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Review

Nanoadjuvants: Promising Bioinspired and Biomimetic Approaches in Vaccine Innovation

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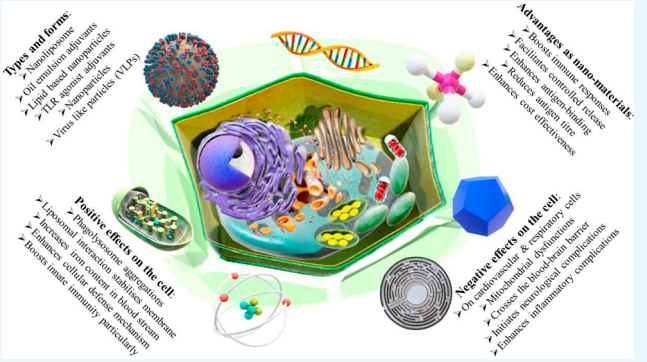
ABSTRACT: Adjuvants are the important part of vaccine manufacturing as they elicit the vaccination effect and enhance the durability of the immune response through controlled release. In light of this, nanoadjuvants have shown unique broad spectrum advantages. As nanoparticles (NPs) based vaccines are fast-acting and better in terms of safety and usability parameters as compared to traditional vaccines, they have attracted the attention of researchers. A vaccine nanocarrier is another interesting and promising area for the development of next-generation vaccines for prophylaxis. This review looks at the various nanoadjuvants and their structure−function relationships. It compiles the state-of-art literature on numerous nanoadjuvants to help domain researchers orient their understanding and extend their endeavors in vaccines research and development.



INTRODUCTION

Vaccine technology and manufacturing are humanity's momentous achievements in the modern world.[1−3] Vaccines have not only significantly reduced deaths or possible induced lifetime disabilities by neutralizing the effect early but also have helped eradicate numerous crippling diseases including the smallpox.[4] Vaccination strategies formulated to prevent child mortality are a giant step forward for a healthy world. Other than the antigens, epitope, or immunogens, vaccine development strategies involve adjuvants as additional components in specific cases. An adjuvant (adopted for the Latin word "adjuvare" which means to help) is an eliciting substance that enhances the immunogenicity of the antigen/immunogen when injected mixed with an antigen or immunogen.[5] They are often used to boost the immune response when an antigen has low immunogenicity or when only a small titer of an antigen is available.[5] The adjuvant concept was proposed at the beginning of the 20th century when the traditional purified diphtheria and tetanus toxoids failed in eliciting an immune response effectively.[6] The understanding of adjuvants and their broad applications have undergone transformation from natural ingredients to artificial synthetic compounds since then. The effect of aluminum salts as an adjuvant in 1926 was a milestone discovery in this regard.[6]

With a deeper understanding of immunology and various advancements in molecular biology, developing novel and more effective vaccines is possible. However, the immunostimulatory properties of live/attenuated new generation vaccines seem to be lacking.[7] Nanotech-inspired formulations offer numerous vaccine development advantages. Nanoaluminum salts, nanopolymeric particles, liposomes, lipid nanoparticles, virosomes, etc. are promising carriers that help in vaccine-antigen uptake by the immune cells thereby enhancing the efficiency of a vaccine.[7] While focusing on targeting the antigen to antigen-presenting cells (APCs) for enhanced targeted performance, such nanocarriers also improve vaccine stability by protecting the vaccines from degrading. Nanotechnology involves the structure, device, and/or system manipulated at the nanoscale level to design, characterize, produce, and apply those that expectedly possess at least one superior trait or property.[8] A major area of application of nanotechnology is drug and vaccine delivery. Nanomaterials are particles, emulsions, liposomes, micelles, dendrimers, etc. that typically have high therapeutic indices compared to traditional adjuvants.[9−11] Carrier systems effectively enter the APCs and

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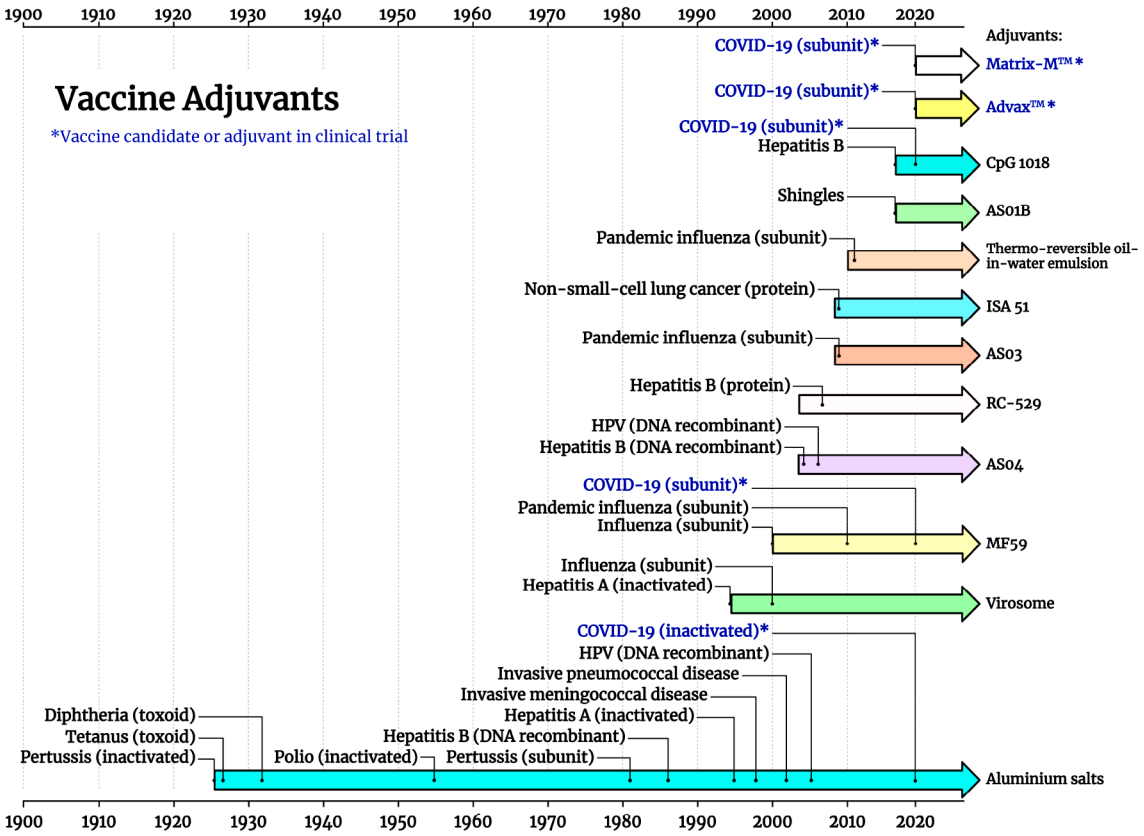

Figure 1. Timeline of adjuvants used in human vaccines development.

deliver antigens in the right quantity.[12] Adjuvants could be built and improved for increased immune responses against specific diseases in the systems. Two alternate ways to achieve this are, first, by coentrapping an immunomodulator and the antigen and, second, by surface engineering the antigen or the carrier particle to activate innate and adaptive immunity. An NP-based delivery system can deliver a need-based site-directed antigen to promote effective antigen presentation and processing. Such systems seek to address the poor immunogenicity and stability issues of recombinant protein vaccine candidates. A major attraction of NP-based delivery systems is modulating the immune response and generating a cytotoxic T-cell response. Nanotechnology system based vaccine delivery for both humoral and cellular immunity is necessary to combat intracellular infections in diseases like malaria and tuberculosis. Fine-tuned NP-based vaccine delivery systems offer tremendous opportunities as viable solutions for enhanced immune responses.

Nanosize particulates entrap vaccine candidates and nano-adjuvant (nanoimmunomodulator) with a dendritic cell activator, supporting vaccine development opportunity. They are particularly useful in treating intracellular infections where T- and B-cell mediated antibody responses for protective immunity are needed, wherein immunization with a suitable nanosystem helps. Nanoparticle delivery systems could be useful in genetic immunization and for delivery of antigens along with immunomodulators and toll-like receptors (TLRs). With increasing vaccination success against several infectious diseases, the focus on modern strategies for chronic non-infectious diseases like allergies, cancer, inflammatory diseases, and contraception problems has grown. Nanotechnology-based vaccine delivery systems could be a game-changer in new and efficient vaccine development strategies through deeper research.

Figure 1 provides a compiled timeline of adjuvants used in the development of human vaccines. Viral vector based carrier molecules like viral genome, virus-like protein and virosomes, and nonviral vector based adjuvants like lipid nanoparticles and nanoemulsions have gained prominence in vaccine development.[13] Nanobased formulations have been promising in developing vaccines against cancers for better targeting, identification, and destruction.[14] A nanoparticles-based approach to counter public health emergencies like the current COVID-19 pandemic has been recommended.[15−17] Notably, a biocompatible nanobased approach is cost-effective with reduced toxicity.[18] Nanomolecules demonstrably have unique characteristics like a higher surface-to-volume ratio, surface charge, size, shape, and optical, biological, and functional properties.

RESULTS AND DISCUSSION

Cellular Effects of Nanoadjuvants. Sustained research and development on adjuvants has been greatly facilitated through our understanding of the innate immune responses triggered by the highly conserved molecular patterns on microbes and recognition receptors on the immune cells. Activation of adaptive immunity to induce protective antibody responses thus ensues. Numerous adjuvants for live and viral vaccines have been frequently used to elicit innate responses through the TLRs.[19,20] The development of new generation adjuvants aims at better and effective antigen-specific immune responses with a sustained effect on cellular differentiation and metabolic activity while ensuring high safety levels. Despite their widespread use, the molecular mechanisms in human cells

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of most of the adjuvants are not yet well-understood. Specific instances of these are elaborated later in the article under specific dedicated heads.

Certain adjuvants as ligands could activate TLRs synergistically and engage multiple DC subsets, even direct B-cell activation. This fact paved the way to produce adjuvants as ligands to multiple TLRs, inducing a persistently healthy specific antibody response, reactions at the germinal center, response of the follicular T helper cells, and enduring plasma cells.[21]

Aluminum Hydroxide NPs as Nanoadjuvants. Alum adjuvants have been in use for decades in vaccine manufacturing. Some routinely used alum adjuvants include aluminum hydroxide, aluminum phosphate, and amorphous aluminum hydroxyphosphate (AAHS). These have been effective in stimulating the humoral immune system as compared to the cell-mediated immune response. Aluminum hydroxide is a common vaccine adjuvant and has been used in numerous human and animal vaccines. Although the safety profile of aluminum hydroxide is relatively quite high, it reportedly could induce only weak to moderate antigen-specific antibody responses.[22] Alum allegedly stimulated the Th2 immune cells that resulted in increased antigen-specific antibody production but was incapable in stimulating robust Th1 or against cytotoxic responses.[23] The actual size of the primary aluminum hydroxide fibers is in nanometers. However, when dispersed in an aqueous solution, it forms aggregated particulates of 1−20 μm size.[24,25] Aluminum phosphate is another well-known aluminum salt adjuvant, the size of which is about 20 nm, and it also shows similar aggregation properties in aqueous solution like aluminum hydroxide.[24,25] Growing evidence suggests that nanoparticles of about 200 nm have more potency as adjuvants and as vaccine or antigen carriers than larger particles.[22]

Numerous studies determining the efficacy and utility of aluminum nanoadjuvants have been reported. In one such study, ovalbumin (OVA) and *Bacillus anthracis* were used as protective antigens to induce inflammatory responses by adsorbing them on aluminum hydroxide NPs, which showed a stronger OVA-specific antibody immune response as compared to bigger-sized OVA-adsorbed larger aluminum hydroxide microparticles.[22] It was observed that aluminum hydroxide NPs induced milder local inflammatory reactions than the microparticles.[22] Thus, the potent adjuvant activity of aluminum hydroxide NPs was closely associated with their ability to more efficiently facilitate the uptake of the adsorbed antigen by the antigen-presenting cells.[22] The overall efficiency of the conventional aluminum hydroxide adjuvant was particle size dependent, and its nanometer size has a greater potential to generate a better immunity response.[22] The same concept was also used later for administering *Mycobacterium tuberculosis* antigen EsxV in mice subcutaneously. The efficacy of aluminum hydroxide NPs in inducing the release of IFN-γ and activating Th-1 response from the immune system was significantly high as compared to the antigen alone.[26] The efficacy of alum adjuvants appears to be low, especially with subunit and low molecular weight vaccines. Also, nanoalum has a larger surface area and could accommodate more antigens.[27] Aluminum oxide microparticles were constructed into alum NPs of around 30 nm size and used in pulmonary vaccine as an adjuvant-based antigen delivery system. It elicited a stronger immunogenic response as noticed through the presence of IgG and IgA in salivary, nasal, respiratory, and vaginal fluids. Thus, it showed prospects of use in vaccines as an efficient tool for simplified delivery systems.[28] Initial research findings on alum-based nanoadjuvant are positive and may guide the development of a better and more effective adjuvant for antigens or subunit vaccine delivery.

Aluminum-Based Adjuvants. Along with increasing the efficacy in eliciting the differentiation of CD^{4+} T helper cell (Th2) and antibody responses in humans, alum is a prevalent adjuvant primarily due to its availability and its low-cost manufacturing.[29,30] The adjuvant effect of alum is TLR-independent, although adjuvant activated TLR-mediated innate immunity enhances the immunogenicity of a vaccine.[31] Alum reportedly stimulated adaptive immunity by causing cell death at the site of injection, thereby activating the inflammatory DCs to release cytokines and chemokines. Studies report the use of alum nanoparticles (Al-NPs) as adjuvants in robust and durable immune responses especially in lymph nodes and tumors (anticancer vaccines).[28,32] The available larger surface area also increases the carrying capacity of Al-NPs, allowing use of multiple adjuvant/antigen in a single vaccine; e.g., CpG is coated onto Al-NPs for better delivery of anticancer vaccines as in Sipuleucel-T, Provenge.[32]

As the use of alum as an adjuvant becomes prevalent, there is an urgent need to assess the relevance of stress signals from tissue damage, its role in activating cells of different origins, the metabolic and nutrient sensing pathways involved, the innate receptors other than TLRs, and the signaling mechanisms leading to antibody production and T helper cell differentiation for optimum efficacy.[33] Upon administration into the host, alum particles form aggregates that are engulfed by phagosomes, and the antigen entrapped or adsorbed is presented to the phagosomes.[34] However, formation of an aluminum depot is seen upon phagocytosis, and, contrary to the popular assumption, this depot was not cleared completely by the hydrolyzing enzymes in the phagolysosomes.[33] As these macrophages divide, the aggregated aluminum particle is carried to lymph nodes and is dispensed for years. Phagosomal degradation of the engulfed particles is mostly driven by the activity of the v-ATPases and hydrolytic enzymes.[35] As the initial pH of an undifferentiated phagosome is neutral, the aluminum particles get accumulated (alum particles solubilize only at lower pH). Even when the phagosomes mature and the pH goes below 5.0, the alum particles do not clear off, leading to increased concentration of alum inside the vesicle modulating the innate properties of phagosome,[36] a glaring bioaccumulation and biomagnification instance. Aluminum ions interact with biomolecules like carboxylates, phosphates, and nucleotides. They reportedly also interact with proteins and alter their 3-D structure, rendering them ineffective or reducing their bioactivity. Also, alum ions compete with biologically useful ions involved in important metabolic pathways (like Fe^{2+}, Fe^{3+}, Zn^{2+}, and Ca^{2+}) and modulate their function.[37]

Alum ions affect the membrane polarity and potential of mitochondria. Production of cellular reactive oxygen species (ROS) is majorly mitochondria and cellular respiration regulated.[38] Cellular ROS results in a dysfunctional mitochondrion. ROS release is associated with phagosome maturation due to enhanced activity of the NO_X^2 complex. Human macrophages are classified into M1 type of inflammatory phenotype and M2 macrophages of a regulatory/anti-inflammatory phenotype.[33,34] Excess ROS production drives the differentiation and polarization of an immature

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macrophage toward the M1 fate, leading to increased inflammatory burden and further inflammatory complications. Aluminum ions rendered stability to the produced ROS in mammalian cell lines, highlighting that alum-based adjuvants might be significant in persisting inflammatory conditions.[33] After the membrane ruptures and alum ions are released to cytosol, they extensively affects organelles, biological pathways, and biomolecules. Interacting with proteins, these increase misfolding in them, leading to an increased demand for chaperone-mediated protein refolding. Increased activation of chaperone proteins and subsequently the DAMPs induces an immune response, which leads to increased accumulation of inflammatory cytokine.

Alum ions also reportedly activate the inflammasome pathways. This activation is facilitated by cytosolic pattern recognition receptors that trigger signaling pathways downstream thereby producing and secreting inflammatory cytokine IL-1β.[38,39] After the aggregated alum is phagocytosed, IL-1β activation induces the NLRP3 inflammasome pathway. NLRP3, a cytoplasmic pattern recognition receptor (PRR), belongs to the family of the nucleotide-binding oligomerization domain (NOD) like receptor and is characterized by central nucleotide binding and leucine-rich repeat pyrin 3 domains. Activated NLRP3 inflammasome pathway activates caspases that express proinflammatory cytokines, such as IL-1β and IL-18. Continued activation of the pathway ultimately leads to cell clearance through apoptosis. ROS reportedly regulates NLRP3 inflammasome activation and is a key byproduct of the administration of alum-based adjuvants. It could drive the activation of the NLRP3 inflammasome pathway.[33,34,38] Alum ions could also disturb the tricarboxylic acid (TCA) cycle, increase succinate accumulation, and activate hypoxia inducible transcription factor 1α (HIF-1α). HIF-1α leads to metabolic reprogramming of macrophages by controlling the expression of glycolytic enzymes and proinflammatory signaling.[40] Phagosomal rupture may activate maturation of the macrophages toward a M1-biased phenotype and increased inflammation upon receiving the vaccine.[38]

Alum reportedly affects iron metabolism in mammals. Alum ions enter the blood as the vaccine is administered, bind to readily available transferrin, and go systemic in the bloodstream. Transferin, Fe^{3+}, and Al^{3+} enter the cell and kickstart the transferin cycle through receptor mediated endocytosis. By activating proton pumps like divalent metal transporter 1 (DMT1), endocytosed Fe^{3+} is transported to cytoplasm along with alum ions,[41] taken up by mitochondria and subsequently neutralized, and the cycle goes on (Figure 2). The neutralization cycle burdens mitochondria through ROS production, DNA damage, and sentinel cell proliferation, and also leads to the loss of viability of the affected cell.[42]

Oil-in-Water Emulsion. An emulsion is a mixture of two immiscible liquids whose stability is maintained through mechanical shear and surfactant.[43] Oil-in-water emulsions as vaccine adjuvants have shown better results than water-in-oil emulsions. The most commonly used oil-in-water adjuvants are MF59 (Novartis) and AS03 (GlaxoSmithKline). Both droplets are squalene-based emulsions of around 160 nm size. MF59 is approved and AS03 has already been licensed for H5N1 and H1N1 influenza vaccines.[44,45] MF59 has Tween 80 and Span 85 (two surfactants), and AS03 has polysorbate 80 and α-tocopherol (vitamin E), with a squalene base in common.[46] MF59 could recruit local monocytes, macrophages, and dendritic cells for antigen uptake and can activate CD4 T

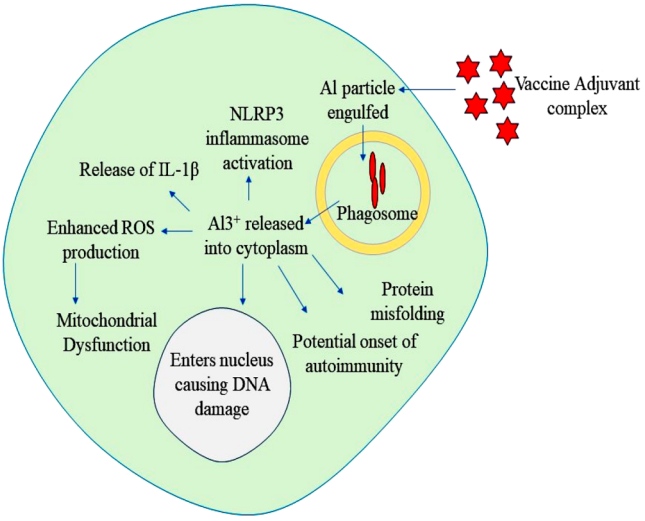

Figure 2. Metabolic and immunostimulation of alum-based adjuvants in animal cells.

cells.[47,48] Contrarily, AS03 could nonspecifically activate the immune system in mice.[49] MF59 and AS03 could be good nanoadjuvant candidates due to the <200 nm size and biocompatibility trait. However, only small variants of oil-in-water emulsions have also been tested so far. Theoretically small-size adjuvants (of about 20 nm) would be more efficacious, although the opposite finding is also reported. It is found that commercial adjuvants of about 160 nm size were more effective for in influenza vaccine administered in mice as compared to 20 nm oil-in-water emulsion adjuvant.[50] EAS, another novel squalene-based oil-in-water emulsion adjuvant of approximately 105 nm particle size, was compared with commercially available MF59 adjuvant for subcutaneous administration of swine influenza vaccine,[51] and it was found that the novel EAS adjuvant had comparable properties to the established MF59 adjuvant. EAS elicited strong IG1 and IG2a antibodies response with a high Th1 response involving IFN-γ and IL-2 cytokines compared to vaccine/antigen alone.[51] Various oil-in-water emulsion based adjuvant studies show the potential to develop an effective subunit vaccine. However, deeper insights into the properties and side-effects need to be considered before its wider application.

Differentiating between oil-in-water emulsion and water-in-oil emulsion approaches is vital. The oil is the dispersed phase in the oil-in-water emulsion distributed in water as the continuous phase, while the roles switch in the other. A better way to comprehend is that milk is an example of an oil-in-water emulsion, while butter is a water-in-oil emulsion. Nevertheless, due to the highly mobile water droplets that enhance coalescence, flocculation, and/or sedimentation, the water-in-oil emulsion often is less stable.

Oil Emulsion Adjuvants. These include an array of oil-in-water or water-in-oil emulsions. Due to their high ranking on the reactivity scale, water-in-oil adjuvants are deemed unfit for use in humans.[29] Owing to their relatively low reactivity, oil-in-water emulsions are often used, although they exhibit side-effects. They are known to induce a strong inflammatory response at the site of injection and to induce inflammasome activation. Oil based adjuvants like compound 81 increase cytokines production by reducing mitochondrial membrane potential and inducing mitochondrial stress, which lead to decreased basal and ATP-linked respiration.[52] This adjuvant

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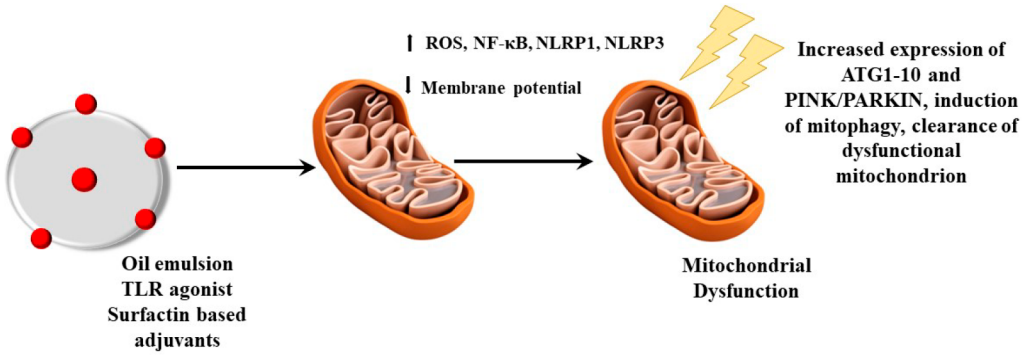

Figure 3. Overall effects of nonalum based adjuvants on mitochondria.

induced mitochondrial stress further led to mitophagy to clear the damaged mitochondria and activation of the cytokine release cascade. Oil emulsion adjuvants reportedly also regulate mitochondrial ROS production in a dose-dependent manner ultimately driving the activation of NF-κB driven inflammatory pathways and activation of IFN regulatory factors (Figure 3).[52]

TLR Agonist Adjuvants. TLR adjuvants include a wide range of pathogen-derived substances like proteins, lipopeptides, glycolipids, and their synthetic equivalents. The TLR agonists activate the inflammatory transcription factor nuclear factor NF-κB through TLR adaptor proteins TRIF and MYD88.[53] Studies revealed that R848, a TLR7 agonist, increased the level of mitochondrial ROS leading to mitophagy and inflammation when loaded on to a zirconium-based metal organic framework and injected into mice.[54]

Surfactin. Surfactin is natural lipopeptide used as a vaccine adjuvant.[55] Its usage pattern varies, and the mechanism of the immune response is yet to be thoroughly explored. As an adjuvant it activates the inflammasome NLRP1 and NLRP3 mediated by endogenous danger signals.[56] Surfactins reportedly increase ROS production inside mitochondria of macrophages, thereby initiating ROS-mediated activation of the NF-κB pathway and triggering inflammation. The presence of ROS simultaneously triggers apoptotic genes as a result of mitochondrial stress that leads to the loss of cell viability.

Nanoliposome. Liposomes are made of a double-layer phospholipid shaping as a sphere within water through self-assembly under the driving force of hydrophobicity.[57] Liposomes are made of various amphiphilic phospholipids that could combine with lipids, such as cholesterol, to provide membrane stability. PEGylation also increases the stability of liposomes. PEGylation prevents liposome adhesion, thereby preventing fusion or coagulation. PEGylation also prevents liposomes from binding with plasma proteins but allows binding with other specific plasma proteins, thereby increasing the *in vivo* circulation time of the nanoparticle.[58]

Freeze-drying of liposomes with a liposome cryoprotectant like sugar is an alternate strategy to increase its structural stability.[59] Among various nanocarriers, liposomes are frequently used in the delivery of drug or as vaccine adjuvant. According to liposome size, immune reactions may vary when used as a delivery system or adjuvant. Subcutaneous injection or oral inoculation of small-sized liposomes (<150 nm) influences the development of the Th-2 type response, and larger particles (>200 nm) promote IFN-γ and Th-1 type responses more.[60] The phospholipids in liposomes are known important mediators of innate and adaptive immunity.[61] Liposome has the built in capacity to activate innate immunity but could also effectively present the carried antigen to APCs. According to their physiochemical properties, these could stimulate both naïve T-lymphocytes into CD^{4+} and CD^{8+} cells, and β-lymphocytes to initiate the humoral immune response.[61]

Very small particles of <20 nm may distribute and disperse into the tissues and become systemic by entering the circulating blood with little scope of lymphatic uptake.[57] However, bigger (>100 nm) particles have a better chance to be trapped in tissue, thus creating a depot effect at the site of injection. Through this it is easily identified and picked by APCs which would then mature, present the required epitopes, and migrate to the draining lymph nodes for adaptive immune response further.[57] Monophosphoryl lipid A, a detoxified part of endotoxin lipid A, along with a TLR4 ligand is identified as a safe and effective lipid-based adjuvant to use in developing emerging vaccines against infectious diseases like malaria, AIDS, leishmaniasis, and others, as also to treat several cancers.[62,63] The approved adjuvant for "Mosquirix" (malaria)[64] and "Shingrix" (herpes zoster)[65] vaccines, AS01 is liposome-based system containing 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) and saponin QS-21 as immunostimulants.[66]

Immune-Stimulating Complexes (ISCOM). ISCOM is an important lipid-based adjuvant or vaccine delivery system. Like liposomes, the lipid particles here are also made of cholesterol and phospholipids; however it also contains some protein antigens devised by using saponin Quil A found in soapbark tree *Quillaja saponaria*.[67] They are 40−50 nm sized cages, and several APCs uptake them because of their lipid-based structure. The antigens carried by the ISCOM are presented to both MHC I and MHC II (major histocompatibility complexes). ICOSAMATRIX is an ISCOM without an antigen.[45] High affinity between saponin and cholesterol that are part of ISCOM and ICOSAMATRIX provide high stability.[68] They could elicit a balanced Th1 and Th2 response maintaining durable antibody titer and effective cell-mediated immunity. The presence of saponin that could be toxic is a disadvantage of these carrier molecules, and both ISCOM and ICOSAMATRIX are approved only for veterinary purposes as of now. However, the development of influenza and human papilloma virus vaccines using ISCOM is under research.[45]

Toll-like Receptors (TLRs). TLRs, pattern recognition receptors, are crucial for microbial detection (Table 1). Many adjuvants are built on PAMPs that bind to TLRs. TLRs on cell surfaces like TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are capable of detecting extracellular antigens, whereas TLRs like TLR3, TLR7, TLR8, TLR9, and TLR10 found inside of cells are capable of detecting internal intruders like viruses.

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Table 1. TLRs Ligands and Their Sources

TLRs	agonist	source	antagonist	source
TLR 2	Lipoglycans, Lipoteichoic Acids, Peptidoglcans, Synthetic Lipoproteins, Zymosan	InvivoGen	OxPAPC (TLR 2 and TLR 4 inhibitor)	InvivoGen
TLR 3	Virus ds RNA/polyinosinic-polycytidylic acid (Poly I:C)	Sigma-Aldrich, InvivoGen	614310-TLR3/dsRNA Complex Inhibitor − Calbiochem	MerckMillipore
TLR 4	Lipopolysaccharides, Monophosphoryl Lipid A	InvivoGen, Sigma-Aldrich	*Rhodobacter sphaeroides* lipopolysaccharide (LPS-RS)	InvivoGen
TLR 5	Flagellin	InvivoGen, Sigma-Aldrich	Soluble ectodomain of TLR5 of human with engineered Fc region fusion (hTLR5-Fc)	InvivoGen
TLR 7/8	ssRNA/Resiquimod, Imiquimod VaxxiGrade Imidazoquinolinone or Imidazo quinolin gallamide (IMDG)	InvivoGen Bharat Biotech	-	-
TLR 9	Unmethylated CpG DNA (intracellular sensor of bacterial DNA)/Class A CpG ODNs (ODN 1585, ODN 2216, ODN 2336); Class B CpG ODNs, Class C CpG ODNs	InvivoGen	ODN 2088	InvivoGen
TLR 13	23S rRNA, ORN Sa19	InvivoGen	-	-

TLR13 is also reported, although not in humans. Activating transcription factors like AP-1, NF-B, and interferon regulatory factors begins by stimulating TLRs by the related PAMPs (IRFs). Creating interferons (IFNs), pro-inflammatory cytokines, and effector cytokines that control adaptive immune response are only a few cellular responses triggered by TLR signaling. NF-B, MAP kinase, and IRF3 are three important transcription factors activated by all TLRs except TLR3. In addition to the activation enzyme caspase-1, NF-B and MAPK activate the genes for three important proteins IL-1, IL-6, and TNF-α. Another transcription factor, IRF3, which is activated by the TRIF complex, turns on the antiviral cytokine Interferon beta gene. TLR ligands include synthetic substances causing professional APC to mature and be activated and to release inflammatory cytokines and chemokines. TLR-7 and TLR-7/8 are ligands, respectively, for small-molecule nucleoside analogues imiquimod and resiquimod. Imiquimod is approved to treat HPV and basal cell carcinoma in addition to leishmaniasis (BCC).[69,70] Although the precise mechanism of action of imiquimod is unknown, it is believed that its activity as a TLR-7 agonist mimics that of a microbial antigen and induces the expression of cytokines. These include IL-1, IL-6, IL-12, IFN-α, and TNF-α which stimulate or enhance both the innate and the cell-mediated immune responses while enhancing the migration of Langerhans' cells from the dermis to local lymph nodes. Table 1 details the TLRs as ligands and their sources.[71,72]

Virosomes. Virosomes, made up of membrane lipids that are actually part of the viral envelope and viral glycoproteins (but completely devoid of genetic information), are quite similar to liposomes.[11] They are US FDA-approved lipid nanomolecules for use as vaccine delivery systems. Their surface could be modified and used for the fusion of proteins/antigens during vaccine designing. Virosome-based vaccines have demonstrated a better response in terms of both humoral and cell-mediated immunity.[73] Virosomes are ever more beneficial in designing subunit vaccines, wherein microbial proteins of low molecular weight need an efficient carrier or adjuvant molecule for an enhanced vaccine effect.[74]

Virosomes could carry antigen determinant sites of pathogens by allowing attachment to the viral coat in the virosome.[11] As adjuvants, virosomes present antigens to both MHC I and MHC II, and thus produce both humoral and cell-mediated immunity.[75] Many novel vaccines of recent times use virosome nanoadjuvants. Some such approved ones are influenza virus vaccine Inflexal V in Europe and hepatitis A virus vaccine Epaxal in Asia.[76] Virosomes in these vaccines were derived from an influenza viral coat containing influenza hemeagglutinin protein.[77] The Inflexal V vaccine is reportedly safe for all age groups.[45] A nanocurcumin-based hybrid influenza virosome was designed to treat cancer. These were more efficient in terms of drug release and targeted delivery, thus enabling their use in the delivery of herbal medicine and treating diseases including cancer.[78] Virosome technology which has proved to produce higher titers of neutralizing antibodies could be applied to design vaccines like the currently available SARS-CoV-2 vaccines of AstraZeneca AZD1222 given its low immunogenicity.[79] Virosomes have high fusogenic properties, enabling them to attach to host cells and transport the drug/protein into cells through endocytosis. They are efficient drug delivery systems and could be used in the development of an array of vaccines.[80]

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Virus-like Particles (VLP). VLPs are suggested as a safe alternative as a live (or even attenuated) virus could be reactivated, thereby triggering infections. VLPs are viral proteins including capsid, envelope, and others. VLPs are produced, purified, formulated, and administered using microbial and nonmicrobial vehicles like bacteria, yeasts, plants, insect cells, mammalian cells, etc. Effectively, they have antigenicity (or immunogenicity) without pathogenicity. VLP technology could be applied to counter emerging infections and cancers.[81]

VLP is a noninfectious self-assembled structure of viral envelope proteins without genetic material. As it is a viral derivative, it possesses several properties very similar to those of the host virus. This makes it an efficient elicitor of the immune response when administered as an adjuvant. Like virosomes, VLPs are also seen to elicit cell-mediated as well as humoral immunity.[82] Plasmids containing the genes for viral structural proteins infect the cell lines, and the generated proteins are filtered and separated from the culture to be used as VLPs. Two vaccine cases where VLPs are successfully used are recombinant hepatitis B and the human papilloma virus vaccines.[83] VLPs of, say, foot and mouth disease (FMD) virus were used alongside silica nanoparticles to produce an effective vaccine that induced long duration (three months) high antibody titer in pigs.[84] VLPs allegedly induced a better immune response than vector- and virosome-based vaccines. VLPs, thus, could make approved vaccines such as Inflexal V against influenza more efficient.

Dendrimers. Derived from Greek "dendron", dendrimer literally means tree. These are versatile, derivable, well-defined, compartmentalized chemical polymers resembling protein biomolecules in size and physicochemical properties,[85] synthesized either through divergent or convergent routes following a series of well-directed polymerization.[86] Dendrimers generate from small monomeric units from a central core by polymerizing.[86] Thoroughly branched globular macromolecules similar to globular proteins are generated. Nanosize dendrimers (10−100 nm) look similar to a ball with a capacity to trap molecules in the interstitial space of the branched structure and the multiple functional groups expressed on the surface.[7]

After their discovery in the last century, the importance of dendrimers has increased and has been put to several applications. One such significant application in biological sciences is as a vaccine adjuvant and for drug delivery. The potential of dendrimers as adjuvants for antigen delivery in cancer is quite evident. These could be used in making anticancer drugs, in efficient drug delivery, as dendritic sensors, in phototherapy for precise anticancer targeting, gene therapy, etc.[87]

A branched dendrimeric chain could be altered or modified, which is helpful in adaptations for specific use.[7] Antigens could be loaded onto dendrimers through complexation or chemical bonding at end-branch points. Polyamidoamine (PAMAM) is recognized as a significant dendrimer formed by repetitively adding branched units to ammonia or ethylene diamine.[7] Research has identified specific dendrimers like glycodendrimers, phosphorus dendrimers, and several others including PAMAM with potential use in cancer nanotheranostics with the ability to activate the immune system.[88] Mesoporous silica nanoparticle dendrimers as adjuvants are highly stable and safe for use as subunits in VLP-based FMD vaccines for ruminants.[89]

Silk Fibroin (SF). Silk fibroin is a polymeric protein whose potential as a drug delivery system that has been under study for a long time.[90] Due to certain encouraging traits like low toxicity, better biocompatibility, and few structural properties, silk fibroin nanoparticles (SFNPs) seem efficacious.[91,92] Silk fibroin forms hydrogel matrices.[93] It is made of disulfide-bound heavy (370 kDa) and light (25 kDa) chains.[94] The core structure is of 12 hydrophobic protein domains with a high propensity to form an antiparallel sheet (physically cross-connected structure). These domains are alanine and glycine residues rich. There are 11 tiny hydrophilic amorphous domains dividing the blocks, which demonstrated valuable immunological traits, a factor to consider while implanting protein-based materials.[95] pH, temperature, shear, vortexing, electricity, and sonication based techniques are used to make silk hydrogel.[93,96−98] Silk-based biomaterials could stabilize and release proteins.[96,99] It suggests integrating proteins, either trapped or free, into silk films. Reversible interactions between free protein and silk allow its quick escape from the matrix, and irreversible connections in the trapped protein may need destroying the silk matrix to allow the release.

Loading sensitive therapeutic molecules like peptides and proteins into SF needs gentle aqueous processing conditions, which is an encouraging feature of SF for drug delivery.[100] Additionally, SF protein has amino acids with amine and carboxyl groups that could facilitate attachment to numerous materials.[100] In preventing urinary tract infections (UTI), the use of SFNPs adjuvant for vaccine delivery validated that their biocompatibility, biodegradability traits, and capability in controlled release of antigen is useful to formulate an efficient vaccine.[101] SFNPs have garnered increased interest in recent times due to their unique properties of tunable size, high surface area to volume ratio, and high drug loading capacity.[102]

A suitable adjuvant should be risk-free and should be devoid of negative effects. Also, adjuvants should be chemically neutral, biodegradable, and quickly eliminated from the body. As a natural biopolymer, SF undergoes proteolytic breakdown to nontoxic byproducts.[100] The biodegraded products of SF have a negligible or nonexistent effect on the human body.[103] They must also be accessible and affordable for commercially viability.[104] The techniques for SF extraction and purification are simple and cost-effective. Due to these, SF is a highly biocompatible polymer with distinctive physical and functional traits. By enhancing the particle size and structure of SFNPs, these could be good nanoadjuvant candidates.

Polycationic CpG Nanoparticles. Antigen-adjuvant complex loading on nanoparticles is precisely affected by cationic polymer polyethylenimine (PEI), an electrostatically forming biological macromolecules complex.[105] As compared to most other nanoparticle formulations, fabricating an assembled PEI−protein nanoparticle is simple, fast, and biomolecule-friendly. PEI increases the immunogenicity of the vaccines involving DNA and protein.[106] Like damage-associated molecular pattern (DAMP) adjuvants such as $Al(OH)_3$ or alum ($K_2(SO_4)\cdot Al_2(SO_4)_3\cdot 24H_2O$), Th2 dominates PEI-induced immunity.[107] Due to the lack of IFN-γ cytokine induction, PEI could not induce cellular immune responses such as cytotoxic T-lymphocytes (CTLs). Yet, PEI-adjuvanted subunit H1N1 HA protein protected mice challenged by the homologous influenza virus. The negatively charged viral glycoproteins formed nanoscale complexes, induced by PEI.[108]

Higher mucosal antibody and cellular immune responses could be induced by polyanhydride NPs-induced coencapsu-

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lation of inactivated or killed soluble swine influenza (KAg + CpG-nanovaccine) antigen and the toll-like receptor (TLR)-9 agonist (CpG-ODN). A cross-reactive IgA antibody response, higher lymphoproliferative response in mononuclear blood cells, and elevated secretion of IFN-γ were noticed in an antigen-induced recall response in intranasally delivered prime-boost KAg + CpG-nanovaccine.[108] Oligodeoxynucleotides (ODNs) with unmethylated cytosine-phosphate-guanine (CpG) motifs (CpG ODNs), which are TLR9 agonists, are promising adjuvants and are already used in humans. Th1-type immune responses and antigen-specific IgG2 production could appropriately be induced by oligodeoxynucleotides with unmethylated cytosine-phosphate-guanine (CpG) motifs as adjuvants. CpG ODN is a synthetic small fragment ssDNA with an immunostimulatory CpG motif that binds to TLR9 at the endosomes of the dendritic cells after uptake. TLR9 primarily binds to the unmethylated CpG DNA motifs that are commonly encountered in bacterial and viral DNA, thus being central to viral immunity and autoimmune disorders.[109]

Lipid Nanoparticles (LNPs). A lipid molecule is amphiphilic and has a polar head, a hydrophobic tail, and a linker between them. Cationic and ionizable lipids have been successfully tried in mRNA delivery. mRNA is a novel agent in therapeutics and prophylaxis. To protect from degradation and allow its *in vivo* release after a cell takes it up, mRNA needs an effective, safe, and stable delivery system. LNPs seem to be promising vehicles across pharmaceutical industries to deliver mRNA and other therapeutics. As a milestone in mRNA-based intervention, LNPs-mRNA vaccines were successfully tried for the first time against COVID-19.[110] LNPs were the key in developing the COVID-19 mRNA vaccine for effective protection and transportation. Liposome, an early LNPs version, is a versatile nanomedicine delivery platform (Figure 4). Lipid nanoparticles are successfully used as an mRNA delivery system. Many liposomal drugs are approved and are in use in medical practice.

An enhanced physical stability with a more complex architecture was exhibited in subsequent lipid-nanocarrier innovations in the form of a nanostructured lipid carrier, cationic lipid-nucleic acid complex, and solid-lipid NP. LNPs hold great promise in treating an array of diseases due to their encapsulation and site-specific delivery ability and sustained release of contents.[111] The delivery of polymeric particles encapsulated nucleic acids and the delivery of exogenous mRNA into a host cell with liposome were successfully demonstrated long ago, in 1976[112] and 1978[113] respectively.

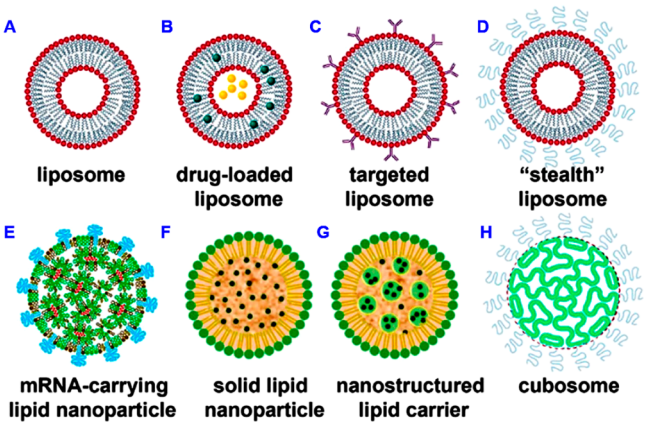

Figure 4. A schematic presentation of liposome (A), drug with liposome-encapsulation (B), targeting ligand-functionalized immuno-liposome (C), inert polymer functionalized and sterically stabilized "stealth" liposome (D), NPs inside inverse lipid micelles containing the organized nucleic acids (E), solid-lipid NPs (F), nanolipid carrier (G), NPs-lipid in a continuous bilayer phase cubosome (H). (Source: Tenchov et al. 2021; further permissions related to the material excerpted should be directed to the ACS).[111]

Cationic lipids are commonly used in formulating mRNA-based vaccines. The headgroup in cationic lipids has a permanent positive charge.[114] Usually, quaternary NH_4^+ lipids, i.e., 1,2-di-*O*-octadecenyl-3-trimethylammonium-propane (DOTMA), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), are used in lipid NPs-based vaccines.[115−117] Spleen-targeted DOTMA−mRNA lipoplexes are developed as systemic cancer vaccines.[118] Antigen-specific CD4 regulatory T cells proliferate by such vaccines that lead to superior immunosuppression with reduced clinical signs.[119] DOTAP-based cationic nanoemulsions could deliver antigen mRNA against viral, bacterial, and parasitic infections.[120−123] Dimethyldioctadecylammonium bromide (DDAB), a quaternary NH_4^+ lipid as an mRNA vaccine adjuvant, forms mRNA complexes that stimulate innate immunity.[124] Biodegradable lipids are more and more being accepted in the vaccine world. It helps the rapid elimination of lipid NPs from tissue and plasma through which their safety and tolerability are improved. COVID-19 mRNA (mRNA-1273 and BNT162b2) vaccines involving biodegradable lipids have widespread acceptance. Lipids themselves could have adjuvant properties boosting the vaccine efficacy alongside being a delivery vehicle.[125−127]

Other than being used for vaccines against infectious diseases, mRNA vaccines have a promising future, including their use in cancer. LNP-based mRNA vaccines have had significant progress in cancer therapy.[128,129] They are a safe and tolerable treatment option in cancer therapy with high-specificity. New-age personalized cancer vaccines could treat solid tumors like colorectal and lung cancers, preclinical studies demonstrate. By activating TLR subtypes, a combination of diverse adjuvant types could synergize an immunostimulating effect. Combined adjuvants in LNPs could further improve mRNA-mediated immune responses. Costimulation of several TLR subtypes by combined adjuvants may expedite the evolution of mRNA-based LNP formulations for cancer immunotherapy.[130]

Nanoadjuvants in Treating Respiratory Diseases. The world has an enormous respiratory disease related health burden, and quite a large population suffers from asthma, obstructive pulmonary disease, TB, pulmonary fibrosis, pneumonia, lung cancer, occupational lung disease, and pulmonary hypertension that are chronic in nature. Billions are exposed to such risks owing to exposure to biomass burning toxicity, outdoor air pollution, and smoking of tobacco. Chronic respiratory diseases prematurely kill millions of people annually. Lack of suitable treatments for many of these has demanded an urgent need to develop desired vaccines against them. New generation vaccines to induce immunity against these through attenuated or killed entire organisms, subunits, and peptides or RNA or DNA vaccines have recently developed. Efficient delivery of the vaccine molecules to the target site and the need for a prime-boost vaccination regime with other immunogenic agents present

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challenges like their low infection risk, ability to elicit immune response against specific pathogen, and cost effectiveness.

Efficient vaccine delivery to the target site requires a competent delivery cargo system and an adjuvant to potentiate immunogenicity, avoiding rapid degradation under a hostile environment. An effective vaccine delivery mechanism is required to get through the challenges mostly as it gets the vaccine molecule to the target site where it could trigger long-term immunity but also has few side-effects. Further, there is a growing need to create next-generation composite vaccines that function both as an adjuvant and immunogen. Nanotech-based formulations have several advantages in developing a new generation vaccines. Using nanocarrier-based delivery methods, vaccine antigens could be encapsulated in the nanoparticles or decorated on the surface for antigen presentation with more precision, improving stability, protecting vaccines from early degradation and having superior adjuvant qualities (Table 2).

Nanoparticles-based vaccines against various animal and human respiratory tract infections against bovine parainfluenza virus, swine influenza virus, influenza virus and respiratory syncytial virus (RSV) etc. are being manufactured. Nanotech-based vaccines are injectable or inhaled formulations that may or may not need a cold chain, are fast acting with better safety and usability characteristics compared to traditional vaccines like the live attenuated vaccines, inactivated vaccines, nucleic acid based vaccines, subunit vaccines, recombinant viral vaccines, carbohydrate vaccines, conjugated vaccines, and toxoid vaccines.[173] Viral infections having pandemic potential still pose a huge threat to human health globally despite the path-breaking advances in science and technology. Nanobased technologies could further facilitate to resolve the key antiviral treatment related issues.

Diversified nanoparticles are poised to revolutionize especially the medical sciences, while the safety assessment remains challenging. Nanoparticle absorption is a vital assessment of the safety of nanobased products. Studies show that nanoparticles influence immunity and the respiratory tract, could create oxidative stress, and could even be genotoxic.[174,175] Nanoparticle uptake is dependent on its physicochemical characteristics intrinsically and on the organism's health status extrinsically.

Success of nanobased formulations developed specifically against COVID-19 and MERS-CoV as delivery carriers, vaccine transfer platforms, and as nanodrugs has encouraged research in antigen and adjuvant delivery, antigen agglomeration prevention, and improved immune responses in booster immunization.[176] Ag-NPs with stabilizers were effective with anti-TGEV action, and the Ag_2S nanoclusters prevented RNA synthesis and budding as for instance in porcine epidemic diarrhea vaccine (PEDV). Ag-NPs as inhalable nanotherapeutics against SARS-CoV-2 have also been suggested. Their stability, surface functional chemistry, and biocompatibility make Au-NPs good antigen conjugation candidates. Non-metallic NPs like the organic curcumin carbon dots (CCM-CDs) and polylactic-*co*-glycolic acid (PLGA) are demonstrably effective against PEDV.

SWOT Analysis. Keeping the growing demand particularly in medical applications and their universal value in view, nanoadjuvants offer tremendous prospects in biobusiness in international trade and commerce.[177−179] In light of this, it is essential to discuss and analyze the strengths and opportunities (as the positive factors), and weaknesses and threats (as the negative factors) of the commercial implications of nano-adjuvant-based vaccine development with the former as either a vaccine carrier or as a performance elicitor. Thus, a schematic presentation of the SWOT analysis is provided in Figure 5 for better and comprehensive understanding for readers. Needless to mention, the strengths are internal positive factors, the

Table 2. Application of Nanocarriers in Treating Respiratory Diseases

nanocarrier	immunogen/antigen	disease/ etiological agent	reference
Liposome	Polysaccharides	Pneumonia	131
	Bacterial toxin and parasitic protein	Cholera and Malaria	132
	Fusion protein	*Helicobacter pylori*	133
	M. Tb Fusion protein	Tuberculosis	134
Chitosan	Live virus	Newcastle disease	135
	Hep B surface protein	HepB	136
			137
	Mycobacterium lipids	Tuberculosis	138
	T-cell epitope and tyrosine kinase-3 ligand	Tuberculosis	139
Gold nanoparticle	Viral protein	FMV	140
	Membrane protein	Influenza	141
	Plasmid DNA	HIV	142
Poly(D,L-lactic-*co*-glycolic acid) nanosphere	Antigenic protein	Anthrax	143
	Tetanus Toxoid	Tetanus	144
	Hep B surface antigen	HepB	145
VLPs	Capsid protein	Norwalk virus infection	146
			147
	Influenza virus structural protein	Influenza	148
			149
			150
			151
			152
			153
	Nucleocapsid protein	Hepatitis	154
	Fusion protein	Human papilloma virus	155
			156
			157
			158
			159
	Multiple proteins	Rotavirus	160
			161
	Virus Proteins	Blue tongue virus	162
	Enveloped single protein	HIV	163
			164
			165
			166
Nanoemulsion	Antigenic protein	Cystic fibrosis	167
		Anthrax	168
Polypeptide nanoparticle	Viral protein	SARS	169
	Plasmodium berghei circumsporozoite protein	Rodent malarial parasitic infection	170
Iron oxide nanoparticle	Merozoite surface protein	Malaria	171
ISCOMs	Surface protein from Eimeria falciparum sporozoites	Diarrhea	172

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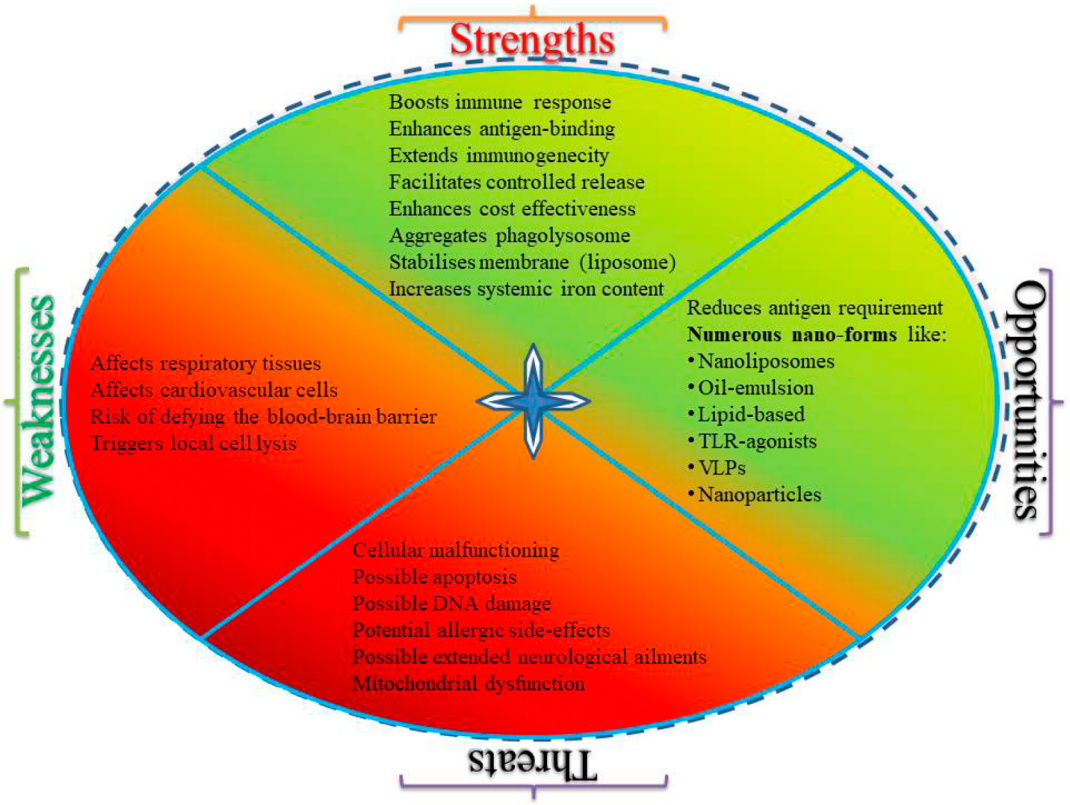

Figure 5. SWOT analysis.

opportunities are external positive factors, the weaknesses are internal negative factors, and the threats are external negative factors.

It is also pertinent here to elaborate on and dissect the two terms mentioned in the title of the article, which are "bioinspired" and "biomimetic" approaches. The biomimetic approach often falls under radical innovation, and its consequences could be revolutionary. On a broader sense, biomimicry focuses on inspiration, ideation, and education to reconnect with nature primarily with a sustainability (global sustainable development) goal in focus. While biomimicry is about precise imitation of a biological system, bioinspiration seems more encompassing on the other hand, considering the artificial synthesis of a material inspired by a natural system. Essentially, it synthetically mimics a natural system. In order to ensure the needed sophistication and function level are on par with those of a biological system, bioinspiration obviously needs extensive research and validation.

■ CONCLUSION AND FUTURE DIRECTIONS

Adjuvants other than those based on alum include oil emulsions, TLR agonists, and surfactin. Such nontraditional adjuvants could affect the metabolic state of the host though they are successful in eliciting an immune response. Increased ROS production is a common side-effect in them that leads to activating inflammatory pathways mediated by the NF-κB transcription factor. An increase in ROS is also a cellular signal to activate the pathways associated with autophagy and mitophagy in which the cell or dysfunctional mitochondria are cleared off, leading to reduced cell viability. Endogenous danger signals also are potent activators of inflammasome pathways like NLRP1 and NLRP3 which further increase the metabolic dysfunction and inflammatory load.

Despite increasingly favorable evidence of the potential of nanoparticles as a drug delivery vehicle, there are only a few such carrier molecules and vaccines designed so far. Nano-based vaccine strategies majorly work on stimulating the APCs and DCs in antigen uptake and processing, thereby stimulating both humoral and cell-mediated immunity. Owing to the lack of adequate data, nanomolecule-based vaccines and delivery systems have failed to gain acceptance in pharmaceutical market although they appear extremely beneficial.[180] Thus, it is increasingly vital to augment studies that attempt to understand the characteristics of nanobased vaccines such as their administration route, interaction with host biomolecules, general and immunological toxicity, bioavailability, inter- and intracellular signaling, and cellular uptake mechanisms, among others.

The immense potential of biologics and biosimilars in biopharmaceutical products in general and vaccines development in particular has been recognized in recent times and highlighted.[181] Similar, or even better, has been the scenario for the applications of bioinspired and biomimetic nanomaterials especially in vaccine research and development. As lessons learned during the recent COVID-19 pandemic, keeping in mind the potential increase in the number and the intensity of the evolving strains of the infectious viral entities, dedicated research and development in vaccines is emergent and urgent. This review shall surely provide researchers a bird's-eye-view of recent trends in a compiled manner, and also as essential leads for furthering their research endeavors.

■ MATERIALS AND METHODS

The study began with a systematic search of electronic/digital databases like PubMed, Scopus, and the Web of Science for the

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recent relevant literature. It included searching the databases for all original scientific publications in English without date limit, with strings as nanoadjuvant, nanoadjuvant in vaccine discovery, nanotechnology in vaccine development, aluminum-assorted adjuvant, nanoparticle as adjuvant, oil emulsion adjuvant, TLR agonist adjuvant, nanoliposome, lipid nanoparticle, virus-like particle, adjuvant in human vaccine development, cellular effect of nanoadjuvant and nanoadjuvant in treating respiratory disease.

Two of the designated coauthors independently read the titles and abstracts and collated the literature to include in the work. Other authors joined to exclude the seemingly irrelevant or less meaningful articles that could potentially defocus the writeup. While facts and figures from each selected article were meticulously extracted, one author independently reviewed those to ensure the veracity, accuracy, and relevance. 181 articles were shortlisted from a total of more than 994 searched, references validated, and listed manually.

It followed critical dissection of the articles, corroboration of facts and figures, secondary data analyses and synthesis, building-up a free-flowing focused article, and giving a contextually meaningful title. Thus, the workflow followed was Identify the relevant database → search for literature online systematically → collate literature → exclude less meaningful (seemingly less veracity, accuracy, and irrelevant) articles → critically dissect selected articles → corroborate the facts and figures → analyze and synthesize the presented data → construct a free-flowing focused article → give a contextually meaningful title to the final writeup → validate references → list the references manually.

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DND, **RV**: Conceptualized and provided the draft framework; **BG**, **PM**: Did the initial literature search and compilation; **BG**, **AM**, **PM**, **PP**, **NCM**, **AJO**, **VK**, **AAR**: Drafted the major portion of the paper and provided necessary technical inputs in the tables and figures; **SM**, **RKM**: Spearheaded the work from front, corrected the technical inputs and the language in the draft, and added valuable and necessary inputs for the completeness of the final draft. All authors have seen the manuscript, participated in the revision, and approved it for publication.

Notes

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