

Coordinating Poly(I:C) and Aluminum within Liposomal Vesicles for Enhanced Innate Immune Activation

Ning Zhong

Tabor Academy, Marion, MA, United States

nzhong25@taboracademy.org

Abstract. Poly(I:C) is a double-stranded RNA formed by two complementary ribonucleic acid chains: inosine and cytidine. It is a synthetic analog RNA that mimics the viral genome. Poly(I:C) can activate downstream signal pathways to trigger interferon (IFNs) production after it is uptaken by antigen presenting cells and recognized by the Toll-like receptors (TLRs) located in the endosomal system. The productions of interferon have the functions such as enhancing antigen presentation and immune modulation, and stimulating innate immune responses. However, the delivery of Poly(I:C) into tumors is hampered by RNases present in physiological conditions, which leads to enzymatic degradation. To address the delivery challenge, we encapsulated and stabilized the Poly(I:C) into liposomes, which are lipid-bilayer nanoparticles that enclose and carry drugs inside the core, using the coordination of metals and phosphate on ribose. By using aluminum as the coordination metal, an adjuvant often used with vaccines to enhance immunity, we found that Poly(I:C) synergized with coordinating aluminum to activate macrophages and increase the production of interferons. The results proved that the coordinating liposomes Al-Poly(I:C) allowed for evasion from enzymatic degradation and enhancement of innate immune response.

Keywords: Immunology, Cancer, Poly(I:C), Liposome

1. Introduction

Immunotherapy is a form of biotherapy that boosts the immune system to fight against cancer cells. Immunotherapy has the advantage of high specificity, fewer side effects, and wide adaptability, enabling control of multiple types of tumors (1). Immunotherapy involves the use of engineered immune cells, monoclonal antibodies, and/or immune modulators to activate suppressed immune system by tumor cells. Immunotherapy against cancer has been evolving, and new therapeutic agents are actively being pursued in modern medicine. Immune agonists are potential agents for stimulating the immune system in the fight against cancer, as they activate the innate immune system upon recognition by pattern recognition receptors within antigen-presenting cells.. Immune agonists have shown great potential in a series of clinical investigations (2).

Poly(I:C) is one of the most promising immune agonists, a double-stranded RNA formed by two complementary ribonucleic acid chains: inosine and cytidine. It is a synthetic analog of RNA that mimics the viral genome. Upon uptake by immune cells, it can be recognized by the Toll-like receptors (TLRs) located in the endosomal system, leading to activation of the pathways of the toll/interleukin-1 receptor/resistance protein domain (TRIF). Activation of TRIF-dependent signaling pathway will then induce production of IFN- β along with polarization of MSC into MSC1. The TRIF domains are widely distributed in living organisms, including plants, animals, and bacteria: they play a role in innate immune pathways in plants and animals, and some interfere with the host of innate immune pathways in bacteria. This activation can lead to interferon productions (IFNs), which have the function of enhancing antigen presentation and immune modulation, stimulating innate immune responses (3). However, the delivery of Poly(I:C) is hampered by RNase present in physiological conditions, requiring an appropriate carrier for protection from enzymatic degradation. We aim to encapsulate the Poly(I:C) into liposomes using coordination chemistry to solve this problem, specifically the coordination of aluminum and phosphate on ribose. Aluminum adjuvant is often used in vaccines for enhancing immunity, and we hypothesized that it is able to boost Poly(I:C)'s ability to

activate immunity. Therefore, we used it as the coordination cation to synthesize liposomes, which are lipid-bilayered nanoparticles that enclose and carry drugs inside the core (4).

Liposomes are lipid bilayer vesicles that allow for protection of payload, prolongation of circulation, and enhancement of accumulation. Hence, we speculated that liposomes could protect the delivery of PolyIC from being degraded by RNase, as the liposome structure can encircle PolyIC inside and isolate it from the surrounding environment. Consequently, encapsulation of PolyIC into liposomes can increase the efficiency of PolyIC in immunotherapy. In addition, aluminum has been reported to enhance immunotherapy via unknown reasons. Therefore, we also speculate that aluminum can enable coordination with phosphate groups on ribonucleic acids and enhance immune activation, such as adjuvant, thus enhancing the efficiency of PolyIC. To synthesize coordination liposomes, we first coordinated PolyIC with aluminum in a reverse emulsion. Then, we coated helper lipids onto the bare nanoparticles to assemble them into a bilayered liposome. The liposome is formed by biocompatible phospholipids and cholesterol using nanotechnology, which enables the physical isolation of PolyIC from RNase and clearance. PolyIC-mediated immune activation first requires immune cells internalizing PolyIC into their endosomal systems. Then, PolyIC liposome will dissociate in response to the acidic environment in the late endosome and release free PolyIC for TLR3 binding, which locates in the inner membrane of endosomes in innate immune cells. Ultimately, the subsequent signal pathways can be activated to initiate interferon production. The IFNs produced can boost the presentation of antigens. The importance of our research is to solve the challenges of PolyIC delivery and confirm whether aluminum coordination can enhance the efficacy of PolyIC, inspiring new treatment strategies for multiple types of cancer (5).

2. Materials and Methods

2.1. Synthesis of liposome with coordination chemistry

To synthesize PolyIC-liposome, we added an aqueous solution of PolyIC (2 mg, 10 mg/mL) to a 5 mL of 0.3 M Triton X-100/1.5 M 1-hexanol in cyclohexane, and then stirred it vigorously for 15 minutes in the presence of 4 mg (200 mg/mL in CHCl_3) DOPA (1,2-dioleoyl-sn-glycero-3-phosphate). Next, we added an aqueous solution of 20 mg (100 mg/mL) $\text{Al}(\text{NO}_3)_3$ to a 5 mL of 0.3 M Triton X-100/1.5 M 1-hexanol in cyclohexane, and stirred it vigorously for 5 minutes. The $\text{Al}(\text{NO}_3)_3$ -containing microemulsion was then added dropwise into the PolyIC-containing microemulsion, and we stirred it vigorously for 30 minutes at room temperature. Afterwards, we added 10 mL ethanol and precipitated PolyIC-bare after centrifugation at 14,000 rpm. The resulting pellet was then washed twice with THF/ethanol and finally resuspended in THF. Subsequently, we added a THF solution (80 μL) of PolyIC-bare, cholesterol DOPC, DSPE-PEG2k (1:0.4:0.8:1.5 by mass) to 500 μL of 30% ethanol/water at room temperature to obtain liposome by self-assembly. This mixture was stirred for 20 minutes, and THF/ethanol was then be completely evaporated under nitrogen.

For characterization of PolyIC-liposome, we dropped PolyIC-liposome and control liposome onto copper grids and imaged the nanoparticle with transmission electron microscopy. The hydrodynamic radius was measured with dynamic light scattering.

2.2. Cell culture

To use PolyIC-liposome for immunotherapy, we started the cell culture by thawing RAW 264.7 cells and growing RAW 264.7 using RPMI-1640 complete medium (supplemented with 10% Fetal Bovine Serum, 1% penicillin and streptomycin). We grew the cells in 37°C incubator and monitored their confluency. When the cell confluency reached 70-80%, we passaged the cells using 0.25% of EDTA-trypsin. Briefly, we trypsinized RAW 264.7 cells at 37°C for 3 minutes and stopped digestion with complete medium containing serum. Then, we collected the cells by centrifugating them and resuspending them in fresh medium.

2.3. Confocal laser scanning microscopy

For Laser Scanning Confocal Microscopy, we first seeded 200,000 cells/mL in a 35 mm² glass-bottom dish and cultured them overnight for cell adhesion. Then, we removed the medium and replaced it with medium containing fluorescently labelled (Ce6) liposomal PolyIC, and cultured for another 24 hours to allow for endocytosis uptake. To visualize the cellular uptake, we removed the medium, washed with ice-cold PBS, and stained them with Hoechst 33342 on ice for 15 minutes. Afterwards, we visualized the cells using confocal microscopy (bright field, nuclei/DAPI, Alexa Fluor 488).

2.4. ELISA of IFN- β production

We started by trypsinizing the RAW 264.7 cells at 37°C for 3 minutes, then centrifuged the cells down and counted them using a hemocytometer. Next, we seeded 50,000 cells/well into a 6-well plate and cultured them overnight for cell adhesion. After the adhesion, we removed the medium and replenished it with drug-containing medium, then cultured the cell for another 24 hours. Afterwards, we collected the medium supernatant containing secreted IFN- β and measured the concentration of IFN- β using a Mouse IFN-beta ELISA kit following the product instructions.

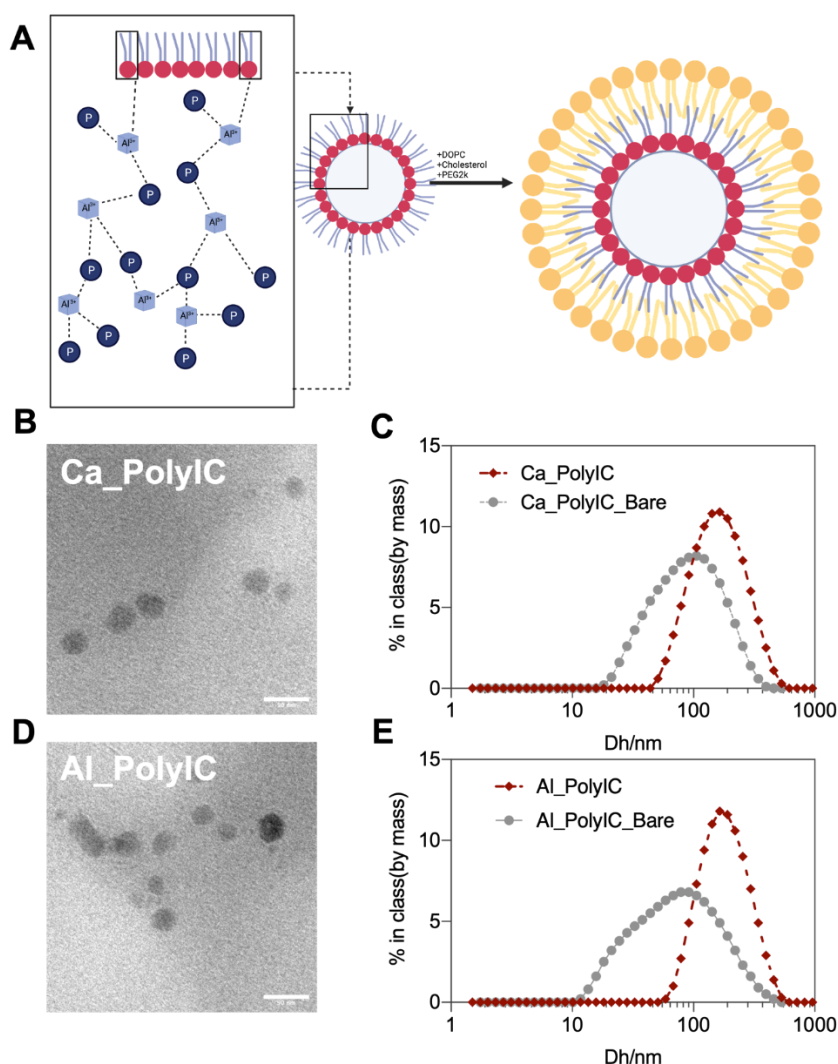


Figure 1. Characterization of coordination liposomes. (A) Synthesis of Ca_polyIC and Al_polyIC includes two-step: coordination and self-assembly. (B) TEM micrograph of Ca_PolyIC; (C) Dynamic Light Scattering of Ca_PolyIC and Ca_PolyIC without lipid coating; (D) TEM micrograph of Al_PolyIC; (E) Dynamic Light Scattering of Al_PolyIC and Al_PolyIC without lipid coating. Scale bar, 50nm.

Table 1: Z-average and PDI of coordination liposomes

	Z-average	PDI
Al_Bare	55.71nm	0.295
Al_PolyIC	140.7nm	0.196
Ca_Bare	66.17nm	0.391
Ca_PolyIC	173.3 nm	0.242

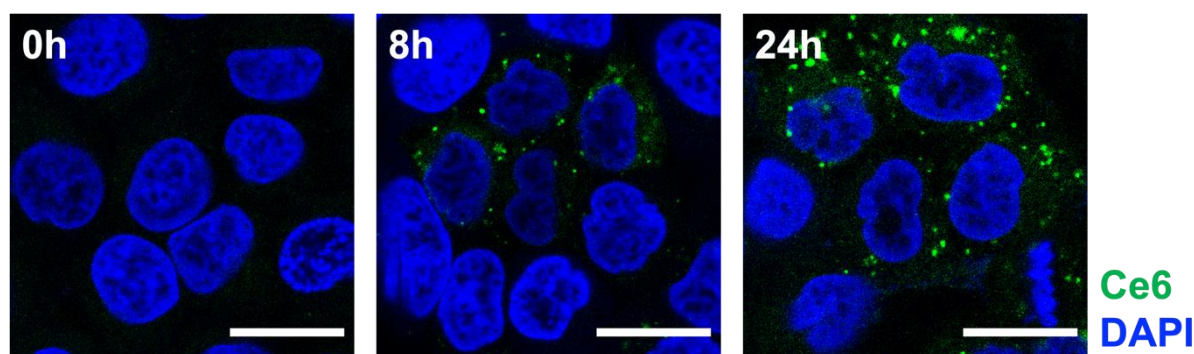


Figure 2. Confocal laser scanning microscopy figures of uptake of Al_PolyIC by Raw 264.7 cells. The uptake of nanoparticles increases over 24-hour period.

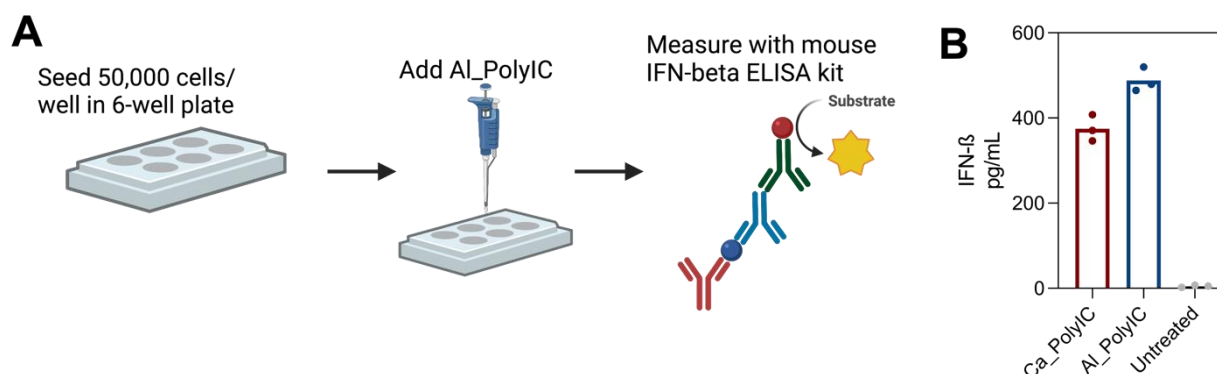


Figure 3. IFN- β production by liposomal PolyIC and improved immune response mediated by Aluminum coordination. (A) Experiment design; (B) IFN- β production induced by different coordination liposomes in Raw 264.7 cells.

3. Results

3.1. Characterization of coordination liposomes

The synthesized liposomes contain a metallic core formulated with PolyIC and biocompatible metals, and a bi-layered lipid membrane, as shown in Fig. 1A. To visualize their morphology, we imaged the nanoparticles with transmission electron microscopy (TEM). We found that the liposomes were monodispersed with spherical structures for Al_PolyIC and Ca_PolyIC (Fig. 1B & 1D). Because of the low electron density of the lipid bilayer, only the electron-dense core is visible under TEM. The TEM images of Al_PolyIC and Ca_PolyIC confirmed the successful formation of coordination liposomes by presenting the spherical self-assembly.(6)

To quantitatively evaluate the size of the formulated nanoparticles, we measured their hydrodynamic diameter using Dynamic Light Scattering. The size distributions were shown in Fig. 1C and 1E. We found that before membrane coating, the bare particles have average hydrodynamic diameters of 55.71 nm for Al_bare and 66.17nm for Ca_bare, as shown in Table 1. Unsurprisingly,

the hydrodynamic sizes of Al_PolyIC and Ca_PolyIC increased to 140.7nm and 173.3 nm after coating with DOPC and DSPE-PEG. This increase was resulted from both lipid chain length and poly-ethylene glycol. The PDI, which reflects molecular mass distribution and dispersion, is 0.196 for Al_PolyIC and 0.242 for Ca_PolyIC. Usually, an acceptable PDI should have the value below 0.7 (7). For drug delivery applications, which the liposomes in this study were used for, a PDI of 0.3 and below is considered favorable (8).

3.2. Internalization of Al_PolyIC by RAW 264.7

After successfully synthesized the coordination liposomes, we wanted to confirm whether they were able to be internalized by immune cells to activate Toll-like receptors located within the endosomal system. Hence, we used confocal laser scanning microscopy to confirm the internalization of Al_PolyIC by RAW 264.7 cells. In order to trigger an innate immune response with PolyIC encapsulated in liposomes, the nanoparticles must first be internalized by antigen-presenting cells to enter the endosomal system. Upon uptake by cells, PolyIC liposomes can then be dissociated by the acidity of late endosomes, releasing free PolyIC to bind with Toll-like receptors to initiate interferon production. According to the confocal laser scanning microscopy figures, uptake by immune cells gradually increased within the 24-hour span, as shown by the green color representing the internalized ce6-stained Al_PolyIC liposomes. We confirmed the successful delivery of Al_PolyIC liposomes into immune cells, allowing PolyIC to be recognized by pattern recognition receptors. These results also demonstrated our speculation that liposomes could protect the delivery of PolyIC from being degraded by RNase because of their protective supermolecule structure.

3.3. Al_PolyIC induces interferon production

After confirming that the Al_PolyIC liposomes were internalized by antigen-presenting cells, the Toll-like receptors located on the inner membrane of the immune cells would activate the interferon production for immunity. Interferon in immune cells can increase the production of cytokines and chemokines, thereby enhancing the innate immune response. According to the ELISA data of IFN- β production, Al_PolyIC produced 519.846 pg/ml, 464.999 pg/ml, and 479.307 pg/ml IFN- β , while Ca_PolyIC had 407.898 pg/ml, 346.361 pg/ml, and 370.566 pg/ml IFN- β produced. The untreated control group had undetectable production for all three replicates of data. Al_PolyIC triggered a much larger production of interferon compared with Ca_PolyIC, and both were much more effective in triggering IFN- β production for innate immune reaction than untreated PolyIC. This result demonstrated an effective protection for delivery of PolyIC from being degraded by RNase. It also proved aluminum's ability as an adjuvant in enhancing immune activation. The data proves our speculation regarding aluminum's ability to coordinate with phosphate groups on ribonucleic acids and enhance immune activation as an adjuvant.

4. Conclusion and Discussion

Because PolyIC's ability to trigger innate immune cells can be hampered by RNase dissolution, we synthesized liposomes using coordination chemistry as a nanocarrier for PolyIC. Both Al_PolyIC and Ca_PolyIC synthesized had spherical morphologies and high monodispersity. In vitro tests of the nanoparticles confirmed their internalization by RAW 264.7 cells. After the particles are introduced into cell bodies, they induced IFN- β production. Both Al_PolyIC and Ca_PolyIC were proven to have the ability to activate IFN- β production, while Al_PolyIC had a higher amount of interferon produced. The results overall have demonstrated that liposomes could be effective for protecting PolyIC against RNase dissolution, and aluminum could enhance immune activation.

The significance of double-stranded RNA in activation of immunity has been actively investigated. PolyIC is a synthetic dsRNA, an agonist of TLR3, which mimics natural dsRNA and is often used as an adjuvant. PolyIC serving as an adjuvant could result in the maturation of antigen-existing cells, induction of type I IFNs (IFN- α/β), and lead to the activation of innate immune reaction (9). However,

the delivery of PolyIC is particularly challenging because RNA could be sensitive and vulnerable in systemic circulation. RNase could diffuse PolyIC before it is delivered, and quick clearance of PolyIC could happen after it is delivered.

On the other hand, aluminum adjuvant has been used in vaccines to induce immune responses that are mainly antibody-mediated, but its mechanism has seldomly been investigated. Nowadays, the idea of innate immune systems directing and enhancing adaptive immune responses is generally accepted. Therefore, aluminum should be able to potentiate the PolyIC induced immune responses. However, the systemic exposure of both PolyIC and aluminum could lead to systemic toxicity. To protect them, we intend to synthesize liposomes using nanotechnology and coordination chemistry, which will also ensure the co-delivery of aluminum and PolyIC for cancer immunotherapy. We also speculate that this liposome-based system can enhance innate immunity.

The significance of this research is to address the challenges that arise in the delivery of PolyIC and the potential neurotoxicity associated with aluminum exposure in the human body using liposomes. Additionally, we to study aluminum's potential in enhancing PolyIC's ability of innate immunity induction.

There are still many limitations and areas for improvement in our research. Firstly, we did not optimize the ratio for aluminum and PolyIC, which can affect their potential synergistic effect on immune activation. To fully apply this coordinated liposomal vesicle in clinical use, understanding the optimal dose ratio is important. Second, similar tests should be conducted with human cell lines. In this research, we only performed *in vitro* tests for the liposomal vesicles in RAW 264.7 cells, which is a macrophage cell line established from a tumor in a male mouse. Third, *in vivo* tests are necessary for future studies, such as primary models of anti-cancer efficacy experiments and/or patient-derived xenografts on rodents. Finally, to test the delivery efficiency of PolyIC particles, we should study the pharmacokinetics and pharmacodynamics, which refers to the time course relationship between drug concentration and the action of the drug on organisms (10). Our goal is to increase the drug exposure in the organism while lowering the systemic toxicity. To prove the successful isolation of PolyIC using liposomes, we would need pharmacodynamics data to show the systemic toxicity and efficiency of drug in organisms.

In conclusion, our research has made significant progress in addressing the challenges of delivering PolyIC and the potential neurotoxicity associated with aluminum exposure in the human body. Although there are limitations, our results have demonstrated the potential of liposomes as a nanocarrier for PolyIC, as well as the ability of aluminum to enhance PolyIC's ability to induce innate immunity. Our findings pave the way for future research to improve the liposome-based delivery system for clinical use in cancer immunotherapy.

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