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Current strategies in cationic liposomal vaccine development for anti-cancer therapy

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ABSTRACT. Nanomedicine is currently at the forefront of technology. Nanovaccines are a relevant development derived from this field and comprise nanoparticles ranging from 50-250 nm to deliver antigens and other immunomodulatory agents. Their formulation can include liposomes, which are widely known as safe and allow their engineering to be cationic, conferring a superior immunostimulatory effect. This promising strategy for vaccine delivery has gained interest in cancer as it provides higher targeting efficiency, increased antigen stability, prolonged circulation time, and enhanced uptake by antigen-presenting cells, mainly dendritic cells. Therefore, this minireview discusses recent research on cationic liposome-based vaccine delivery systems for anti-cancer therapy. Pubmed, Science Direct, and Google Scholar were screened for original and review papers published in the last ten years. The antigen association with the cationic nanoparticles either by electrostatic interactions or complementary coiled coil peptide pair strategy were found as the most promising strategies. The work also highlights the potential of this therapeutic platform for enhancing the T-cell immune response against cancer through mRNA-containing formulations for different routes of administration, providing a detailed physicochemical characterization of the reported nanosystems.

Keywords: Adjuvant; antigen-presenting cells; drug delivery; nanovaccines; vaccine delivery

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INTRODUCTION

Nanovaccines are a new generation of vaccines containing nanoparticles of 50-250 nm size as carriers and/or adjuvants (Hayat & Darroudi, 2019), overcoming the known limitations of conventional subunit formulations (*e.g.*, viral vector, attenuated organisms, proteins combined with adjuvants) (Yin *et al*., 2022). These systems exhibit higher targeting efficiency, increased antigen stability, prolonged circulation time, and remarkably, an enhanced uptake by the antigen-presenting cells (APCs), mainly dendritic cells (DCs), which ultimately activate naïve $CD8^+$ and $CD4^+$ T-cells (Heuts *et al*., 2018; Das & Ali, 2021). Noteworthy, liposomes, a well-known clinically safe type of nanoparticles, have been engineered to be cationic, which confers them a superior immunostimulatory effect (*i.e.*, adjuvant role) compared to the neutral and anionic counterparts, and thus, representing a promising strategy for vaccine delivery in cancer. For instance, the positive charge facilitates the binding and uptake by APCs, enhancing antigen and drug delivery and increasing therapeutic efficacy and immune response. However, toxicity should be closely monitored due to the interaction with the negatively charged cell membrane (Du *et al*., 2017; Heuts *et al*., 2018).

Among their composition (Fig. 1), cationic liposomes contain positively charged lipids in their lipidic bilayer membrane, which are essential for the interaction with negatively charged biomolecules (*e.g.*, DNA, RNA) to form the lipoplexes (Ewert *et al*., 2021). The mentioned lipids are usually synthetic with positively charged or ionizable functional groups. For instance, DOTAP,

DOPE, DOTMA, and DC-Chol stand out as some of the most widely used lipids for this kind of liposomes (Liu *et al*., 2020). Although these nanocarriers have been extensively explored in the delivery of several active pharmaceutical ingredients (APIs) (Akombaetwa *et al*., 2023; Sun *et al*., 2023), their capacity to encapsulate antigens within the aqueous core or lipid bilayers combined with the possibility of surface decoration and functionalization have turned the interest into them for vaccine development. Furthermore, the interaction with toll-like receptors (TLRs) expressed in APCs has been demonstrated to promote the production of cytokines and chemokines, leading to the recruitment and activation of T-cells and B-cells (Ponti *et al*., 2021).

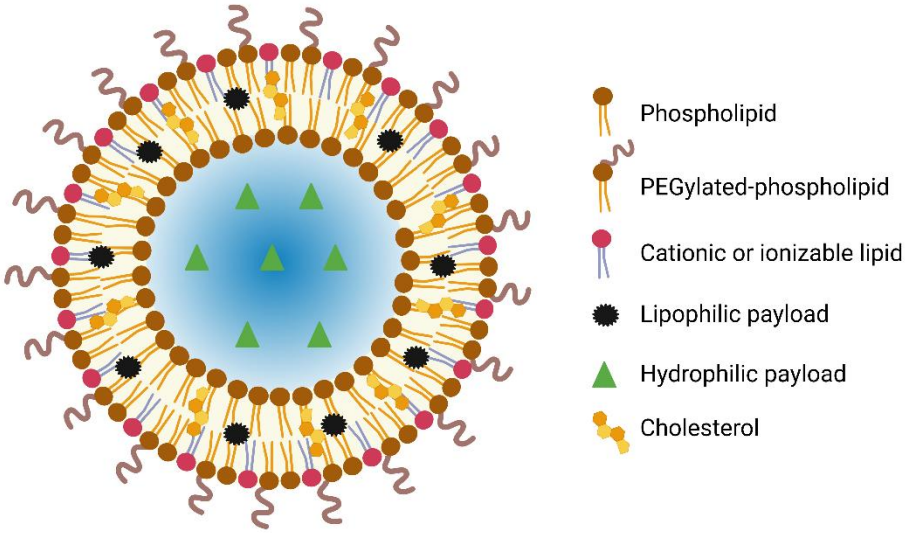

Fig. 1. Cationic liposome's basic structure (Castillo-Henríquez *et al*., 2023)

Despite liposomal vaccines jumping into the public eye due to the COVID-19 formulations developed by different manufacturers, studies have reported that the effectiveness decreases six months after complete vaccination (Feikin *et al.*, 2022). However, other approaches may rely on this platform, modifying it to target cancer cells and provide more extended immune stimulation to fight the disease. Therefore, this minireview aims to critically analyze 32 scientific papers on cationic liposome-based vaccine delivery systems for anti-cancer therapy published in the last ten years, discussing their characterization, mode of action, the uptake by DCs, and the influence on T-cell immune response (Fig. 2). To the best of our knowledge, this paper presents a unique brief overview of novel strategies with the potential to be extensively exploited in the following years, being antigen association by electrostatic interactions and the complementary coiled coil peptide pair, which can also be suitable for mRNA delivery.

MATERIALS AND METHODS

Pubmed, Science Direct, and Google Scholar were screened for original and review papers published in the last ten years using the following search strategy: Cationic AND liposomes AND vaccines, nanovaccine AND anti-cancer AND therapy. For the study selection, the publications were screened by title and abstract. Literature concerning topics other than cationic liposomes for anti-cancer vaccines or irrelevant for the scoping of this minireview were excluded, as well as duplicated papers and articles written in other languages than English.

RESULTS AND DISCUSSION

Antigen association strategies. Cationic liposomes have been explored with great interest due to the ease of incorporating antigens through different strategies. Antigen association with this type of nanocarrier through electrostatic interactions has been considered one of the most straightforward

procedures (Li *et al*., 2022b). Following this premise, the approach by Su *et al*. (2021), describes self-assembling hybrid polymer-lipid cationic liposomes utility for melanoma immunotherapy, composed of PEAD cationic polymer, egg yolk lecithin, and cholesterol (Z-ave: 86.32 ± 0.60, PDI: 0.29, ζP: ~ 40 mV). The nanovaccine comprises a combined delivery of 1-methyl-tryptophan (1-MT) and the inhibitor of indoleamine-2,3-dioxygenase (IDO) (*i.e.*, an immune checkpoint). Additionally, two anionic immunomodulators were included: An antigen made by the conjugation of D8 peptide with the SIINFEKL melanoma-specific epitope, and a potent TLR-9 agonist (CpG). The physicochemical characterization denoted a significant increase in particle size (453.0 ± 3.8), a relatively monodisperse system (PDI: 0.33), and a negative surface charge (ζP: ~ -5 mV) conferred by the anionic antigen and CpG.

Nevertheless, electrostatic approaches for liposome-based vaccine development have also resulted in low antigen loading, accelerated desorption under physiological conditions, and loss during liposome purification (Hamborg *et al*., 2014; Tretiakova & Vodovozova, 2022). Given the previous, another approach, reported by Leboux *et al*. (2021) presents an alternative method of antigen association to cationic liposomes. The innovation consists of a liposome based-strategy previously used for targeting cells (Yang *et al*., 2016; Kong *et al*., 2020), comprising a complementary coiled coil peptide pair upon the interaction of peptide K (pepK) and peptide E (pepE). The first peptide, coupled to cholesterol, was encapsulated in the lipid bilayer, whereas pepE was covalently linked to the restricted epitope of ovalbumin OVA323 (pepE-OVA323). The liposomes containing DSPC, DOTAP, and cholesterol were prepared by the dehydration-rehydration method and presented a hydrodynamic diameter after functionalization of 176.3 ± 14.4 nm, PDI of 0.071 ± 0.047, and ζP of 44.9 ± 6.2 mV.

In terms of cell studies, hybrid liposomes are usually studied using bone marrow dendritic cells (BMDCs), displaying non-toxic effects when the liposomes' concentration is below 40 $mg.mL^{-1}$ (Li *et al*., 2022a). No pathological abnormalities in major organs are found when administered subcutaneously in mice, meaning good biocompatibility (Knudsen *et al*., 2015). On the other hand, functionalized liposomes seem to present higher association efficiencies with the antigens upon simple mixing, which has led to the hypothesis of an improved uptake by BMDCs (Wang *et al*., 2016). Indeed, several works have reported an antigen uptake of almost 100% when associated with functionalized liposomes (Tada *et al*., 2015; Varypataki *et al*., 2015). Furthermore, biodistribution studies in zebrafish have supported this finding, given that a high percentage of the functionalized signal is usually co-localized with the antigen, in contrast to the low percentages provided by the association with non-functionalized liposomes (Arias-Alpizar *et al*., 2021). The latter represents the foundation for a potential translation to *in vivo* complex tissues to increase antigen presentation efficiency in cancer patients (Leboux *et al*., 2021).

Although coiled-coil constructs have been reported to enhance by 18-fold the immunogenicity of the antigen, thereby, inducing a higher $CD4^+$ T-cell activation after immunization, these vaccines lack an immune potentiating effect for $CD8^+$ T-cells when evaluated using BMDCs (Neefjes *et al*., 2011). Liposomes inability to endosomal escape implies that unbound antigen molecules are responsible for T-cell proliferation (Gao *et al*., 2017; Lee *et al*., 2022). In contrast to this anomalous outcome, hybrid liposome vaccines have shown a great delivered amount of antigen to the cytoplasm of DCs, proving the system's capability to escape from the lysosomes, and promoting a strong antigen-specific $CD8^+$ T-cell response that could kill melanoma tumor cells *in vivo* (Cebrian *et al*., 2011). Additionally, this type of formulation enables the expression of CD86 surface markers inducing BMDCs maturation and causing an increase in the recruitment of $CD8^+$ and $CD4^+$ T-cells infiltrated in the tumor (Su *et al*., 2021).

mRNA delivery. Aside from peptides and other antigens, cationic liposomes have also been designed as nanocarriers and adjuvants for the delivery of mRNA in cancer vaccines, as presented in Table 1. These formulations provide different benefits compared to peptide-based preparations such as direct mixing with the negatively charged mRNA, and simultaneous encoding of multiple antigen

sequences (Heuts *et al*., 2018). The previous is illustrated in the research performed by Zhang *et al*. (2020) and Mai *et al*. (2020) for treating lung carcinoma. The first group focused on the development of a potentially universal mRNA vaccine composed of DOTAP liposomes (Z-ave: 90.74 ± 10.21 nm, PDI: 0.213, ζP: 47.56 ± 3.45 mV) combined with a novel cholesterol-modified cation peptide (DP7-C) to form DOTAP/DP7 liposomes (Z-ave: 100.23 ± 7.50 nm, PDI: 0.231, ζP: 53.02 ± 5.51 mV) by the thin-film dispersion method. The incubation with *in vitro* transcription (IVT) mRNA caused a further increase in particle size (130.45 ± 9.32 nm) without significantly affecting the PDI (0.201), and logically, reducing the ζP (34.67 ± 7.45 mV).

Table 1. Recent cationic liposomal vaccine formulations for mRNA delivery against cancer

Vaccine composition	Model	Characterization	Ref.
-Autologous total tumor mRNA -pp65 full length lysosomal associated membrane protein (LAMP) -DOTAP	Adult glioblastoma	Data not available, clinical trial in Phase I	(University of Florida, 2023)
-Luciferase-mRNA and synthetic modified tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mRNA -Polyethylenimine cationic polymer	C-57 mice *in vivo* animal model for Glioblastoma	18% transfection after 24 h	(Peng *et al*., 2022)

Mai *et al*. (2020) work provides a new approach, adapting the cationic liposome mRNA vaccine technology for intranasal administration. The group focused on the delivery of the antigen to the nasal-associated lymphoid tissue to promote an immune response against pulmonary cancer. The cationic liposomes were developed using DOTAP, cholesterol, and DSPE-PEG2000 by the thin film hydration method. Remarkably, protamine was used to condensate mRNA-encoded cytokeratin 19 (*i.e.*, a widely distributed protein on epithelial cell membranes and present in lung cancer cells) into nanosized complexes to have a complete encapsulation in the nanoparticles and to protect it from nuclease degradation. Therefore, a complete encapsulation was achieved at a liposome/mRNA/protamine 10:1:1 molar ratio (Z-ave: 170 nm, PDI: 0.180, ζP: 10 mV).

To overcome the low immune response that has characterized mRNA vaccines, different strategies can be applied to induce higher intracellular delivery (Korsholm *et al*., 2014; Chaudhary *et al*., 2021). For instance, DOTAP/DP7-C liposomes enhance mRNA transfection into DCs owing to the penetrating properties of DP7-C, which has proved to be more efficient and less cytotoxic than some commercially available gene transfection reagents. In addition, DP7-C performs as an immune adjuvant promoting a higher maturation of DCs and inducing the production of $CD103^+$ DC, thus contributing to antigen presentation. Lewis lung carcinoma LL2 cells exposed to these cationic liposomes have revealed their greater tumor growth inhibition compared to DOTAP/mRNA liposomes. Noteworthy, the DP7-C-modified liposomes displayed a higher ability for endosomal escape through the clathrin and caveolin endocytosis pathways (Zhang *et al*., 2020).

However, increasing the immune response through the nasal mucosa presents several challenges, where low antigen uptake by the epithelial cells and their incapacity for prolonged effect demand higher consideration (Tada *et al*., 2015). Some research groups have been able to improve this situation by immunizing tumor-bearing mice with their intranasal vaccines once a week for several weeks. Most of these studies reported no tumor growth, suggesting the potential use of these vaccines as novel antitumor therapies (Pardi *et al*., 2018). Moreover, $CD4^+$ and $CD8^+$ T-cell recruitment are also significantly incremented as DCs maturation and antigen uptake by these cells is increased compared to other preparations, such as the protamine-mRNA complex and plain liposomes-mRNA vaccines (Cong *et al*., 2020). These encouraging results are derived from the mRNA protection by the nanocarrier upon exposure to endosome nucleases, which leads to enhanced expression efficiency (Ahmad *et al*., 2021).

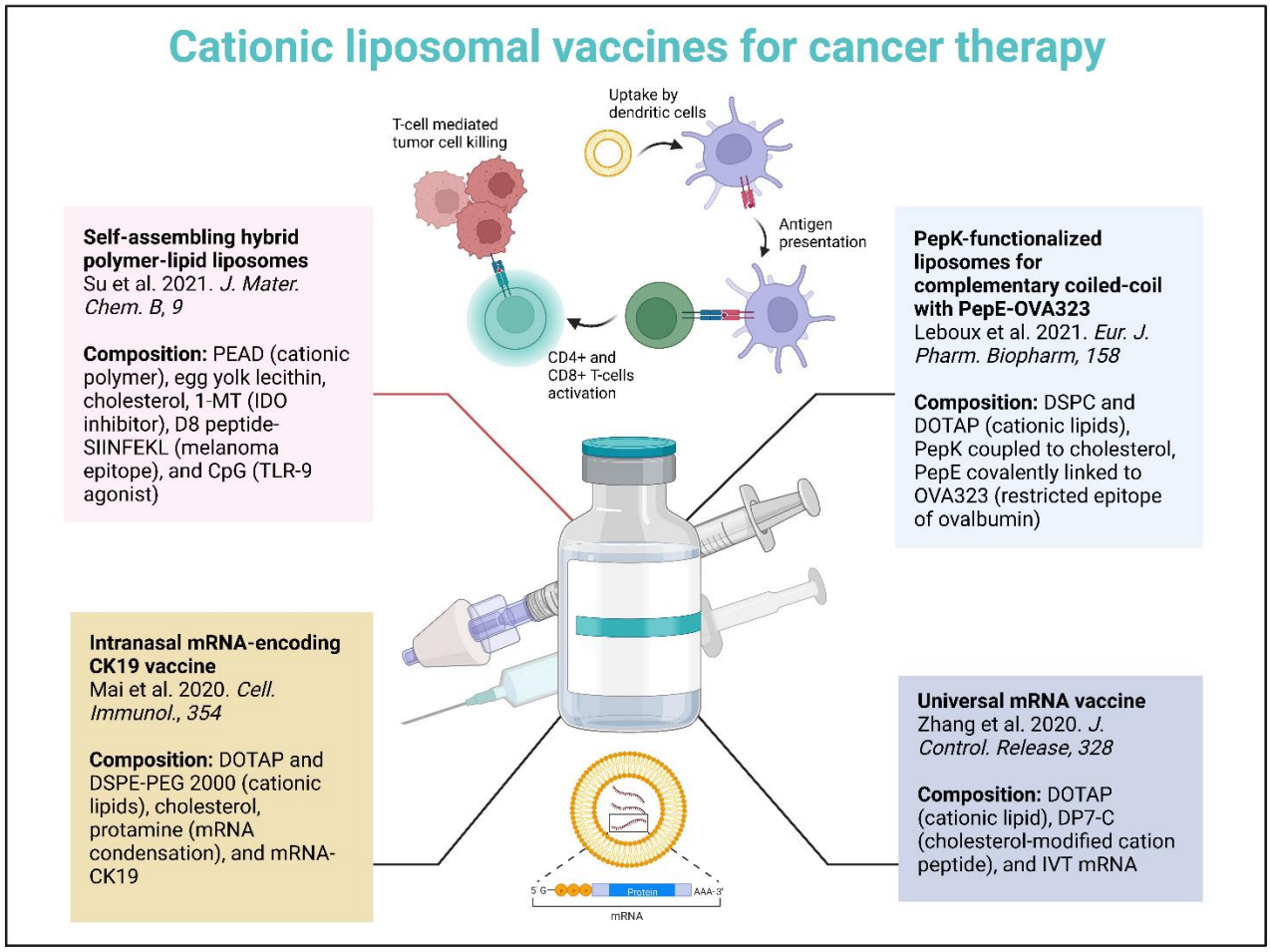

Fig. 2. The graphical abstract of this manuscript (Castillo-Henríquez *et al*., 2023)

CONCLUSION

The promising approaches discussed here highlight the potential of cationic liposomes for anti-cancer vaccine development. The reviewed studies comprise formulations considered as low toxic for DCs, simple to prepare, and suitable for different antigens. Additionally, their dual function as carriers and adjuvants makes them an outstanding platform for vaccine delivery, enhancing the immune cell response by antigen-specific T-cells and promoting a pro-inflammatory response *via* different cytokines and chemokines. However, it is also necessary to draw attention to different aspects not addressed in these works, such as formulation scale-up, the influence of the protein corona, and a better understanding of the immunogenic response for a proper translation from bench-top to bedside.

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