Review

Poly-*N*-acetyl Glucosamine Gel Matrix as a Non-Viral Delivery Vector for DNA-Based Vaccination

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Abstract. *Intramuscular administration of plasmid DNA vaccines is one of the main delivery approaches that can generate antigen specific T cell responses. However, major limitations of the intramuscular delivery strategy are the low level of myocyte transfection, resulting in a minimal level of protein expression; the inability to directly target antigen presenting cells, in particular dendritic cells, which are critical for establishment of efficacious antigen-specific immune responses. Although several viral vectors have been designed to improve plasmid DNA delivery, they have limitations, including the generation of neutralizing antibodies in addition to lacking the simplicity and versatility required for universal clinical application. We have developed an inexpensive non-viral delivery vector based on the polysaccharide polymer poly-N-acetyl glucosamine with the capability to target dendritic cells. This vector is fully biocompatible, biodegradable, and nontoxic. The advantage of the application of this delivery system relative to other approaches is discussed.*

Cancer immunotherapy is a promising means for treatment of cancer and prevention of recurrence through generation of T cell memory responses. The recent identification and characterization of genes coding for tumor-associated antigens has enabled the design of antigen-specific cancer vaccines based on the use of plasmid DNA (pDNA) (1). Successful DNA-based vaccination depends not only on targeting antigen delivery to dendritic cells (DCs) but also on the activation status of these cells (2). Although immature DCs are efficient in antigen uptake, they are poor in the antigen presentation process, leading in most cases to T cell tolerance and generation of regulatory T cells (3, 4). Therefore, activation of DCs with inflammatory cytokines such as IL-12, which induces DC maturation (5), is essential for DCs to cross-prime antigen to T cells and generation of effective immunity (6). Thus, induction of inflammatory cytokines at the site of injection to induce activation of DCs is an important requirement for efficient immune response.

Although intramuscular (i.m.) administration of DNA vaccines is the main delivery method (7), it induces low levels of myocyte transfection, resulting in a minimal level of protein expression, and is incapable of directly targeting antigen presenting cells such as DCs (2), which are essential for the antigen-specific immune responses. Several viral vectors have been designed to improve pDNA delivery (8). However, the generation of neutralizing antibodies, in addition to their lacking the simplicity and versatility, limits their universal clinical application. Non-viral vectors have been developed to overcome these hurdles (9-12); however, only modest progress has been achieved in the development of a reliable and inexpensive technology with the capability to target DCs. In the following sections, the specific limitations of viral and non-viral vectors that limit their application in DNA-based vaccination are listed, and a new vector is discussed with its advantages that present it as a potential non-viral DNA delivery vector.

Limitations of DNA-based vaccination using naked DNA. DNA vaccines encoding a variety of antigens have been shown to induce cell-mediated immune responses resulting in a measurable anti-tumor immunity. Several parenteral methods of administration of DNA vaccines have been used, including

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needle injection into muscle or skin and DNA-covered particle bombardment using a 'gene-gun' (2). Delivery of a DNA vaccine via the i.m. route, the most popular method, represents a simple and effective means of inducing both humoral and cellular immune responses (13). There are three potential pathways responsible for antigen presentation after i.m. injection of DNA. First, muscle cells may take up the DNA, express the encoded protein antigen, and present it to T and B cells (14). Second, DCs attracted to or already at the site of injection may take up the DNA, express the encoded protein, and then perform MHC class I and class II-restricted presentation of antigen to T and B cells (6, 15). Third, myocytes may take up the DNA and express the protein antigen and then transfer it to professional antigen presenting cells such as DCs for subsequent presentation (1). The transfection efficiency of the i.m. delivery of pDNA, however, is low due to the rapid degradation of DNA by nucleases and poor cellular uptake when delivered in aqueous solutions. The i.m. delivery of DNA also does not directly target DCs, neither does it activate significant number of these cells. Delivery of antigen into DCs in the absence of inflammatory signals, however, induces tolerance rather than stimulation of immune responses. This can explain the very modest immunogenicity in cancer patients after vaccination with naked DNA (16-18). Large quantities (5-10 mg) of DNA are required to induce only modest immunogenicity (19-21). Therefore, developing a DNA delivery system that can protect DNA from degradation and sustain its delivery, and with the capability to target and elicit DCs activation and maturation would significantly improve DNA-based vaccination.

Limitation of DNA-based vaccination using viral vectors. The limitation of naked DNA vaccines has stimulated the development of vectors, including viral systems such as retroviruses and adenoviruses, or non-viral systems such as liposomes and polymers. Although viral vectors have shown high transfection efficiency as compared to non-viral vectors (22, 23), they have a number of drawbacks.

• The pre-existence of T cell and antibody-mediated immunity to the viral vector limits the ability of subsequent administration of recombinant viruses to further boost immune responses (24-27). Although the heterologous prime-boost vaccination regimen, in which the same antigen is delivered in sequence using different vectors, can overcome the generation of neutralizing antibodies against the repeated vaccination with the same vector (28), this approach is laborious and requires preparation of large amounts of different vectors which raises safety concerns during preparation and after administration.

• Administration of recombinant viruses induces immune responses toward the viral vector proteins, reaching about a 20-fold higher level than those induced against the transgene itself (29, 30). This antigen competition limits the immune responses to the transgene itself.

• Viral-based delivery of recombinant vaccines has a risk for the interaction of a vector and a host-cell genome.

• Most of the viral vectors are degraded by serum nucleases and consequently almost 90% of injected viral vectors are degraded within 24 hours even before reaching the target cells (31, 32).

• Lytic viral vectors, including human adenovirus and herpes simplex virus families, destroy the infected cell after replication and virion production (8, 12).

• Some viral vectors such as retroviral vectors have low transfection efficiency due to their inability to transduce non-dividing cells (8, 12).

• Some viral vectors such as adenoviruses induce short term expression, limited transduction of the cells with reduced or no expression of attachment and internalization receptors, transient expression of foreign antigen genes, and immunogenicity (33).

Limitation of DNA-based vaccination using non-viral vectors. Non-viral vectors, including lipids (lipoplex), synthetic polymers (polyplex) (9, 10), and chitosan (34), although characterized by low toxicity, compatibility with body fluid, the possibility of tissue-specific gene transfer, unlimited clone capacity, and immunogenicity (35-38), have a number of limitations:

• The construction of lipoplexes is very demanding and needs formulation of DNA into the vehicle (9, 10).

• The interactions of liposomes with DNA and the subsequent lipoplex formation are dependent on several physical factors such as pH and charge as well as structural characteristics of the liposomes (9, 10).

• Similarly, polyplexes consist of cationic polymers and their production is regulated by ionic interactions. In addition, most of the polyplexes are not able to release intracellular DNA into the cytoplasm, and thus require co-transfection with endosome-lytic agents (inactivated adenovirus) (39).

• The kinetics and efficiency of lipoplex and polyplex assembly could affect the structural integrity of the pDNA and thereby transfection efficiency, resulting in inefficient wrapping of plasmid into the lipoplex shell and thus affect the interaction of lipoplexes with cell surfaces. Therefore, even though a high lipoplex-mediated delivery of genes to the nucleus can be accomplished, it can be associated with a very poor transcription (40).

• The level of transduction using lipoplexes is relatively low due to their rapid clearance from the circulation (9, 10).

• Chitin- and chitosan-based products have been advanced for a wide variety of applications, including DNA delivery. However, medical product development with these materials has been hampered by the chemical and physical heterogeneity of the polymer products and contamination of preparations by proteins and other components (41).

F2 gel matrix as a potential non-viral delivery vector. Defining a non-viral vector that can induce higher transfection efficiency than those induced by viral vector but without inducing toxicity

is of a great significance. Ultimately, the ideal feature of a vector for DNA delivery includes reducing the frequency of administration of the integrated components, while maintaining the therapeutic concentration at a desired level, and targeting and activating DCs. As stated above, applications of viral and non-viral vectors for the delivery of DNA are limited in the gene therapy in general and in the vaccination setting in particular. The inability of these approaches of targeting DCs due to the lack of induction of inflammatory microenvironment at the injection site represents a barrier to advance the field of DNA-based vaccination. Avaccine delivery system utilizing a gel generated from poly-*N*-acetyl glucosamine nanofibers (p-GlcNAc) purified from marine diatom cultures has recently been characterized (42).

The main component of F2 gel matrix is a partially deacetylated high molecular weight (MW=2.8×10^6 Da) linear polymer derived from fully-acetylated poly-*N*-acetyl glucosamine (pGlcNAc) nanofibers. The individual polymers in the fibers are tightly bound to one another by interchain hydrogen bonding in a parallel (β-structure) orientation. P-GlcNAc fibers are generally 80-100 μm in length with 2-4 nm diameter (43), and consist of ~80 polymer molecules per fiber. The β-pGlcNAc fibers are isolated from microalgal cultures in a highly pure chemical form with preservation of the native supramolecular structure (41). Microalgal pGlcNAc fibers are the single and unique component of several FDA cleared medical device products currently in the topical hemostasis market (43-48). β-pGlcNAc fibers can be disassociated into their individual poly-*N*-acetyl glucosamine polymer components with strongly chaotropic (hydrogen-bond breaking) solvents. Also, microalgal-derived β-pGlcNAc fibers can be deacetylated to varying extents to obtain soluble, partially deacetylated or fully deacetylated cationic polymers that can be formulated as hydrogels (F2 and F3 gels, respectively). Comparison of the properties of the poly-*N*-acetyl glucosamine fiber-derived materials with chitin, chitosan, and commercial chitosan-based products showed that β-pGlcNAc material has a unique ultrastructure in comparison to the other materials; a fine (~50 nm diameter, ~80-100 um length) fibrous structure. The β-pGlcNAc fibers in FDA approved haemostatic products are organized in nonwoven fabric patches and resemble the dimensions of natural fibrin networks (43).

P-GlcNAc has been carefully evaluated and found to be fully biocompatible, biodegradable, and nontoxic. Membrane patch and lyophilized pad formulations of p-GlcNAc nanofibers have been FDA approved and are in commercial production as a topical hemostatic agent (41-49). A particular formulation of the p-GlcNAc polymer gel matrix, designated the F2 gel, possesses unique properties that are ideal for the *in vivo* delivery of pharmaceuticals, and biological response modifiers such as 5-fluorouracil, taxol and erythropoietin (50-52). F2 gel can also be formulated into a stable matrix in combination with antigenic peptides and GM-CSF, IL-2, IL-12 cytokines (51-54). Importantly, formation of an F2 gel emulsion does not require vigorous sonication, and labile proteins are efficiently incorporated without denaturation. F2 gel loaded with these cytokines can create a vaccine delivery system capable of providing the sustained release of antigenic peptide and cytokines *in vivo*, establishing a potent microenvironment for antigen presentation (54) through stimulation of macrophages and DCs to produce the inflammatory cytokines IL-12, TNF-α, and IFN-α (42, 51, 55). This results in enhanced antigen-specific $CD8^+$ T cell responses and protection from lymphoma, melanoma, and mesothelioma tumors as compared to vaccination with systemic cytokine administration (42, 49-51, 53, 54, 56, 57). F2 gel-based vaccination has been used in the schistosomiasis infection setting. Schistosomiasis is a tropical diseases caused by the trematode *Schistoma mansoni*, resulting in liver fibrosis and failure due to the deposition of the egg and the formation of granulomatous reaction (52). It was found that vaccination with F2 gel matrix loaded with both IL-12 protein and an adult worm antigen can lead to beneficial effects toward amelioration of the granulomatous reaction in the liver as compared to the systemic delivery of IL-12 and the adult worm antigen (52), confirming that the use of F2 gel matrix as a potential non-viral delivery vector of vaccine components such as peptides and proteins.

Studies have shown that F2 gel matrix can also be used as a vector for DNA-based vaccination (58). It was found in a non-transgenic mouse model that incorporation of IL-12 into the F2 gel matrix loaded with DNA plasmid can dramatically enhances the immune response to the coded antigen (58). In these studies, naked DNA using HIV-1 gag DNA plasmid was used as a model, integrating HIV p37 gag DNA plasmid into F2 gel matrix with or without IL-12 p70 protein and then vaccinated naive BALB/c mice were vaccinated with gag pDNA; gag pDNA/IL-12 pDNA; F2 gel/gag pDNA; F2 gel/gag pDNA/IL-12 vaccines. It was found that vaccination with F2 gel/gag pDNA/IL-12 was far superior to vaccination with gag pDNA/IL-12, resulting in higher levels of the Th1 cytokines IFN-γ after single and two vaccinations. It also induced higher levels of the Th1 cytokines IL-2 and TNF-α, but lower levels of the Th2 cytokine IL-5 measured by the effector cells measured 2 week after vaccination (58). Of note, F2 gel-based DNA delivery was more effective than F2 gel-based protein delivery to induce immune responses as evidenced by the higher numbers of IFN-γ-producing cells. Taken together, these results suggest that the F2 gel matrix has potential as a non-viral delivery vector not only for proteins but also for naked DNA.

Advantages of F2 gel matrix as a unique non-viral delivery vector. Previous studies showed that the F2 gel acted not only as a slow-release depot system, but also as a trigger for inflammation resulting in influx of the inflammatory cell populations, including activated DCs (51, 54). Therefore, F2

gel matrix provides a danger signal via the innate immune system and at the same time serves as a sustained simultaneous release vehicle for antigen and paracrine IL-12 DNA delivery at the vaccine site (57). This creates a potent microenvironment for efficacious antigen presentation, where IL-12 enhances activation of the APCs and IFN-γ production (57). Since systemic toxicity is currently a major limitation to the use of IL-12 in human clinical trials, the lower serum levels of IL-12 (51) associated with its paracrine administration are likely to be associated with a significant reduction in its toxicity (52). The relatively simple and inexpensive production of F2 gel matrix, as well as its efficacy and potential for significant reduction in toxicity, provide rationale for the clinical translation of this approach.

The following advantages of the F2 gel matrix present it as unconventional and exceptionally innovative approach that can overcome the hurdles listed above for the application of viral and non-viral vectors in DNA-based vaccination.

• The polymer component of the F2 gel is inexpensive and has been certified to be fully biocompatible, biodegradable, and nontoxic; a membrane patch and lyophiolized pad formulation of its diatom-derived precursor nanofiber has been FDA approved and is in commercial use as a topical haemostatic agent.

• Formation of an emulsion of F2 gel matrix and pDNA stable for vaccination does not require vigorous sonication, and labile proteins are efficiently incorporated without their denaturation.

• The positive charge of the F2 gel allows interactions with the negatively charged DNA and forms a stable complex. The positive charge of the F2 gel also allows interactions with the negatively charged cell membrane and thus penetration into the cell is permitted.

• Delivery of F2 gel matrix into skin through subcutaneous injection would target DCs.

• It has been speculated that the cytokine microenvironment created by a local virus infection during boosting is responsible for the effective expansion of effector T cells (59-61). Therefore, the subcutaneous delivery of F2 gel matrix integrated with the pDNA is a simple approach to create a local inflammatory microenvironment during transfection, allowing for a high transfection efficiency of DCs.

• Recent studies provided evidence that DNA-based vaccines can be augmented by co-administration of adjuvant cytokines such as IL-12 either in a protein or a DNA form. Based on our studies, the F2 gel matrix can be loaded with multiple DNAs and injected at the same site to target DCs so that both the antigen and the adjuvant will be concomitantly delivered to these antigen presenting cells. Therefore, one strategy could be the use of two plasmids coding for the antigen and the adjuvant IL-12 to maximize the antigen presentation and the overall activation of the immune cells.

• Given the slow biodegradable feature of F2 gel matrix, it allows for sustained release of the integrated pDNA, increasing the transfection efficiency. It also allows for protection of the integrated pDNA from degradation by the serum nucleases.

• Because F2 gel *per se* is non-immunogenic, it allows for repeated DNA vaccination.

• The application of F2 gel-based DNA delivery in clinical setting is a simple approach. It can be performed as an outpatient medical service, and thus allows for a low-cost effective service.

Mechanisms of F2 gel-based DNA delivery. Subcutaneous vaccination with F2 gel matrix loading with pDNA coding for the antigen of interest and the adjuvant cytokine (IL-12) can target DCs in the skin; Langerhan's cells in epidermis and DCs cells in dermis. As was recently reported (51, 54), the advantage of using F2 gel through subcutaneous injection is 3-fold: (i) induction of local microenvironment by F2 gel itself, (ii) transcription of IL-12 in the skin cells; and (iii) delivering the DNA coded for antigen into the skin cells. The advantages of IL-12 were reviewed in a recent article (57). The simultaneous delivery of these signals at the injection site would markedly induce a favorable microenvironment not only for DNA transfection but also for antigen presentation. These proposed features of F2 gel matrix are based on our published and preliminary studies.

Conclusion

The authors believe that the application of the F2 gel matrix is a simple and an inexpensive means for DNA-based vaccination in different cancer settings and infectious diseases. This technology, without a complicated technological processing, would significantly decrease the resources; expenses, labor, and time required for DNA delivery and improve its application without induction of potential toxicity. Accordingly, the clinical practice of DNA delivery in the vaccination setting will be much easier than it has been thought.

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