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Combining different types of multifunctional liposomes loaded with ammonium bicarbonate to fabricate microneedle arrays as a vaginal mucosal vaccine adjuvant-dual delivery system (VADDS)

Ning Wang [a], Yuanyuan Zhen [b], Yiguang Jin [c], Xueting Wang [b], Ning Li [b], Shaohong Jiang [b], Ting Wang [b,*]

[a] School of Biological and Medical Engineering, Hefei University of Technology, 193 Tun Brook Road, Hefei, Anhui Province 230009, China
[b] School of Pharmacy, Anhui Medical University, 81 Plum Hill Road, Hefei, Anhui Province 230032, China
[c] Department of Pharmaceutical Sciences, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, China

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ABSTRACT

To develop effective mucosal vaccines, two types of multifunctional liposomes, the mannosylated lipid A-liposomes (MLLs) with a size of 200 nm and the stealth lipid A-liposomes (SLLs) of 50 nm, both loaded with a model antigen and NH_4HCO_3, were fabricated together into microneedles, forming the proSLL/MLL-constituted microneedle array (proSMMA), which upon rehydration dissolved rapidly recovering the initial MLLs and SLLs. Mice vaccinated with proSMMAs by vaginal mucosa patching other than conventional intradermal administration established robust antigen-specific humoral and cellular immunity at both systemic and mucosal levels, especially, in the reproductive and intestinal ducts. Further exploration demonstrated that the MLLs reconstituted from the administered proSMMAs were mostly taken up by vaginal mucosal dendritic cells, whereas the recovered SLLs trafficked directly to draining lymph nodes wherein to be picked up by macrophages. Moreover, the antigens delivered by either liposomes were also cross-presented for MHC-I displaying by APCs thanks to lysosome escape and ROS (reactive oxygen species) stimulation, both of which occurred when lysosomal acidifying the liposome-released NH_4HCO_3 into CO_2 and NH_4^+/NH_3 to rupture lysosomes by gas expansion and to cause ROS production by excessive ammonia induction, resulting in a mixed Th1/Th2 type response which was also promoted by liposomal lipid A via activation of TLR4. In addition, vaginal vaccination of the engineered HSV2 antigen gD-loaded proSMMAs successfully protected mice from the virus challenge. Thus, the proSMMAs are in fact a vaccine adjuvant-dual delivery system capable of eliciting robust humoral and cellular immunity against the invading pathogens, especially, the sexually transmitted ones.

1. Introduction

Mucosal immunity is critical for protection against various pathogens which often invade hosts by way of mucosa covering the respiratory, gastrointestinal, or reproductive tracts and can be established by mucosal vaccines administered to mucosa. It is now regarded that mucosal vaccines not only induce strong immune responses concentrated at the application site but also contribute to establishing immunity throughout mucosal systems, including even distal mucosal network, due to cross talk between different mucosal compartments enriched in antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, and mucosa-associated lymphoid tissues (MALTs) [1]. Ideally, mucosal vaccines are expected to have the ability to stimulate the immune system to produce not only sufficient pathogen-neutralizing antibodies (Abs) but also powerful pathogen-specific cytotoxic T lymphocytes (CTLs) [2], which have the intrinsic capacity to eradicate the host cell-hidden invaders through binding to and releasing cytotoxins and chemicals into the infected cells causing eventually apoptosis in them [3]. So far, though the detailed mechanisms underlying the immune protection against many mucosal infections are not well elaborated, it argued that mucosal vaccination can induce production of effector lymphocytes imprinted with the mucosa-homing

Abbreviations: Ab, antibody; Ag, antigen; APC, antigen-presenting cells; DC, dendritic cell; BMDC, bone marrow-derived dendritic cell; MP, macrophage; BMMP, bone marrow-derived macrophage; CTL, cytotoxic T lymphocyte; dLN, draining lymph node; ER, endoplasmic reticulum; MALT, mucosa-associated lymphoid tissue; PAMP, pathogen associated molecular pattern; MPC, mannose-PEG-cholesterol; SPC, soy phosphatidylcholine; SA, stearylamine; OVA, ovalbumin; MAIM, microneedle array inverse mold; MPLA, monophosphoryl lipid A; MLL, mannosylated lipid A-liposome; SLL, stealth lipid A-liposomes; MA, microneedle array; proSMMA, pro-SLL/MLL-stacked microneedle array; OCT, optimal cutting temperature; MD, mean diameter; AE, association efficiency; CTC, controlled temperature chain; ROS, reactive oxygen species; VADDS, vaccine adjuvant-dual delivery system; v.p., vaginal mucosa patching; i.d., intradermal; v.i., vaginal instilling with a pipette; HSV2, herpes simplex virus type 2; gD, glycoprotein D of HSV2 surface antigen.

* Corresponding author.
E-mail address: twangcn@hotmail.com (T. Wang).

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properties by mucosa-derived APCs to seed the mucosa to establish the resident memory lymphocytes, which, together with the circulating memory lymphocytes induced by the subsequent infection, will confer the full protection against pathogens from mucosal invasion [4].

Vaginal cavity is newly found a site suitable for inoculation of mucosal vaccines to prevent sexually transmitted pathogens [5], based on the fact that it has a mild environment, large surface area, the administration convenience and less safety concern, compared to other mucosal sites, such as nasal cavity and gastrointestinal lumen, where vaccination may lead to, possibly, the severe CNS injury or the active ingredient loss [6]. However, vaginal mucosa, like other ones, is also covered with a defending layer of mucus which is a continuously renewed viscous fluid containing various categories of agents, such as antiseptic lysozyme enzymes, proteins and glycoprotein mucins, imposing a potential damage to vaccine antigens and preventing mucosal vaccines from approaching and crossing the epithelial layer, under which the professional APCs reside [7]. Moreover, vaginal mucosa has a stratified squamous epithelium (SSE) possessing the densely-lined epithelial cells with intercellular spaces sealed by tight junctions to form a tough barrier for mucosal vaccines to get access to the professional APCs which are the necessary sponsors for most immunoresponses [8]. To conquer these barriers, numerous technologies have been developed, including incorporating bioadhesive polymers such as chitosan and starch to the vaccine carriers, to achieve prolonged mucosal contact and high agent concentration on the mucosal surface [9]; using permeation enhancers to enhance the bioavailability of protein-containing nanoparticles delivered via mucosa route [10]; modifying nanoparticles with PEG to form so-called mucus-penetrating particles just for sustained topical delivery of agents [5], though PEGylation is argued to be a barrier to accessing particles by most types of cells [11]. Recently, researchers employed biodegradable microneedle arrays (MAs) for delivery of subunit vaccines via oral mucosa patching [12,13], which has been demonstrated to effectively eradicate the above obstacles to mucosal vaccination. In contrast to other methods [6], using MA for mucosal vaccine delivery has a big benefit in that the dose of vaccine that actually enters the body can be accurately controlled, because MA delivery can surpass the mucosal barriers set up by the host as defense works, avoiding the dilution of agents in mucosal secretions and the capture of ingredients in mucus gels, the damage of labile Ags by proteases and nucleases and exclusion of vaccines by epithelial cells [14–16]. Although oral mucosal vaccination with MAs elicited robust mucosal immunity in oral cavity and intestinal lumen, the induced immunoresponse in reproductive duct was too moderate to set up an effective defense against sexually transmitted infections, such as HSV, HIV and HPV. This holistic immunity profile established by mucosal MA vaccines drives us to consider a trial of vaginal vaccination using a MA vaccine which should be elaborately fabricated to fit the vaginal anatomic feature [17].

MAs were first introduced as an effective tool for painless transdermal delivery of drugs and vaccines by Prausnitz et al in the mid-1990s and have ever since widely been employed for improving delivery efficiency, recipient compliance and the convenience of, even self-finished, administration [18,19]. However, the requirements to MAs of no pain-causing and efficient tissue-penetrating remarkably limit the size and density of microneedles and, ultimately, the loading capacity of a MA, exposing in MA application actually a practical dilemma, which may be addressed by enhancing the potency of vaccines to spare the required vaccination dose [14]. It is now recognized that, to have a high potency, mucosal vaccines may be elaborately designed into a well-constructed nanocarrier possessing several structural and functional characteristics, such as being efficiently taken up by a large number of APCs, engendering Ags lysosome escape and cross presentation, and activating TLRs with the carried adjuvants to modulate the immunoresponse pathways [20]. Thus, vaccines may be formulated into a stealth nanocarrier being able to traffic, along with flowing fluids and lymph, directly from inoculation sites to draining lymph nodes (dLNs) wherein to be efficiently picked up by a great number of the densely-clustered APCs. Alternatively, vaccines may be delivered with APC-targeting carriers to be captured at the administration mucosa by the sparsely lurked local APCs which then carry them through migration to dLNs, providing a pathway, though inefficient as a whole in vaccine delivery for function, actually capable of imprinting lymphocytes with the receptors specific to mucosal chemokines to generate mucosa-homing immune cells contributing to the establishment of mucosal immunity [21]. It is demonstrated that the vaccine carriers, including various stealth nanoparticles [22], polymeric micelles, and even the macromolecular conjugates capable of noncovalently associating with and thereby 'hitchhiking' on the endogenous proteins, can deliver in a high efficiency the loaded antigen/adjuvant from the inoculation site to dLNs [23], where the immune responses are highly orchestrated, to enhance greatly the efficacy of a vaccine and thus spare remarkably the antigen dose [24]. Another way to spare vaccine dose is saving the endocytosed Ags from cellular endo-lysosomal degradation, which may be realized by using the functional nanocarriers that are constituted with the ability to cause rupture or leakage in endo-lysosomes before Ag degradation occurrence to render Ags lysosome escape. A variety types of vaccine nanocarriers have been developed to engender lysosome escape based on different mechanisms, such as pore formation in the endosomal membrane, fusion with the lipid bilayer of endosomes, pH-buffering effect of protonable groups [25]. Recently, ammonium bicarbonate (NH_4HCO_3) was encapsulated in PLGA nanoparticles by researchers for constituting the pH-responsive vaccine carriers based on a hypothesis that was proposed by the authors as that acidification of the entrapped NH_4HCO_3 would result in production of ammonia (NH_3) gas and carbon dioxide (CO_2) gas, which would subsequently cause PLGA particle rupture and rapid Ag release [26], completely ignoring two basic facts: one as that acidification of NH_4HCO_3 produces mainly the ammonium ions (NH_4^+) and carbon dioxide (CO_2) gas, and the other that encapsulation in particles separates indeed the interior NH_4HCO_3 from outside protons (H^+), thus preventing the expected acidification of the entrapped pH-sensitive chemicals into gas molecules. Nevertheless, this marvellous piece of work inspired us to further explore using the NH_4HCO_3-sourced gas (CO_2) as a driving force to break up endo-lysosomes to achieve lysosome escape for intracellular Ags delivered by nanocarriers.

In this investigation, two types of the Ag- and NH_4HCO_3-loaded nanovesicles, the mannosylated lipid A-liposomes (MLLs) of 200 nm and the stealth (pegylated) lipid A-liposomes (SLLs) of <100 nm, were fabricated into biodegradable microneedles forming the proSLL/MLL-constituted microneedle array (proSMMA) for vaccine delivery. The design was based on the facts that vaccination of proSMMAs by vaginal mucosa patching (v.p.) implants the nanoparticulate vaccines into mucosa by piercing mucus and stratified squamous epithelia (SSE) recovering the initial MLLs and SLLs; while MLLs deliver the ingredients to mucosal APCs by mannose moiety targeting favouring the establishment of mucosal immunity, SLLs by PEGylation preventing matrix binding and local APC capturing will carry the ingredients directly to the LN APCs which possess relatively high phagotrophic abilities [27] (Fig. 1). After APC uptake, the endo-lysosome-wrapped liposomes will disassembly due to phospholipase-catalyzed lysis to release pH-sensitive NH_4HCO_3 [26], which upon lysosomal acidification immediately decomposes into CO_2 gas and NH_4^+/NH_3 to rupture lysosomes by gas swelling engendering lysosome escape and to stimulate production of ROS by excessive ammonia induction [28], facilitating the antigenic peptide for MHC-I presentation, owing to ROS causing oxidized damages of intracellular materials to release "danger" signals promoting APC maturation and enhancing activation of APC proteasomes for exotic antigen process and cross presentation [29]. Thus, the robust humoral and cellular immunity to the loaded antigens may be set up under the additional promotion of liposome-loaded lipid A via activating APC pattern-recognition receptor of TLR4 (toll-like receptor 4) to guide the adaptive response toward Th1 pathway [30]. Additionally, in this investigaiton, the surface antigen gD of herpes simplex virus 2 (HSV2) was also loaded in the proSMMAs, which successfully protected mice via vaginal vaccination against the virus challenge using HSV2, a virus which sexually