosol without trafficking through the lysosome. Many polymers such as poly (N-isopropyl acrylamide) (PNIPAM), poly (alkyl acrylic acid), or poly (malic acid) have been used to modify the surface of liposomes in order to induce such pH-responsive behavior.[17] pH responsiveness in the liposomes is

important because it promotes the fusion between liposomes and the endosomal membrane[18] causing the release of protein at acidic pH. Interestingly, these pH-sensitive liposomes have been shown to enhance the delivery of antigenic proteins into the cytosol of dendritic cells, thereby causing the induction of an efficient immune response.[16,17]

An important advancement in immunotherapy is therefore the development of physical strategies for the effective cytoplasmic delivery of antigens. Physical approaches such as electroporation[19] and ultra-sonication[20] have already been developed for immunotherapy applications. However, the main drawbacks of these methods are low cell viability and phenotypic changes. Therefore, to improve on the physical method for effective cytoplasmic delivery of antigens, further research is required. To this end, we have previously developed a new freeze-concentration method that can deliver antigens to cells.[21,22] The gradual formation of ice crystals over a temperature range of -5 to -45°C excludes solute molecules, thereby enhancing the solute concentration in the extracellular solution by means of phase separation.[23] This phenomenon is referred to as “freeze-concentration”. In the past, use of the freeze-concentration technology has been limited to the food industry and was used for the production of fruit juices, coffee, and tea-extracts.[24] Our earlier studies showed the effective use of freeze-concentration to enhance the concentration of proteins in the external media close to the cell membrane leading to membrane adsorption[21] and ultimately protein internalization[22] inside the cells. Freeze-concentration offers high cell viability, low cost, and an enhanced interaction between the protein-nanocarrier complex and the cell membrane.

In these previous studies, we also demonstrated the development of pH-sensitive liposomes, generated using a hydrophobic polyampholyte.[22] The hydrophobic polyampholyte nanoparticles were obtained by modification of ε-poly-L-lysine (PLL) with hydrophobic dodecylsuccinic anhydride (DDSA) and succinic anhydride (SA).[21] In the current study, using OVA as a model antigen for immunotherapy, we used both pH-sensitive liposomes and the freeze-concentration method for enhanced protein internalization to demonstrate efficient

endosomal protein escape to the cytosol (**Figure 1**). Cytosolic delivery of OVA to a macrophage cell line resulted in the induction of an immune response involving MHC class I molecules as well as enhanced secretion of cytokines. Our results suggest that through a combination of the use of non-toxic polyampholyte-modified liposomes and freeze-concentration, exogenous antigens may enter the classical class I pathway through the process referred to as 'endosomal escape'.

## 2. Results and Discussions

### 2.1. Synthesis of polyampholytes

The polyampholyte cryoprotectant was prepared using PLL as described in our previous report.[25] A novel polyampholyte cryoprotectant, denoted as PLL-SA, was synthesized by changing the appropriate ratio of amino to carboxyl groups by succinylation with succinic anhydride (65 mol %) (Scheme S1, Supporting Information). This polyampholyte cryoprotectant showed extremely high cryoprotection ability in 10% aqueous solution in a variety of different cell lines.[25] The degrees of substitution of SA was found to be 62 % as determined by $^{1}$H NMR (Figure S1A, Supporting Information). Similarly, a hydrophobic-modified polyampholyte (PLL-DDSA), which had been synthesized previously by substitution of PLL with DDSA (5 mol %) (100°C for 2 h with constant stirring), was then substituted with SA (65 mol %) at 50°C for 2 h to synthesize the new hydrophobic polyampholyte PLL-DDSA-SA (Scheme S2, Supporting Information).[21,22] Similarly, the degrees of substitution of DDSA was found to be 4.4 % determined by $^{1}$H NMR. (Figure S1B, Supporting Information).

### 2.2 Preparation and characterization of unmodified or polyampholyte-modified liposomes encapsulating OVA

In our previous study we prepared two different type of liposomes.[22] One type was a zwitterionic liposome prepared by the combination of lipids such as 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). The

other type was a polyampholyte-modified liposome obtained after the addition of PLL-DDSA-SA to zwitterionic liposomes. We then investigated both the particle size and the zeta potential of unmodified and polyampholyte-modified liposomes. **Table-1** shows the zeta potential and the particle diameter, obtained using the dynamic light scattering (DLS) method. The surface charge of polyampholyte-modified liposomes was -18.43 mV whereas for the unmodified liposomes it was -5.13 mV. The reason for this increased negative value for the surface charge is that polyampholytes contain an excess number of carboxyl groups over amino group in their polymeric backbone. These results clearly indicate that the polyampholytes efficiently modified the surface of the liposome.

The particle sizes of the un-encapsulated liposomes were similar for both unmodified and polyampholyte-modified liposomes; the unmodified liposomes had a mean diameter of 279 nm and the polyampholyte-modified liposomes were slightly larger with a mean diameter of 305 nm. We also evaluated the stability of both the unmodified and the polyampholyte-modified liposomes, both with encapsulated OVA protein, over time under physiological conditions. The polyampholyte-modified liposomes did not change their particle size over time whereas unmodified liposomes appeared to be unstable (Figure S2, Supporting Information). These data suggest that hydrophobic polyampholytes enhance the stability of liposomal membranes because of the presence of hydrophobic polymer chains.

**2.3 Adsorption of protein encapsulating liposomes onto cells under non-frozen and frozen conditions**

To investigate the use of the freeze-concentration approach for cytosolic delivery of antigen proteins, we elected to use RAW 264.7 macrophage cells as representative APCs since these cells are readily cultured and display a robust immune response. We examined the adsorption of OVA-encapsulated liposomes onto RAW 264.7 macrophages with or without freezing.

As shown in **Figure 2**A-B, confocal imaging of cells showed that the fluorescence signal from both unmodified and polyampholyte-modified liposomes loaded with OVA was

significantly higher in the frozen condition compared to the non-frozen condition, indicating enhanced adsorption to the cell surface. These results indicate that freeze-concentration acts as a driving force that enhances the adsorption of liposomes to the cell membrane. Quantification of the fluorescence intensity also showed that under the frozen condition, both unmodified and polyampholyte-modified liposomes increased adherence around the cell membrane (Figure 2C). As a control, we also examined the cell adsorption of free, un-encapsulated OVA protein, with and without the freeze-concentration approach. We found that free OVA protein does not adhere to the cell membrane under the non-frozen condition. OVA protein was found to adsorb to a low extent to the cell membrane after applying the freeze-concentration approach. These data indicate that free OVA has a low association with cells after thawing, thereby restricting its entry into cells (Figure S3A-B, Supporting Information).

In earlier reports, energy-based methods such as electroporation have been frequently used as a physical method for the delivery of protein antigens into cells, but the presence of a strong electrical field creates lethal nanopores in the membrane which disrupt cellular homeostasis and lead to cell damage and a decrease in overall cell viability.[26] Based on this, we examined cell viability following freeze-concentration in the presence of unmodified or polyampholyte-modified liposomes. Cell viability was 93% for polyampholyte-modified liposomes and 89% for unmodified liposomes; this difference was not significant (Figure S4, Supporting Information). Taken together, these data indicate that the freeze-concentration method provides enhanced association of OVA-encapsulated liposomes onto cells while at the same time maintaining high cell viability.

Moreover, the stability of the protein-nanocarrier complex plays a crucial role in therapeutic applications at ultra-cold temperatures. We found that the particle size did not change significantly in either unmodified or polyampholyte-modified liposomes at -80°C[22], indicating that the polymeric cryoprotectant stabilized and reduced liposome aggregation.

Accordingly, we used a polymeric cryoprotectant and a protein-liposome complex for delivery of the protein antigen in conjunction with the freeze-concentration method.

**2.4 Internalization of protein encapsulating liposomes onto cells via non-frozen and frozen**

Following enhanced adsorption to the cell surface by freeze-concentration, the internalization of the protein nano-carrier complex inside the cells is an extremely important step in immunotherapy. In order to examine this, RAW 264.7 cells were frozen in the presence of OVA-encapsulated, unmodified or polyampholyte-modified liposomes and internalization of the liposome and OVA examined (**Figure 3**A-D). Both unmodified and polyampholyte-modified liposomes were efficiently internalized by RAW 264.7 cells following the freeze-concentration process (Figure 3B, D). In contrast, in either of the non-frozen controls, there was very little internalization of the complex (Figure 3A,C). This result demonstrated that freeze-concentration could accelerate internalization of the OVA encapsulated liposomes into cells. Additionally, as shown in Figure 3D internalization was visibly greater when polyampholyte-modified liposomes were used rather than unmodified liposomes (Figure 3C). Quantification of the fluorescein isothiocyanate (FITC) fluorescence intensity derived from the FITC-OVA cargo protein confirmed that freeze-concentration using polyampholyte-modified liposomes was more effective than unmodified liposomes (Figure 3E). One possible explanation for this is that the hydrophobic nature of the polyampholyte might enhance the adsorption and interaction with the cell membrane.[27] Several studies have also suggested that modification of liposomes with polymers enhances uptake and internalization of materials into the cytoplasm compared to unmodified liposomes.[28,29] These results are therefore in good agreement with previous reports.[21,22] In addition, as a control, we examined the internalization of un-encapsulated FITC-OVA protein under the non-frozen and frozen conditions. As for the similar study examining adsorption, we found that uptake of un-encapsulated FITC-OVA protein

without liposomes was low under both non-frozen and frozen conditions (Figure S5 A,B, Supporting Information). It has been shown from various studies that liposomes promote adhesion and increase the fusion and permeability of the cell membrane.[16,17] Therefore, in this study, we confirmed that liposomes are extremely crucial to enhance the interaction between the cell membrane and protein-carrier complexes.

Consistent with our previous studies, protein antigen adsorption and internalization increased after freezing.[21,22] As shown in Figure 2A,B, the freeze-concentration method efficiently induces the adsorption of the FITC-labeled OVA-loaded protein-liposome complex to the cell membrane. This enhanced adsorption is likely due to a combination of the high affinity of the hydrophobic polyampholytes for the cell membrane as well as the freeze-concentration effect.[21,22] In OVA-encapsulated unmodified liposomes, the internalization was also enhanced (Figure 3B,D), although the magnitude was lower than for polyampholyte-modified liposomes (Figure 3D); presumably this reflects the freeze-concentration effect alone.

**2.5 Endosomal escape of proteins from unmodified or polyampholyte-modified liposomes**

Escape of a liposomally encapsulated cargo protein from endosomes is an important event if this approach is to be considered as viable in immunotherapeutic applications. Normally, the majority of an internalized protein remains in the endosomes and is unable to reach the cytosol of cells, thus preventing MHC-class I expression. Therefore, we investigated the ability of OVA to escape from endosomes after freeze-concentration-based internalization. For unmodified liposomes, no green fluorescence was observed in the cytosol indicating that OVA remained in the endosomes (**Figure 4**A). Interestingly, in this study, we found that the freeze-concentration method increased FITC-OVA protein internalization with unmodified liposomes (Figure 3B). However, these unmodified liposomes did not show a significant release of FITC-OVA protein from the endosomes (Figure 4A). We cannot exclude the possibility that after using the freeze-concentration method, a small but undetectable amount of FITC-OVA could be released from the endosomes (Figure 4A). In contrast, it is certain that a strong green

fluorescent signal was observed using polyampholyte-modified liposomes, indicating efficient release of FITC-OVA from endosomes (Figure 4B). These data indicated that the pH-sensitive liposomes released the OVA protein more efficiently than unmodified liposomes.

To understand the pH sensitivity of the unmodified or polyampholyte-modified liposomes we compared release of pyranine, a fluorescent dye, from each type of liposome under different pH conditions. At physiological pH, both unmodified and polyampholyte-modified liposomes did not show any noticeable release of pyranine over time. In contrast, under mild acidic conditions (pH-5.5), polyampholyte-modified liposomes demonstrated a high release of pyranine, whereas unmodified liposomes showed only weak release of pyranine (Figure 4C). We also investigated the effect of pH sensitivity of OVA-encapsulated liposomes using DLS analysis (Figure 4D). The particle size of unmodified liposomes did not change on varying the pH from 7.4 to 5.5 whereas polyampholyte-modified liposomes tended to aggregate at acidic pH and exhibit a larger size.

We found that in polyampholyte-modified liposomes, but not in unmodified liposomes, destabilization of the liposome membrane and release of encapsulated OVA occurs readily at a mildly acidic pH of 5.5 (Figure 4A-D). This is because at acidic pH, the carboxyl group present in the polyampholyte becomes protonated resulting in destabilization of the liposomal membrane and ultimately to release of the cargo protein. Therefore, after endocytosis, the low pH in the endosomes induces the fusion of the liposomal membrane with the endosomal membrane promoting the release of the resident cargo protein into the cytosol. Our findings are in good agreement with previous reports.[16,17,22,30]; in particular, Yuba et al., showed that after modification with succinylated poly (glycidol) and 3-methylglutarylatedpoly (glycidol), liposomes obtained the ability to fuse at acidic pH and deliver their contents into the cytosol through fusion with endosomal membranes.[30] Based on these collective data, we conclude that polyampholyte-modified liposomes release OVA protein more efficiently than unmodified liposomes due to their pH sensitivity.

### 2.6 Macrophage activation using liposomes and the freeze-concentration method

In order to induce an immune response, APCs, such as dendritic cells or macrophages, must present antigenic peptides to MHC class I and MHC class II molecules which then respectively activate CD8 (+) cytotoxic T lymphocytes and CD4 (+) helper T cells.[16, 17] For this reason, we next analyzed the effect of activation of RAW 264.7 macrophages on the expression of MHC molecules in the presence of OVA-loaded liposomes containing monophosphoryl lipid A from *Salmonella minnesota R 595* (MPLA) as an adjuvant (immune activator) in the membrane.[16] RAW 264.7 cells were incubated with unmodified or polyampholyte-modified liposomes under frozen or non-frozen conditions using lipopolysaccharide (LPS) as a positive control. Following this, we examined the cell surface expression of MHC class I and MHC class II molecules using flow cytometry with MHC molecule-specific antibodies (**Figure 5**). As negative controls, cells from the respective samples were included that lacked the appropriate MHC class molecule (Figure 5 A-E). Incubation of RAW 264.7 cells with polyampholyte-modified liposomes under freeze-concentration conditions caused a large increase (almost 3 fold) in MHC class I expression compared to non-frozen polyampholyte-modified liposomes (Figure 5I, J). In contrast, there was virtually no effect on MHC class II expression observed under these or any other conditions (Figure 5K-O). Interestingly, after addition of liposomes under non-frozen conditions, two peaks were observed indicating that some fraction of OVA remains intact inside endosomes (Figure 5G,I). On the other hand, a single high intensity peak was obtained under freeze-concentration conditions, demonstrating that a large proportion of OVA was transferred to the cytosol of the cells. (Figure 5H,J). OVA encapsulated unmodified liposomes also enhanced MHC class I surface expression with the freeze-concentration methodology as compared to the level of MHC class I induced by LPS (Figure 5F,H).

MHC class I surface molecules increased significantly when freeze-concentration was used, particularly with polyampholyte-modified liposomes, but also to a lesser extent for unmodified liposomes. This suggests that the freeze-concentration method results in presentation of exogenous antigens to MHC class I molecules through enhanced delivery of the antigen into the cytosol of cells (Figure 5H,J). In keeping with this, the levels of MHC class II molecules barely changed (Figure 5F-J and K-O). Taken together, the data suggest that the liposomes are internalized through endocytosis and that the OVA protein cargo is released from the endosomes into the cytosol under mildly acidic conditions in the endosome by endosomal escape. Our data clearly show that the polyampholyte-modified liposomes are pH sensitive, but the unmodified liposomes are pH-sensitive inside cells since they also increased MHC class I expression, albeit to a lower extent (Figure 5F-J). In this study, zwitterionic liposomes composed of DOPC and DOPE were used. DOPE is unsaturated and has the ability to acquire a hexagonal phase at low pH and so it provides pH-sensitivity to zwitterionic liposomes.[31] The polyampholyte-modified liposomes have greatly enhanced endosomal escape because of the combination of a membrane-destabilizing polymer and the presence of DOPE which significantly destabilize the endosomal membrane and allows for greater release of cargo into the cytoplasm (Figure 4A-D). Numerous studies have shown that exogenous protein antigens can be presented on MHC class I molecules via a process known as cross-presentation[30,32] The physiological mechanism of cross-presentation remains unclear.[33] In our study, the exogenous liposome-encapsulated antigen (OVA) is internalized through endocytic pathways and, after escaping from endosomes into the cytoplasm through a pH-dependent mechanism, is degraded by proteasomes. While we have not directly proven that OVA-derived peptides are presented in the context of MHC class I molecules present on APCs in this study, we aim to focus on this question in future studies.

A few studies have also reported the phenomenon of greater increases in expression of MHC class I surface molecules compared to MHC class II molecules in immune cells.[34] One

study compared the expression of cell surface molecules using the RAW 264.7 cells following LPS stimulation, and showed enhanced expression of MHC class I compared to MHC class II molecules.[35]

The function of MHC class I molecules is to activate cellular immunity. So, from the viewpoint of cancer immunotherapy, the MHC class I molecules are extremely beneficial in inducing activation of CD8 (+) cytotoxic T lymphocytes (CTLs).[6] CTLs recognize the complex between tumor antigens and MHC class I molecules that are expressed on cancer cells and directly kill tumor cells. The data presented here clearly show that the freeze-concentration method introduces antigens into the cytosol of RAW macrophage cells effectively resulting in increased MHC class I expression.

In order to confirm that the effects on MHC class I expression were specific, we examined the effect of the freeze-concentration method in cells in the absence of liposomes and OVA. There was a slight increase in fluorescence demonstrating that stress caused by freezing induces MHC class I expression compared with that in non-frozen condition (Figure S6 A-B, Supporting Information). From these results, it has been suggested that freezing could affect in expression efficiently.

In conclusion, the freeze-concentration method strongly enhanced cell surface expression of MHC class I as compared to the non-frozen method. In contrast, the cell surface expression of MHC class II was not up-regulated to any significant extent under any of the conditions used in this study (Figure 5F-J and K-O). These results demonstrated that freeze-concentration increased levels of the OVA-loaded liposomes around the cell membrane and triggered their internalization, thereby enhancing the immune response.

**2.7 ELISA studies to examine cytokine secretion**

Cytokines are signaling molecules that are secreted by macrophages, B lymphocytes, and T lymphocytes, and play an important role in the regulation of the immune system. These

pro-inflammatory cytokines are usually induced by LPS, play key roles in the inflammatory response, and are well known to be secreted by macrophages and monocytes as part of the innate immune system.[36,37] IL-1β is a potent pro-inflammatory cytokine that is important in host-defense responses to infection and injury. IL-6 supports the growth of B cells as well as regulatory T cells. TNF-α regulates the function of immune cells and is essential in the control of intracellular pathogens and for stimulating the recruitment of inflammatory cells to an area of infection.

Hence, we next examined the production of immune-stimulatory cytokines such as IL-1β, IL-6, and tumor necrosis factor (TNF)-α following RAW 264.7 macrophage stimulation using OVA-encapsulated liposomes, with or without freezing, with LPS as a positive control. As shown in **Figure 6**A,B, secretion of TNF-α and IL-1β from RAW 264.7 cells incubated with unmodified liposomes or polyampholyte-modified liposomes under the non-frozen state was very low compared to that observed in the presence of LPS. In contrast, RAW 264.7 cells, incubated with either unmodified- or polyampholyte-modified liposomes under freeze-concentration conditions, secreted large amounts of both TNF-α and IL-1β to levels that were similar to those seen for the positive control LPS. However, as shown in Figure 6C, a different trend was seen for IL-6. A large amount of IL-6 was secreted from RAW 264.7 cells incubated with polyampholyte-modified liposomes, almost doubling under the freeze-concentration compared to the non-frozen condition. Interestingly, a large amount of IL-6 was also secreted from RAW 264.7 cells incubated with unmodified liposomes, and freeze-concentration increased IL-6 secretion only marginally.

Both TNF-α and IL-1β secretion were drastically enhanced to similar extents when either unmodified- or polyampholyte-modified liposomes were used under freeze-concentration conditions compared to non-frozen conditions (Figure 6A,B). In contrast, IL-6 secretion was increased only slightly by freeze-concentration in unmodified liposomes but was noticeably increased under freeze-concentration conditions in polyampholyte-modified

liposomes. Compared to TNF-α and IL-1β secretion, these differences in IL-6 secretion might be attributed to the pH-sensitivity property of polyampholyte-modified liposomes, which could allow for antigens to be delivered more efficiently to the cytosol of cells and therefore allow for more cytokine secretion compared to that in unmodified liposomes (Figure 6C). As a control, we also investigated the effect of the freeze-concentration method on RAW264.7 macrophages in the absence of both adjuvant and liposomes. There was no significant effect on secretion of cytokines in only cells with or without freezing. This result indicated that freeze-concentration alone does not activate the cells but requires the presence of adjuvants (Figure 6A-C).

In our study, both unmodified- and polyampholyte-modified liposomes, despite the presence of MPLA as an adjuvant, produced a low secretion of cytokines under non-frozen conditions when compared to LPS (Figure 6A-C). This is perhaps not surprising considering that LPS has been reported to induce inflammatory cytokines to a much greater extent than MPLA. [36,38,39] In contrast, a large amount of cytokine secretion was observed when freeze-concentration was employed (Figure 6A-C).

This enhanced secretion of cytokines might be due to freeze-concentration allowing for an increase in the adjuvant activity of MPLA therefore resulting in more efficient release of the antigen, leading to increased secretion of pro-inflammatory cytokines in the frozen situation compared to the non-frozen situation. However, the data obtained for TNF-α and IL-1β demonstrated that freeze-concentration enhances the secretion in both the unmodified and polyampholyte-modified systems, which suggests the presence of a different mechanism of action which still needs to be explored in future studies.

Regardless, we have developed a new and facile freeze-concentration method that enhances the immune response of macrophages to liposomes encapsulated with the antigen OVA. The freeze-concentration method enhances the adsorption between cells and proteins without any toxicity and cell damage. In our earlier studies, we demonstrated enhanced cellular adsorption and internalization of proteins using this freeze-concentration approach. This study focused on the

effective use of this freezing method in enhancing the immune response in RAW 264.7 macrophage cells. Moreover, endosomal antigen escape, which is of particular use in immunotherapy, can be achieved using delivery of the protein cargo through pH-sensitive liposomes created by modification with hydrophobic polyampholytes.

## 3. Conclusion

In conclusion, we found that freeze-concentration enhances MHC class I expression, and activation of APCs. We have also found that adjuvant-containing liposomes with an encapsulated antigen elicit significantly higher MHC class I expression and cytokine release using the freeze-concentration method compared to the non-frozen system. Moreover, polyampholyte-modified liposomes have a destabilizing property at acidic pH that can lead to efficient endosomal escape, which increases the expression of MHC class I molecules. These results suggest that a combination of freeze-concentration and the use of polyampholyte-modified liposomes containing MPLA is a promising strategy for the safe delivery of antigens that could contribute to the establishment of an effective immunotherapy. However, clinical studies will be required to validate this approach. To the best of our knowledge, this is the first report that uses freeze-concentration as a physical method for effective immunotherapy. Although this technique might be applicable only in an *in vitro* antigenic delivery system it may be suitable for establishing adoptive immunotherapy. This pioneering study therefore offers a new possibility for immunotherapy application that avoids cell damage, is simple, and does not require expensive equipment.

## 4. Experimental Section

*Chemicals and Reagents*: Pyranine was purchased from TCI (Tokyo, Japan). Zwitterionic lipids such as DOPC, DOPE, and fluorescently labeled 1,2-dioleoyl-sn-glycero-3-

phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-PE) were obtained from Avanti Polar lipids (Alabaster, AL, US). Enzyme linked immunosorbent assay (ELISA) kits for the measurement of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were purchased from BD Biosciences (San Jose, CA, USA), and that for measurement of IL-1β was obtained from Life technologies (Carlsbad, CA, USA). Anti-MHC class-I PE, Anti-MHC class-II PE were purchased from BD Bioscience. *p*-Xylene-bis-pyridinium bromide (DPX) was obtained from Molecular Probes (Eugene, OR, USA). OVA protein (45 kDa) and monophosphoryl lipid A from *Salmonella minnesota R 595* (MPLA) were purchased from Sigma Aldrich (St. Louis, MO, USA). 2-(N-morpholino)ethanesulfonic acid (MES) was obtained from Dojindo (Kummoto, Japan).

*Synthesis of polyampholyte cryoprotectant and polyampholyte nanoparticles*: Briefly, an aqueous solution of PLL (25% w/w, 10 mL, JNC Corp., Tokyo, Japan) and SA (1.3 g, Wako Pure Chem. Ind. Ltd, Osaka, Japan) were combined at 50°C for 2 h to convert the amino group to a carboxyl group (Scheme S1). Hydrophobic polyampholytes were prepared according to our previously published methods.[21,22] Briefly, PLL was reacted with hydrophobic DDSA (5% molar ratio, Wako Pure Chem. Ind. Ltd.) at 100 °C for 2 h. Afterwards, SA was added at a 65% molar ratio ($COOH/NH_2$) and allowed to react for 2 h at 50°C (Scheme S2). The polyampholytes were characterized by $^1H$ NMR spectra obtained at 25°C on a Bruker AVANCE III 400 spectrometer (Bruker BioSpin Inc., Switzerland) in $D_2O$.

The degrees of substitution of SA and DDSA were obtained by $^1H$-NMR using equation.

$$\text{Degree of substitution for DDSA (\%)} = (2*A_{\delta 0.74}/3* A_{\delta 1.5\text{-}1.8})*100$$

$$\text{Degree of substitution for SA (\%)} = (2*A_{\delta 2.4}/4* A_{\delta 1.5\text{-}1.8})*100$$

$A_{\delta 0.74}$ is the integral of the methyl peak from DDSA located at 0.74 ppm and $A_{\delta 2.4}$ is the integral of the methylene peak of SA located at 2.4 ppm. $A_{\delta 1.5\text{-}1.8}$ is the integral of the b-methylene peak of poly-lysine at 1.5 ppm to 1.8 ppm.

*Preparation of unmodified or polyampholyte modified liposomes encapsulating OVA*: The appropriate amount of lipid DOPC (5 mg) and DOPE (4.7 mg) were dissolved in chloroform (1 mL) and allowed to evaporate under a steady stream of nitrogen gas to facilitate complete drying. A thin dry lipid membrane consisting of DOPC and DOPE was mixed with 1.0 mL of OVA (1 mg/mL, in Milli-Q water) and the lipid suspension was extruded through a polycarbonate membrane (200 nm pore size) to obtain small unilamellar vesicles (SUVs). For the preparation of hydrophobic polyampholyte modified liposomes, a dry membrane of lipid mixtures with polymer (7:3 w/w) was also prepared by the same method. Ten micrograms of MPLA, which was extracted from lipopolysaccharides, was combined with 5 mg of DOPC and 4.7 mg of DOPE lipids, with or without polyampholytes, for the induction of the immune response.

*Particle size measurement of unmodified or polyampholyte-modified liposomes encapsulated with OVA*: Stability, surface charge and size distribution were measured by DLS using a Zeta sizer 3000 (Malvern Instruments, Worcestershire, UK) with a scattering angle of 135° at a temperature of 25°C. The liposomes were dispersed in phosphate buffer saline without calcium and magnesium (PBS (-)) and the zeta potential was measured at the default parameters (dielectric constant of 78.5, refractive index of 1.6).

*Pyranine release from liposomes*: Pyranine release from liposomes was measured as described in previous reports.[16,17,27] To prepare pyranine-loaded liposomes, unmodified and polyampholyte-modified liposomes were dispersed in aqueous solution containing 35 mM pyranine, 50 mM DPX, and 25 mM MES buffer solution and the pH adjusted to 7.4. The suspension of liposomes with encapsulated pyranine (lipid concentration: 1 x $10^{-5}$ M) was added to PBS at varying pHs at 37°C and the fluorescence intensity of the mixed suspension was followed (excitation at 512 nm, emission from 450 to 600 nm) using a spectrofluorometer (JASCO FP-8600, Hachioji, Tokyo, Japan). The percentage release of pyranine from liposomes was defined as

$$\text{Release (\%)} = (F_t/F_f) \times 100$$

Where $F_t$ is the fluorescence without addition of Triton-X-100 and $F_f$ is the final fluorescent intensity after addition of Triton-X-100 (final concentration: 0.1%).

*Preparation of FITC-labeled OVA protein*: OVA (10 mg) and FITC(1 mg /mL; Dojindo) was dissolved in sodium bicarbonate buffer solution (1 mL; 0.5 M, pH 9.0) with gentle stirring and incubated at 4°C overnight with subsequent dialysis (molecular weight cut off: 3 kDa, Spectra/Por, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) for three days against water and freeze-dried. [17, 22]

*Cell Culture*: Murine RAW 264.7 macrophage cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% $CO_2$ humidified atmosphere. When the cells reached 60% confluence they were sub-cultured by trypsinization with 0.25% (w/v) trypsin containing ethylenediamine tetraacetic acid (EDTA) in phosphate buffer saline without calcium and magnesium (PBS (-)) and were seeded onto new tissue culture plates.

*Adsorption of OVA protein using unmodified or polyampholyte modified liposomes via freeze-concentration*: The FITC-OVA-loaded liposomes containing Rh-PE were prepared as follows. Briefly, lipid containing Rh-PE (0.5 mol %) was dispersed in PBS (-) containing FITC-OVA (1 mg/mL) and prepared by the same method described above. The protein-encapsulated solution was then purified using chromatography on a Sepharose 4B column to remove un-encapsulated proteins. RAW 264.7 murine macrophage cells, at a density of $1x10^6$ cells/mL, were re-suspended in 10% PLL-SA cryoprotective solution (1 mL) including unmodified or polyampholyte-modified liposome encapsulated OVA protein (0.5 mg/mL) in a 1.9 mL cryovial (Nalgene, Rochester, NY, USA) and were then placed in a -80°C freezer for 24 h. After 24 h, the cells were thawed at 37°C and washed with DMEM medium containing 10% FBS. The purpose of using the polymeric cryoprotectant was to protect the cells from damage due to freezing and thereby maintain cell viability. Cell viability was determined by trypan blue

staining solution and cell counting with a hemocytometer. The % viable cells were calculated as the number of viable cells divided by total number of cells. Similarly, for non-frozen, unmodified and polyampholyte-modified liposomes encapsulated proteins were directly added to the cells without using freeze concentration. For analysis of the adsorption under non-frozen and frozen conditions, the cells were washed with PBS (-) and were observed using a confocal laser scanning microscope (CLSM, FV-1000-D; Olympus, Tokyo, Japan).

*Intracellular delivery of OVA protein using freeze-concentration*: After thawing, RAW 264.7 cells were washed three times using cell culture medium with 10% FBS. The cells were then seeded onto 35-mm glass bottom dishes and medium (1 mL) was added. After incubation for 24 h, the attached cells were washed with PBS and internalization of protein/liposomes was observed using CLSM. In order to compare the effect of freezing on internalization with non-freezing, we gently added the appropriate protein-nanocarrier complex to RAW 264.7 cells and incubated them for 24 h to create a 'non-frozen' control. In all cases, liposomes were labeled with Rh-PE labeled lipid and the protein cargo was FITC-labeled OVA. All cells were washed with PBS (-) prior to observation of internalization using CLSM.

*Endosomal escape of OVA protein*: A thawed suspension of RAW 264.7 cells (100 µL) at a density of $1x10^4$ cells/mL containing 10% PLL-SA cryoprotectant with OVA-loaded unmodified or polyampholyte-modified liposomes was washed twice with cell culture medium containing 10% FBS and then seeded into a glass bottom dish. The cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% $CO_2$. LysoTracker Red®DND-26 (Molecular Probes) and Hoechst blue 33342 dye (ThermoFisher Scientific, Waltham, MA, USA) were added and incubated for 30 min before observation. The localization of protein inside the cells was examined using CLSM.[22]

*Flow cytometry analysis*: The cells from both the non-frozen and frozen conditions were scraped and washed with PBS buffer (containing 0.5 mM EDTA and 0.5% BSA/FBS). A mouse monoclonal antibody (mAb; anti MHC-I PE or anti MHC–II PE) in PBS-EDTA (50 µL) was

added to the cell suspension, mixed and incubated at 4°C in the dark for 30 min on ice. The samples were divided into stained which is positive control and negative control that is unstained with mAb. The cells were then centrifuged at 120 *g* for 4 min and re-suspended in PBS-EDTA. The cells were then transferred to a FACS tube and the positive control, non-frozen, and frozen samples were immunostained with fluorescently conjugated anti-mouse monoclonal antibodies. The negative control of each samples were carried out by replacing labeled anti-mouse monoclonal antibody to PBS buffer. Data acquisition and analysis were performed using FACS Calibur instrument (BD Bioscience, Franklin Lakes, NJ, USA). For each sample, 20,000 cells were counted and gated on the basis of 20,000 forward scattering and side scattering events. Stained cells were determined by reference to non-stained cells.

*ELISA measurement of in vitro antigen response*: The levels of TNF-α, IL-1β, and IL-6 in RAW 264.7 from cell culture supernatants were measured by ELISA assay in order to compare the non-frozen and frozen systems. Briefly, a monoclonal antibody specific for the particular assay from each kit was coated onto a 96-well plate. Samples and standard were added, allowed to incubate, washed, and detection antibodies were added. For the removal of excess antibody, Streptavidin-horseradish peroxidase (HRP) was added and incubated for 15 min in the dark at room temperature. The solution was aspirated and thoroughly washed, at least four times, using a wash buffer. After incubation and washing, 3, 3', 5, 5'-tetramethylbenzidine (TMB) was added followed by incubation for 30 min. The reaction was terminated by the addition of 100 µL of stop solution (1 M phosphoric acid); the optical density of the sample was then read at 450 nm using a microplate reader (Versa max, Molecular Devices, Sunnyvale, CA, USA).

*Statistical analysis*: All data are expressed as means ± standard deviation (SD). All experiments were conducted in triplicate. To compare data among more than three groups, a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post-hoc test was used. To compare data between two groups, Student's t-test was used. A P value of $<0.05$ was considered statistically significant.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

**Acknowledgements**

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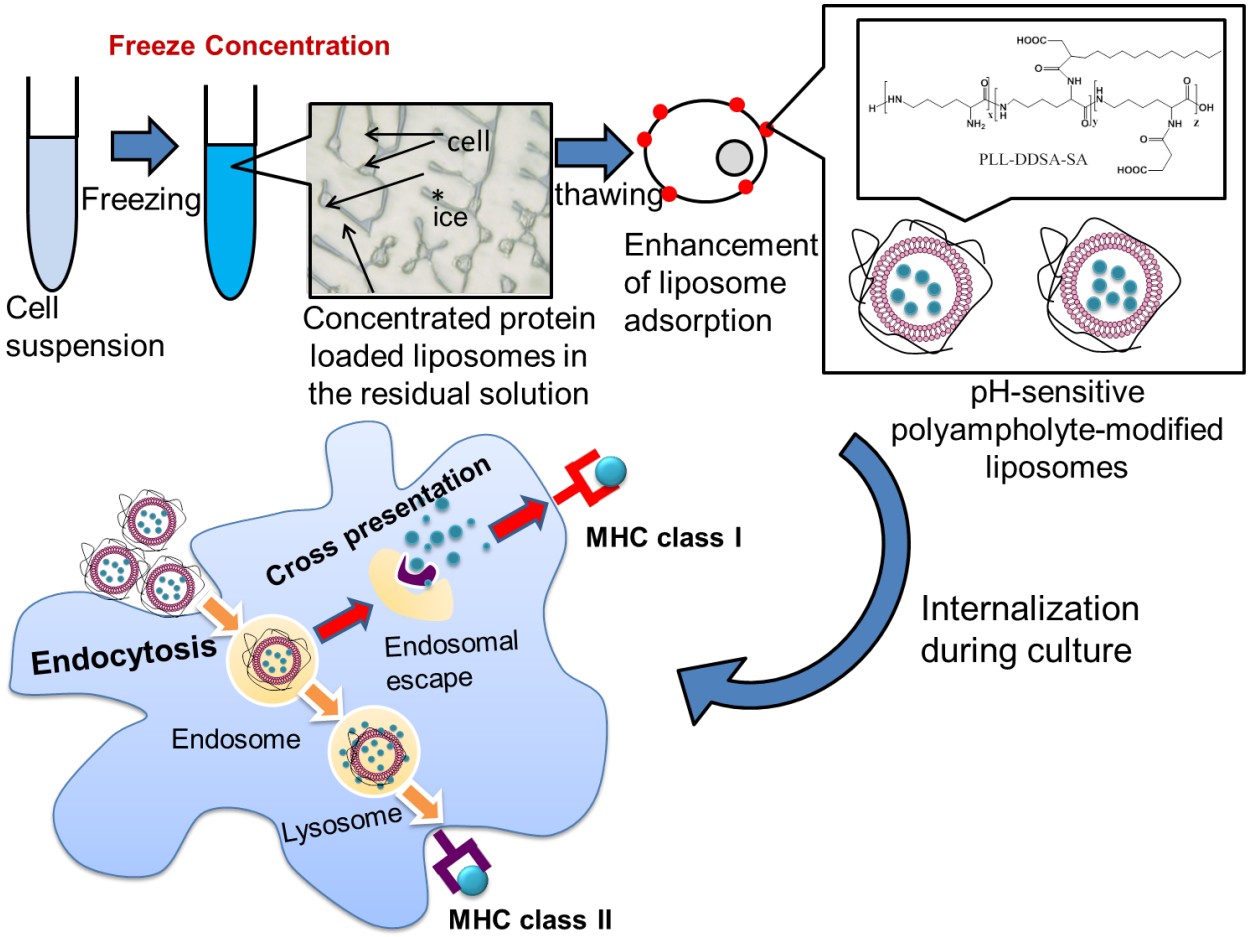
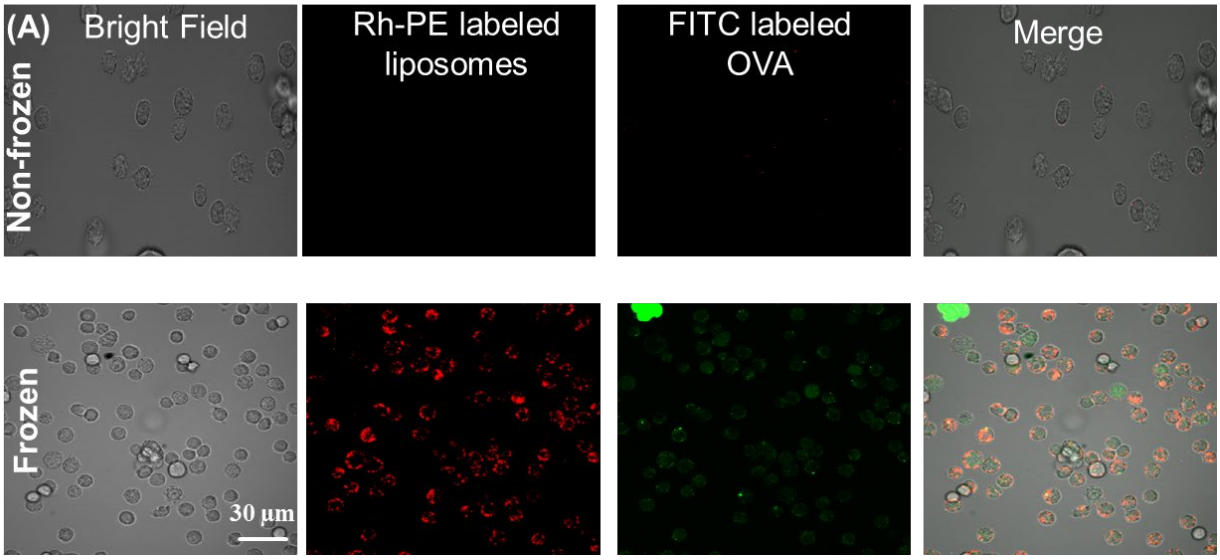
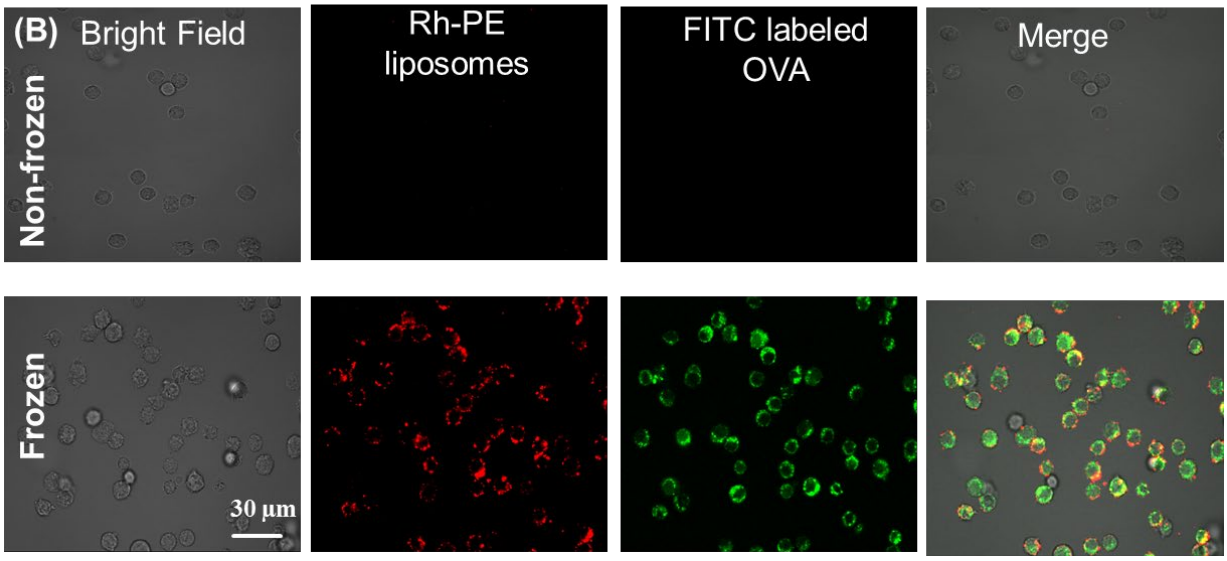



**Figure 1.** Schematic illustration of the expected mechanism of cross-presentation of pH sensitive polyampholyte-modified liposomes for immunotherapy. The polyampholyte-modified liposomes are efficiently internalized through the endocytic pathway after freeze-concentration and then fuse with the endosomes. The pH-sensitive liposomes can escape from the endosomes and release their antigenic protein cargo into the cytoplasm of the cells where they are processed by the proteasome. Cross presentation results in antigen presentation via MHC class I molecules. Any liposomes that do not escape from the endosome are trafficked through the lysosome and peptides derived from the protein cargo are presented via MHC class II molecules.

Unmodified Liposomes




Polyampholyte modified liposomes



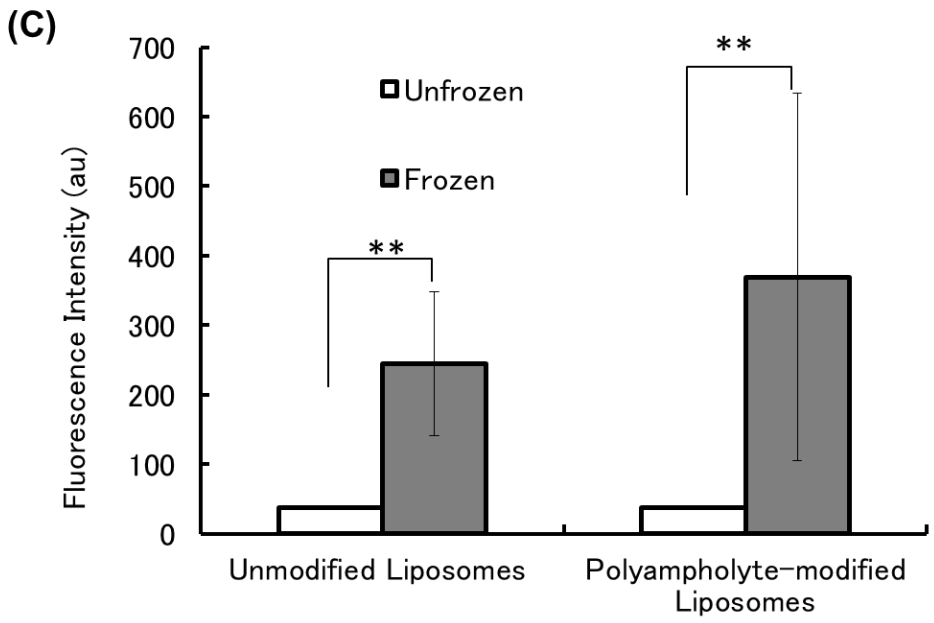
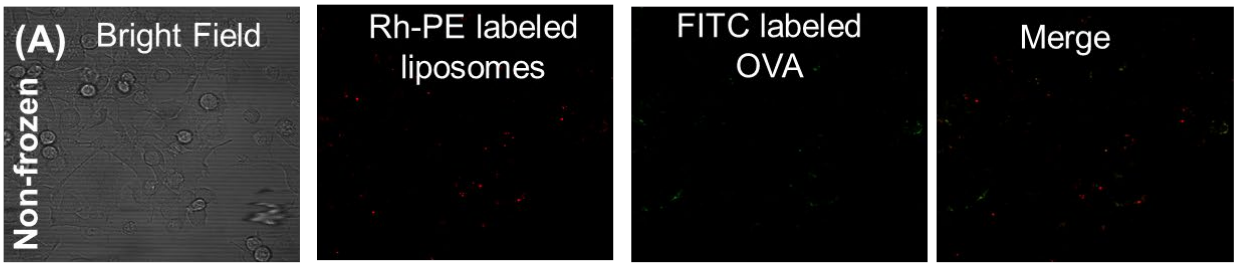


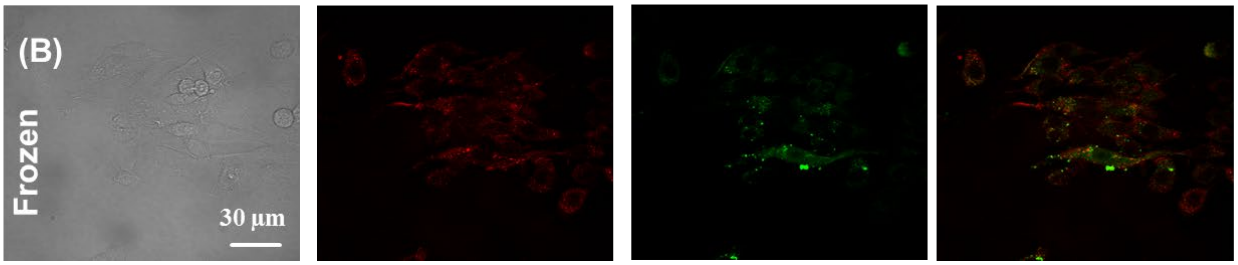

**Figure 2.** RAW 264.7 macrophage cells were cryopreserved using 10% PLL-SA in the presence of unmodified or polyampholyte-modified liposomes at -80°C for 24 h. Liposomes were labeled with 0.5 mol% Rh-PE and the protein cargo (OVA) was FITC-labeled. For the non-frozen samples, unmodified and polyampholyte-modified liposomes were added to cells directly and incubated for 24 h. (A) Unmodified Liposomes (B) Polyampholyte-modified Liposomes. Scale bar: 30 µm (C) Quantification of mean fluorescence intensity obtained from confocal microscopy. Data are expressed as the mean ±SD. **P < 0.01.

**Unmodified Liposomes**

Non-frozen

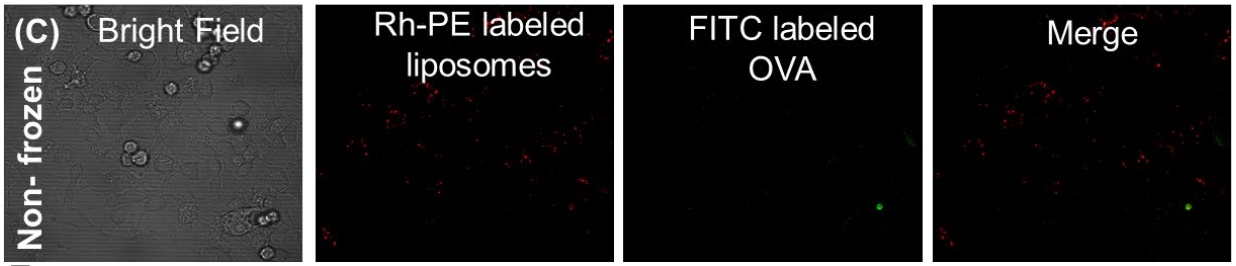



Frozen

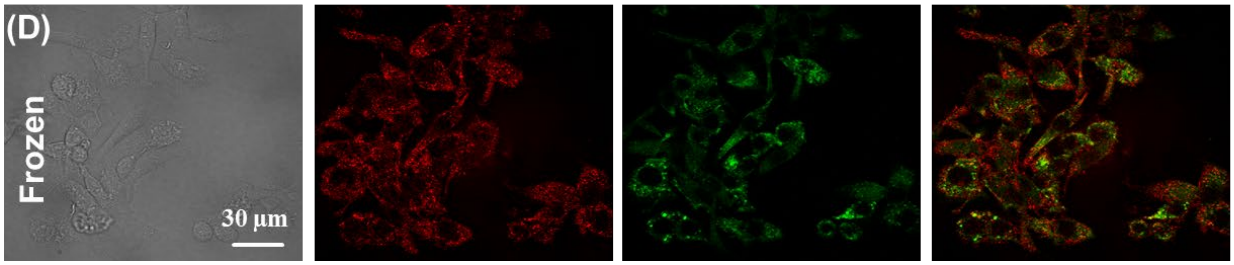



**Polyampholyte-modified Liposomes**

Non-frozen




Frozen



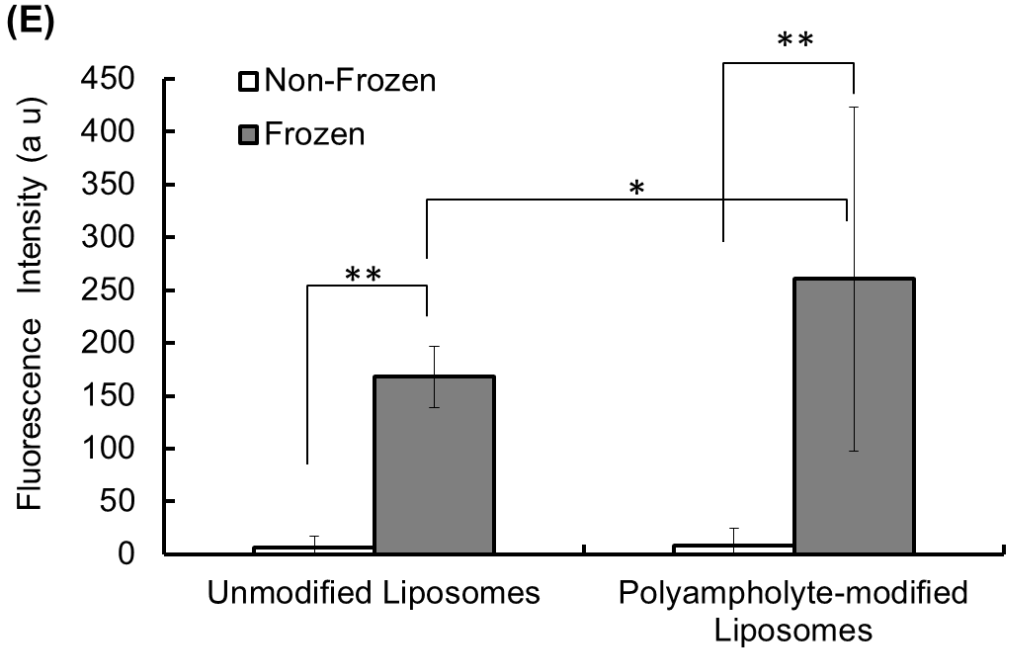



**Figure 3.** Confocal microscopy images showing internalization of OVA in RAW 264.7 cells. (A, C) without freeze-concentration of OVA-encapsulated unmodified and polyampholyte-modified liposomes; (B, D) with freeze-concentration of OVA-encapsulated unmodified and polyampholyte-modified liposomes. Scale bars: 30 μm. (E) Quantification of OVA internalization by fluorescence confocal microscopy in non-frozen and frozen liposomes. Data are expressed as mean ± SD. **P < 0.01, *P < 0.05

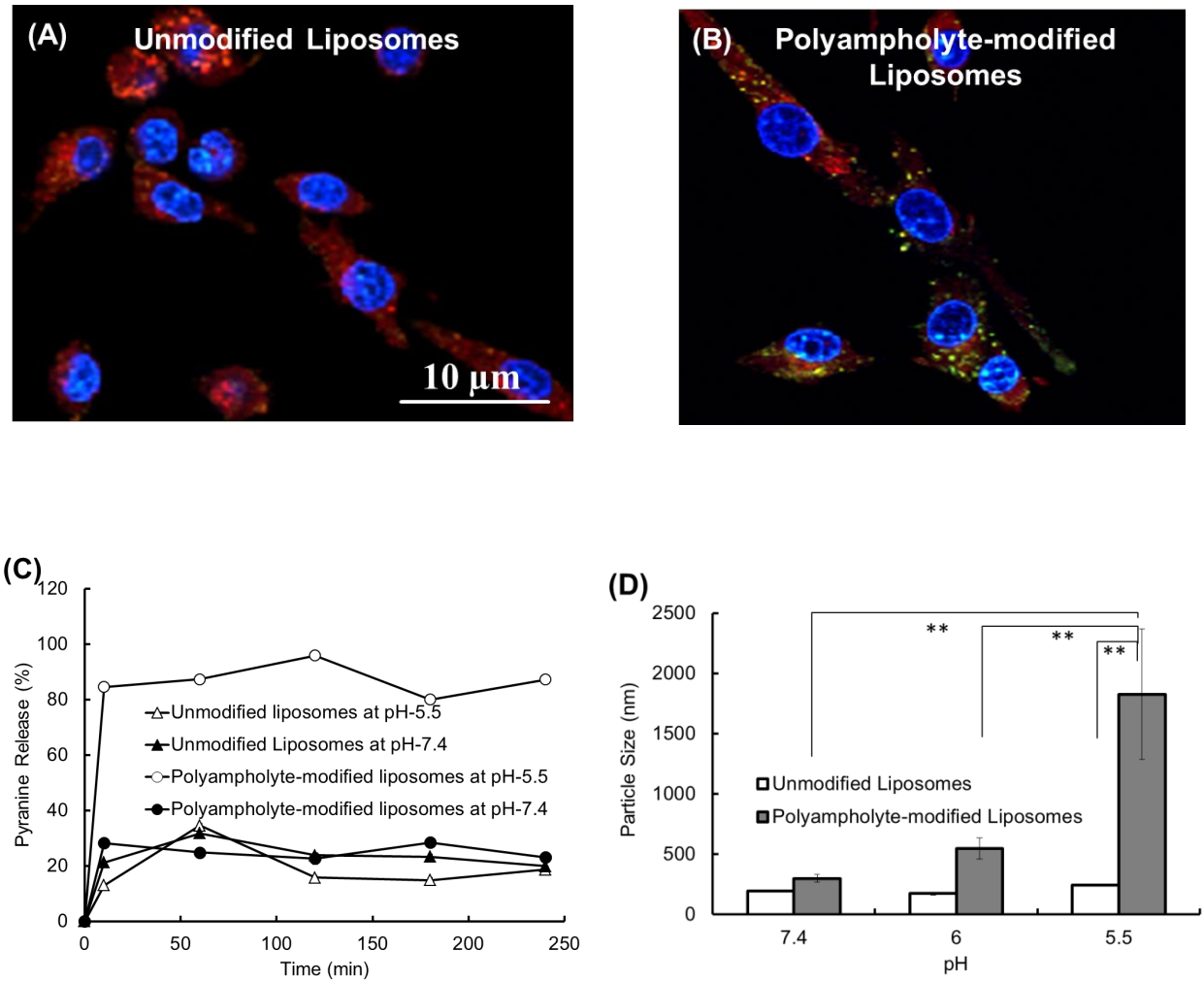
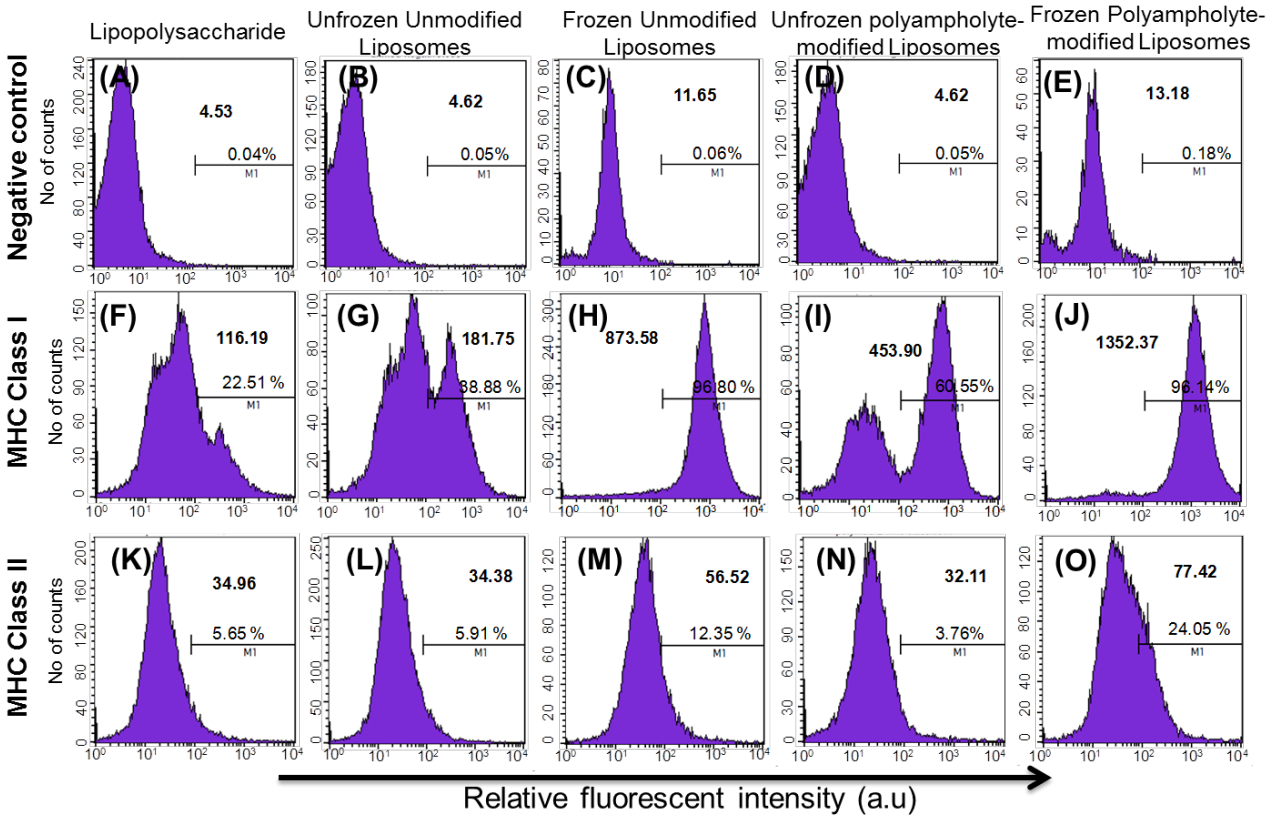
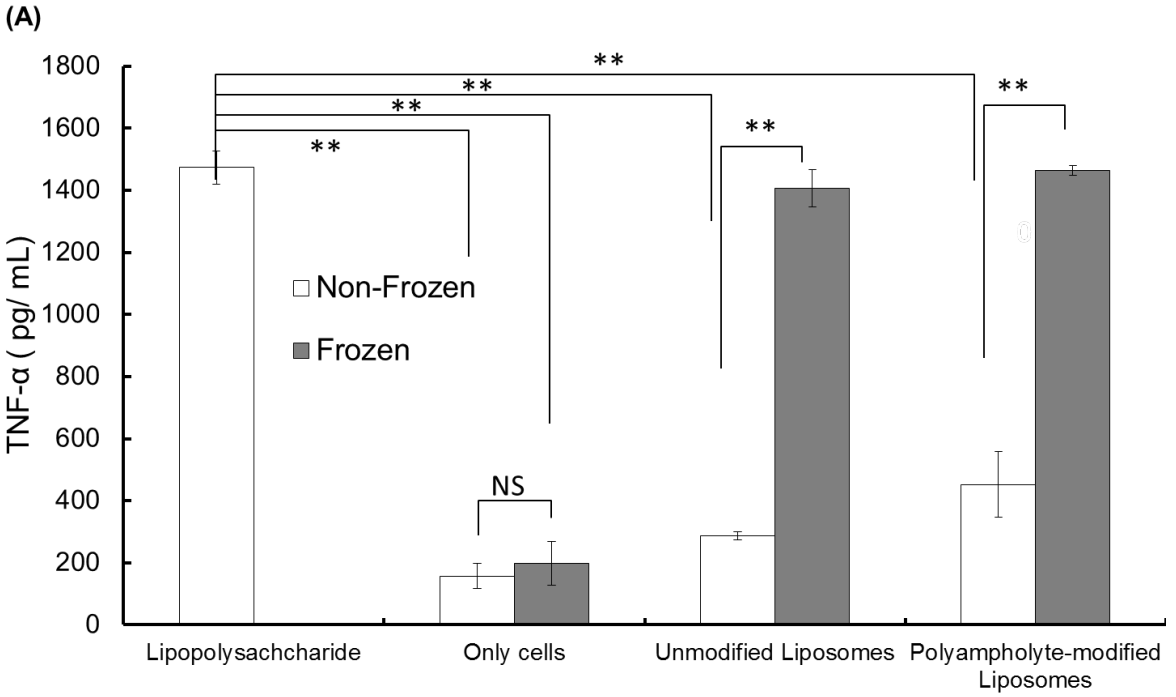


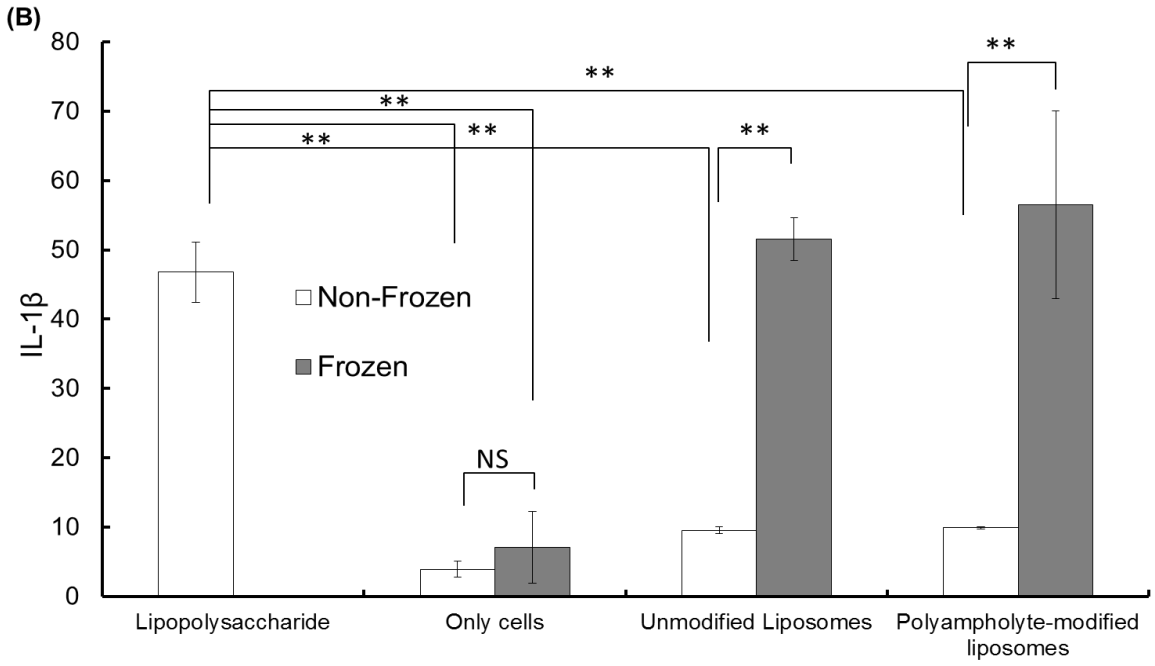

**Figure 4.** Endosomal escape of OVA protein in RAW264.7 cells. RAW264.7 cells ($1x10^6$ cells/mL) were cryopreserved with the polymeric cryoprotectant PLL-SA and OVA protein encapsulated liposomes at -80°C. The cells were thawed and then seeded for 24 h at 37°C. The late endosomes and nuclei were then stained using LysoTracker Red and Hoechst blue 33342 respectively. (A) Unmodified Liposomes (B) Polyampholyte-modified Liposomes. Scale bar: 10 µm. (C) pH- sensitive release of liposome contents. Time course of pyranine release from unmodified liposomes (triangles) and polyampholyte-modified liposomes (circles) at pH 5.5 (open) and pH-7.4 (closed). (D) Particle size of unmodified liposomes and polyampholyte modified liposomes at pH 5.5 and 7.4. Data are expressed as mean ±SD. **P < 0.01.

**Figure 5.** Expression of MHC class I and MHC class II molecules in RAW 264.7 macrophage




cells treated with unmodified- and polyampholyte-modified liposomes under frozen and non-frozen conditions as indicated. LPS (10 µg) was used as a positive control (A, F, K). Cells were stained with either a negative control mAb (A-E), anti MHC class-I (F-J), or anti MHC class-II (K-O). The mean fluorescence intensity is shown as a value on the right hand side of each panel. M1 represents the percentage of stained cells from the histogram. The mean fluorescence intensity for untreated RAW 264.7 cells was 4.43.


(A)
TNF-α ( pg/ mL)
1800
1600
1400
1200
1000
800
600
400
200
0
**
**
**
**
**
**
NS
Non-Frozen
Frozen
Lipopolysachcharide
Only cells
Unmodified Liposomes
Polyampholyte-modified Liposomes


(B)
IL-1β
80
70
60
50
40
30
20
10
0
**
**
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NS
Non-Frozen
Frozen
Lipopolysaccharide
Only cells
Unmodified Liposomes
Polyampholyte-modified liposomes

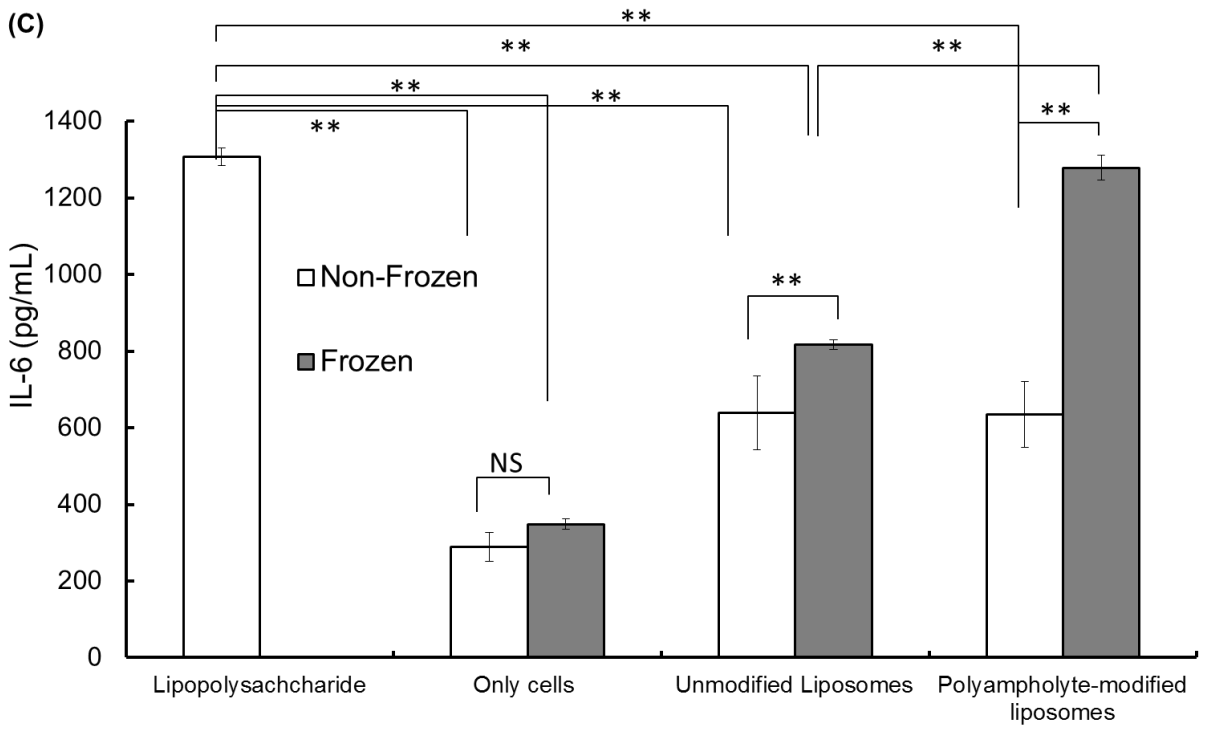



**Figure 6.** Cytokine secretion in RAW 264.7 macrophage cells after 48 h. Cells were treated with OVA-encapsulated unmodified or polyampholyte-modified liposomes. As a positive control, RAW 264.7 macrophage cells were stimulated with LPS. The cell culture supernatant from non-frozen or frozen cells was collected, and the concentration of individual cytokines was measured by ELISA. (A) TNF-α (B) IL-1β (C) IL-6. The experiments were performed in triplicate. Data are expressed as mean ±SD. **P < 0.01. NS: not significant.

**Table 1.** Zeta potential and particle size of unmodified and polyampholyte-modified liposomes. All data are expressed as means ± standard deviation (SD). All experiments were conducted in triplicate.

| Samples | Zeta Potential (mV) | Particles Size (nm) |
|---|---|---|
| Unmodified Liposomes | -5.14 ± 3.1 | 279.4 ± 38.0 |
| Polyampholyte-modified liposomes | -18.43 ± 1.3 | 305.0 ± 71.8 |

A **Freeze-concentration** approach is presented for effective immunotherapy. Antigen internalization by cells is enhanced using a straightforward freezing technique. Moreover, a pH-sensitive polyampholyte-modified liposome was developed that enhances the cytoplasmic delivery of antigen when combined with the freeze-concentration method. The enhanced expression of MHC class I seen following the combination of freeze-concentration and polyampholyte-modified liposomes might be of benefit in immunotherapy.

**Keyword** freeze-concentration, immunotherapy, polyampholyte, liposomes

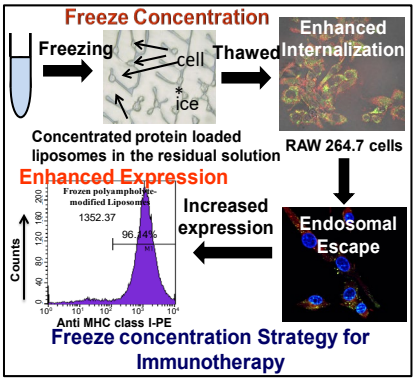
**Authors**

*Sana Ahmed, Satoshi Fujita and Kazuaki Matsumura**

**A Freeze-Concentration and Polyampholyte-Modified Liposome-Based Antigen**

**Delivery System for Effective Immunotherapy**

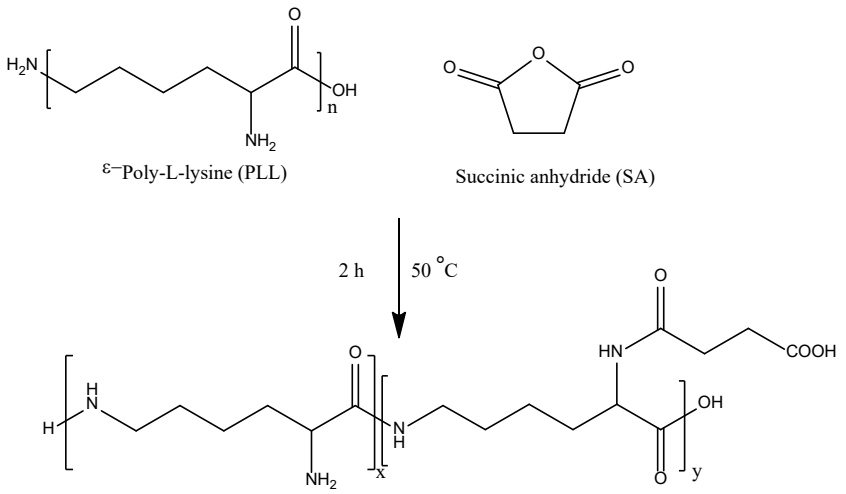
**ToC figure**



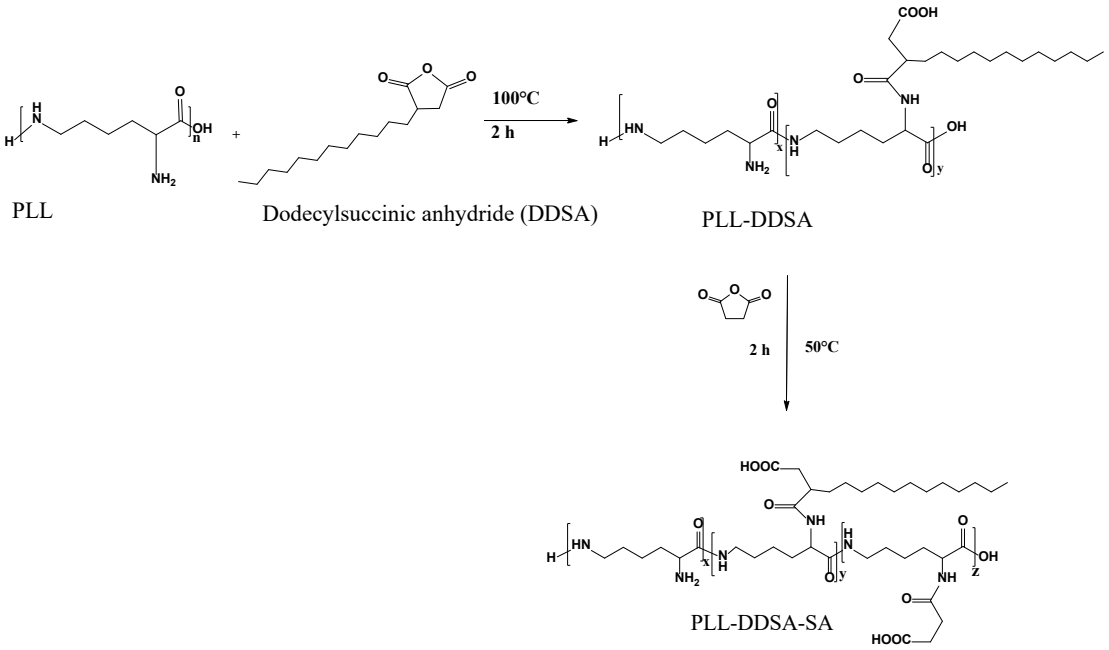
# Supporting Information

## A Freeze Concentration and Polyampholyte-Modified Liposome-Based Antigen Delivery System for Effective Immunotherapy

*Sana Ahmed, Satoshi Fujita and Kazuaki Matsumura**

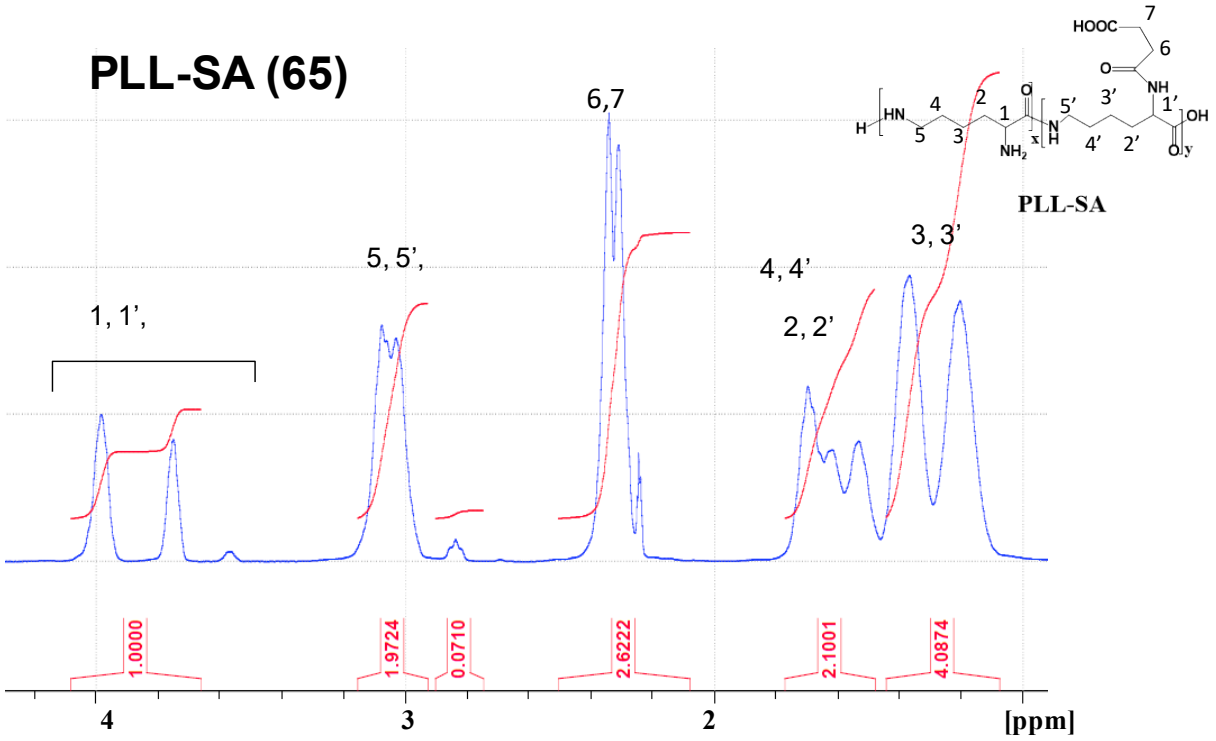



**Scheme S1**. Preparation of polyampholyte cryoprotectant PLL-SA




**Scheme S2**. Synthetic scheme for the preparation of hydrophobic polyampholytes (PLL-DDSA-SA)

(A)




(B)

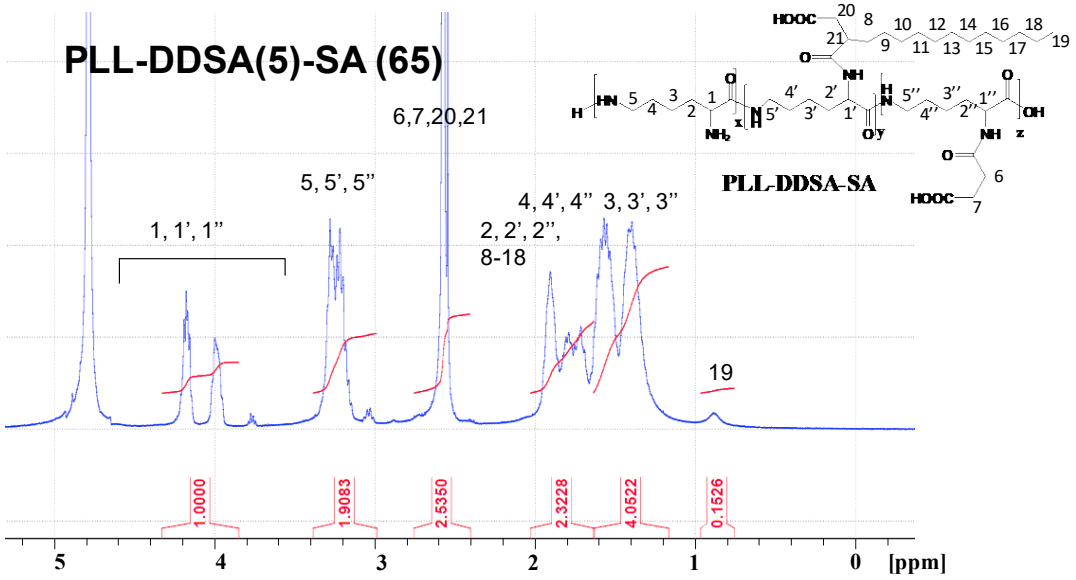



**Figure S1.** (A) $^{1}$H NMR of PLL-SA (B) $^{1}$H NMR of PLL-DDSA-SA

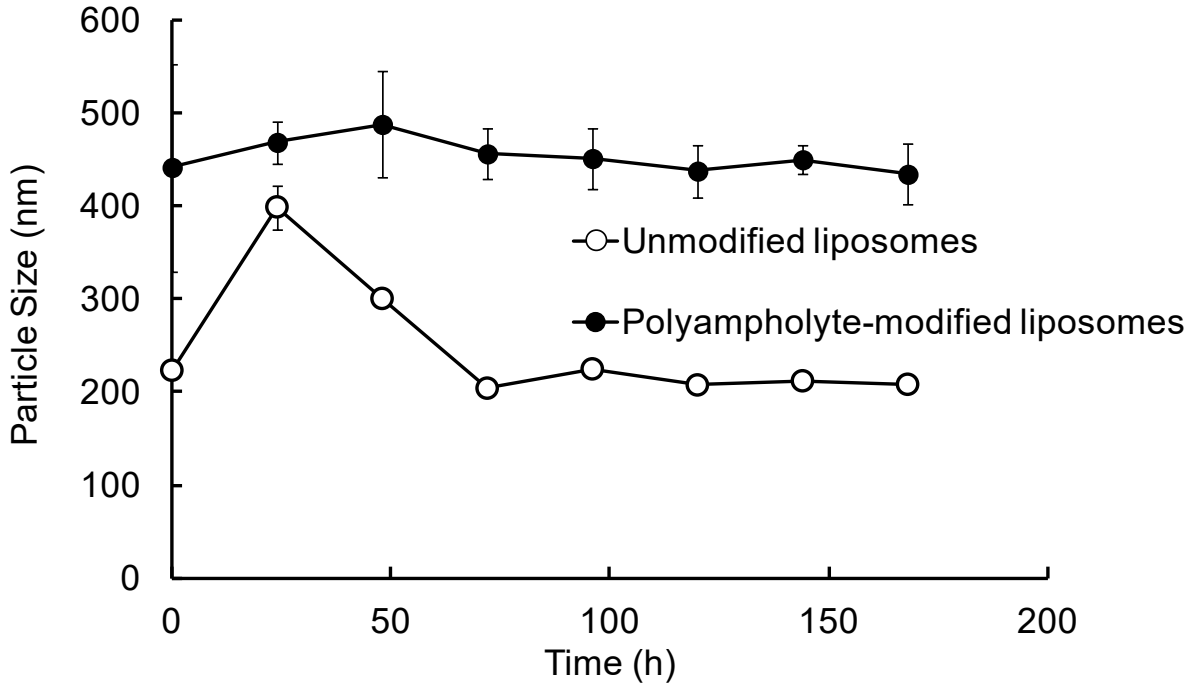



**Figure S2**. Particle size stability of OVA-encapsulated unmodified and polyampholyte-modified liposomes over time at 25°C. Data are expressed as mean ± SD.

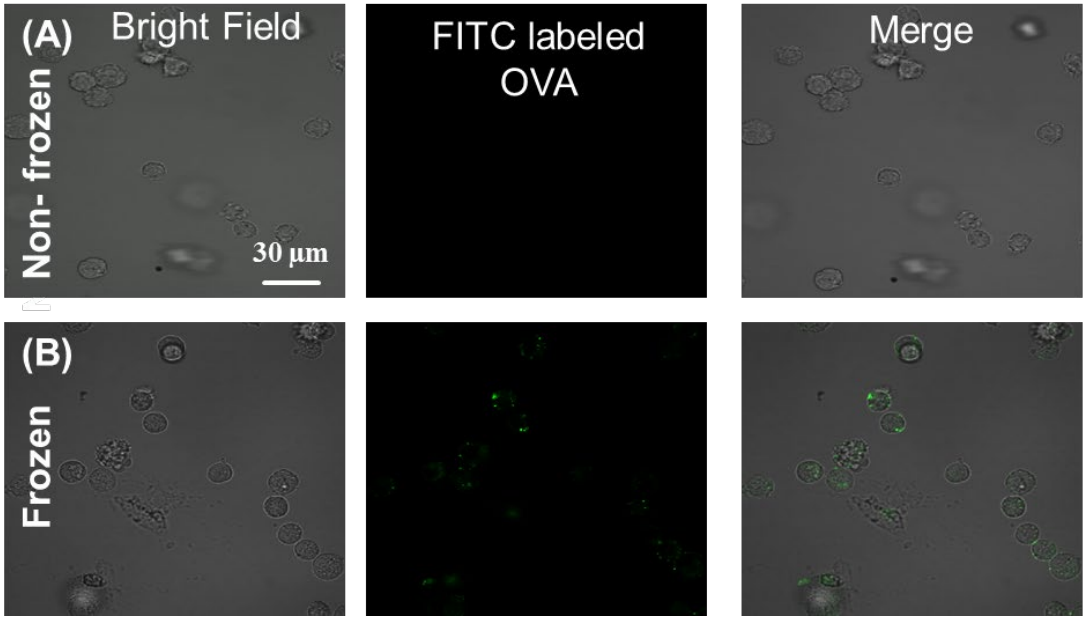



**Figure S3**. RAW 264.7 macrophage cells were cryopreserved using 10% PLL-SA in the presence of FITC-labeled OVA protein at -80°C for 24 h. (A) Non-frozen (B) Frozen. Scale bar: 30 µm

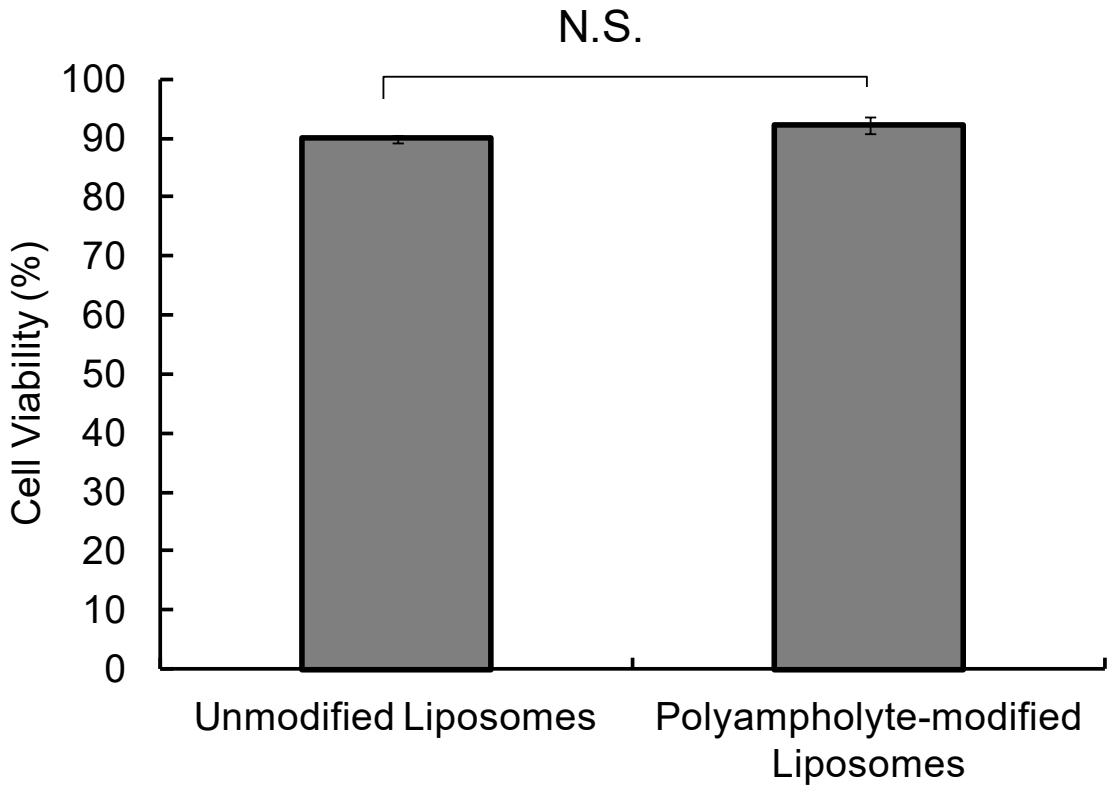



**Figure S4**. Cell viability of unmodified and polyampholyte-modified liposomes after storage at -80°C for 24 h in the presence of cryoprotectant. Data are expressed as the mean ±SD. NS: not significant.

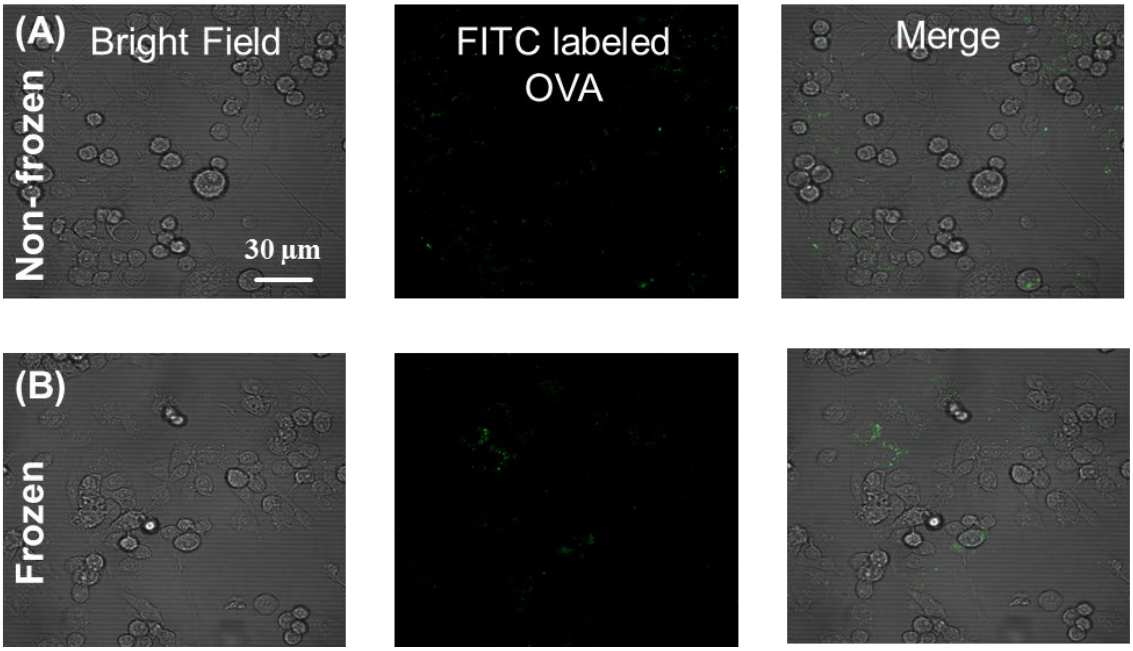



**Figure S5**. Confocal microscopy images showing internalization of OVA in RAW 264.7 cells after 24 h. (A) Non-frozen (B) Frozen. Scale bar: 30 µm

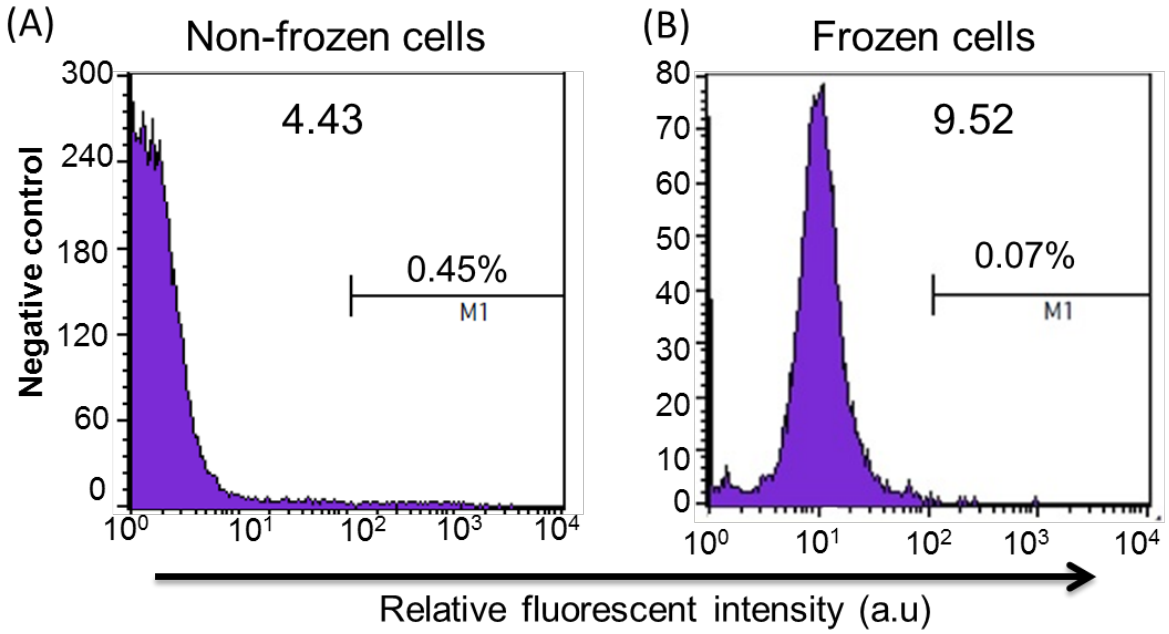



**Figure S6**. Flow cytometry analysis of unfrozen and frozen RAW 264.7 macrophage cells. The cells were unstained with antibody marker (mab). The mean fluorescence intensity is shown as a value on the right hand side of each panel. M1 represents the percentage of stained cells from the histogram. (A) Non-frozen (B) Frozen