Nanomed. J. 7(2):98-107, Spring 2020

REVIEW PAPER

Polymer-based nanoadjuvants for hepatitis C vaccine: The perspectives of immunologists

Piyachat Evelyn Roopngam [1, 2*], Tirawat Wannatung [2]

[1]Visiting Professor, Department of Biotechnology, University of Verona, Veneto, Italy
[2]Faculty of Medical Technology, Western University, Kanchanaburi, Thailand, 71170

ABSTRACT

The hepatitis C virus (HCV) is an infection that affects the liver tissues in humans, leading to the development of effective prophylactic and therapeutic HCV vaccines to prevent a global epidemic. Scientists consider it challenging to produce a therapeutic vaccine for the treatment of hepatocellular carcinoma as opposed to a preventative vaccine. However, several drawbacks are involved with a peptide vaccine, including the low immunogenicity of the protein, significant instability, difficulty in delivery, and inefficient presentation of the antigens. Therefore, the investigation of adjuvants (i.e., immunomodulators) to enhance the efficacy of the vaccine is essential. Nanoparticles could potentially serve as vaccine delivery vehicles, acting as adjuvants for the effective transfer of antigens. The safety and effectiveness of nanoparticles and liposomes in modern vaccinology have also been confirmed. Biodegradable nanopolymers such as polyesters, polylactic acid and the copolymers, polyorthoesters, polyanhydrides, and polycarbonates are commonly used owing to their proper qualities in the combination or loading for the prevention of the degradation of the delivered antigens. The present study is specifically focused on the polymer-based nanoparticles that are mostly comprised a poly (amino acid) based copolymer and poly (D, L-lactic-co-glycolide), which could act as adjuvants or potential immunomodulators for the systems providing effective HCV vaccine delivery.

Keywords: *Adjuvants, HCV, Nanoparticles, Vaccine*

How to cite this article
Evelyn Roopngam P, Wannatung T. Polymer-basednanoadjuvants for hepatitis C vaccine: The perspectives of immunologists. Nanomed J. 2020; 7(2): 98-107. DOI: 10.22038/nmj.2020.07.002

HEPATITIS C VIRUS

The hepatitis C virus (HCV) is an infection that affects the liver tissues in humans. The majority of the global population lack awareness of this health threat, which has infected more than 170 million individuals worldwide often through contact with infected blood. According to the World Health Organization (WHO), approximately 3% of the global population has been chronically infected with the HCV [1]. The main forms of HCV transmission are primarily through the sharing of needles, sexual intercourse, wounds, secretions, blood transfusions, sharing of personal belongings, and exposure at work. In addition, mother-to-child transmission in pregnant women is possible [2]. Most importantly, acute liver disease is caused by HCV infection, which has progressed from mild symptoms to cirrhosis and hepatocellular carcinoma (HCC). The HCV also causes chronic hepatitis C, which leads to death due to liver cirrhosis or HCC. Several factors have been associated with the onset of fibrosis, such as the period of the infection, age, gender, and alcohol consumption. In addition to the high costs, the treatment of the HCV is time-consuming, yet rarely successful [3]. Therefore, it is not expected that antiviral therapies would result in the significant reduction of the number of the carriers in the near future [4].

* *Corresponding Author Email: pyachat@live.com*
Note. This manuscript was submitted on October 13, 2019; approved on February 15, 2020

The HCV structure and the structural proteins

The HCV is embodied in a virus that has an enveloped glycoprotein and belongs to the Flaviviridae family of viruses. Its viral genome is a positive, single-stranded RNA with the approximate viral particle size of 60 nanometers. The virus consists of both enveloped and core proteins.

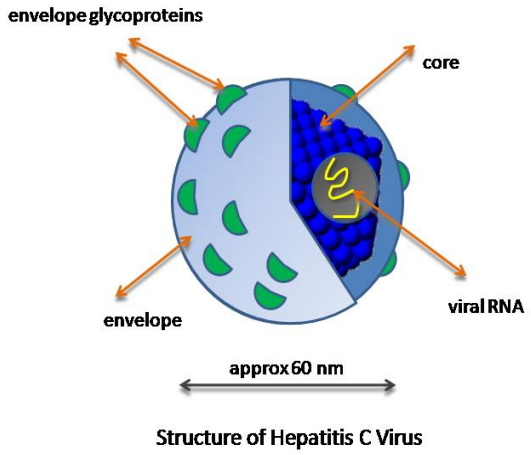

Fig 1. Simplified Structure and Proteins of Hepatitis C Virus Microparticle

Viral glycoproteins are contained in the envelope, and viral RNA is located within the core section of the virus (Fig 1) [5].

The core protein and E1 and E2 enveloped proteins are the major structural proteins of the virus. The mature virion forms the nucleocapsid with the core protein, while the viral enveloped glycoproteins include E1, E2, NS2, NS3, NS4, and NS5, which are known as the non-structural proteins (Fig 2) [6]. Although E1 and E2 are the enveloped glycoproteins, NS2 is the protease, NS3 is the serine protease, and NS5B is the RNA polymerase [7].

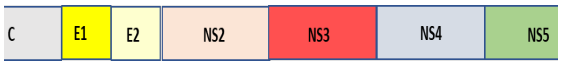

Fig 2. HCV Structural and Non-structural Proteins (C: core structural protein, E1: enveloped protein 1, E2: enveloped protein 2, NS2: non-structural protein 2, NS3: non-structural protein 3, NS4: non-structural protein 4, NS5: non-structural protein 5)

Treatment of the HCV

Although the exact mechanism to inhibit the virus remains unknown, the administration of pegylated interferon or ribavirin and interferon (IFN) to suppress the replication of the HCV is often applied for the treatment of chronic hepatitis C [8]. Moreover, the IFN-related improvement of the host immune response to the cirrhosis virus may partially mediate the clearance [9]. Following that, synthetic materials are used to produce ribavirin (β-D-ribofuranosyl-1H-1, 2, 4-triazole-3-carboxamide), which is a nucleoside analog and an active guanosine that could inhibit the viral structure assembly for the prevention of several viruses *in-vitro*, including Flaviviridae. Progression leading to the cirrhosis of the liver may also be delayed by a combination of interferon alfa-2B and ribavirin [9, 10]. However, 21% of the patients withdrew from the combined therapy prior to the completing 48 weeks of treatment due to the severe complications. Although relapse may occur within 48 hours, the immediate use of interferon could have an impact on the virus, and the agent should be administered three times per week [11]. The limited effects of standard interferon on the virus between doses could be overcome with high molecular weight (40 kDa), containing branched polyethylene glycol (PEG), which is absorbed and cleared at a slower rate [12], resulting in the ability of the IFN to detect the serum of the patients one week following the treatment [13]. The combination of interferon alfa-2B, IFN-α, and ribavirin has yielded more potent outcomes compared to IFN alone through the reduction of hepatic inflammation, which is independent of the virological response. As such, the development of chronic HCV in constant virological responders could be alleviated by IFN-α [14].

HCV Vaccine

Two viral enveloped proteins known as E1 and E2 are found within the HCV [15] with the molecular weight of 33-35 and 70-72 kDa, respectively [16, 17]. These proteins are located in the lipid-protein membrane of viral particles, which contains the nucleocapsid of the viral core protein. The 11 N-linked glycosylation areas, where the most viral particles are conserved, are also the location of highly glycosylated E2.

E2 has hypervariable regions with amino acid sequences, which are approximately 80% different from the HCV genotypes [18]. E2 glycoprotein plays a pivotal role in regulating the interaction between the HCV and viral surface proteins, as well as the binding with the host receptors or human cell membranes (e.g., CD81). Furthermore, an expression of tetraspanin is found in hepatocytes and B lymphocytes [19], as well as various other cells. The truncated forms that are able to bind to the scavenger receptor type B class 1 protein SRB-1 and high-density lipoprotein [20,C 21] are also contained in the CD81. In addition, DC-SIGN and L-SIGN are mannose-binding proteins, which are indicated to have interactions with the E2 proteins of the HCV; however, their role in the

attachment of the virus remains unclear [22] .The HCV E2 possesses glycosylation sites that directly interact with the cell surface receptors, thereby allowing entry to the cell by the virus [23, 24]. The inhibition of the attachment and entry of the virus through inhibiting these proteins is also considered important in the characterization of the E2 envelope [18] and application for *Escherichia coli*-derived hepatitis [25]. To date, no effective HCV therapeutic vaccines have been developed since the majority of chronically infected patients still lack cytotoxic T-cell responses despite their high levels of neutralized antibodies [4, 26] .Therefore, it is essential to produce effective prophylactic and therapeutic vaccines for the HCV in order to prevent a global epidemic [25, 26]. Additionally, scientists consider it challenging to propagate a therapeutic vaccine for the treatment of HCC as opposed to a prophylactic agent. Nevertheless, peptides are still associated with numerous disadvantages, such as the low immunogenicity of the protein, poor stability, delivery system, and inefficient antigen presentation [27].

Therefore, the in-depth investigation of the adjuvants or immunomodulators to increase the efficacy of the vaccine is critical [28].

Adjuvant mechanisms of action

The adjuvant possesses a number of mechanisms that could improve immunity (Fig 3), including the ability to increase the immunological half-life of the immunized antigens and enhancing the delivery of the immunized antigens to the antigen-presenting cells (APCs).

Moreover, the antigens could be processed and presented by the APCs, while effectively triggering the immunomodulatory cytokines (Fig 3).In the successful strategies used for the design of the vaccine, the enhancement of the T-helper type I (Th1) or type II (Th2) immune responses to the vaccine antigens has been achieved using the adjuvant [29].

Several mechanisms of action have been attributed to adjuvants, which should be selected based on the properties of the vaccine and proper administration route.

The mechanism of action for adjuvants is defined as the depot effect found in the gel formation, especially the aluminum hydroxide or emulsion-based adjuvants. For instance, Freund's incomplete adjuvant has been reported to improve the antigen delivery to the draining of the lymphoid tissues, where the immune cells are located, thereby holding the antigens to maintain the immunogenicity of the small antigens, such as the synthetic peptides that are quickly removed from the injection site and draining of the lymph nodes.

Furthermore, the antigen presentation could be improved using adjuvants.

The action of immunological adjuvants could directly or indirectly react to the APCs, especially the APCs that are professional (e.g., dendritic cells) [30].

Moreover, emulsion-based adjuvant MF59 has recently been considered as an emulsion-based adjuvant, which could be internalized by dendritic cells [31].

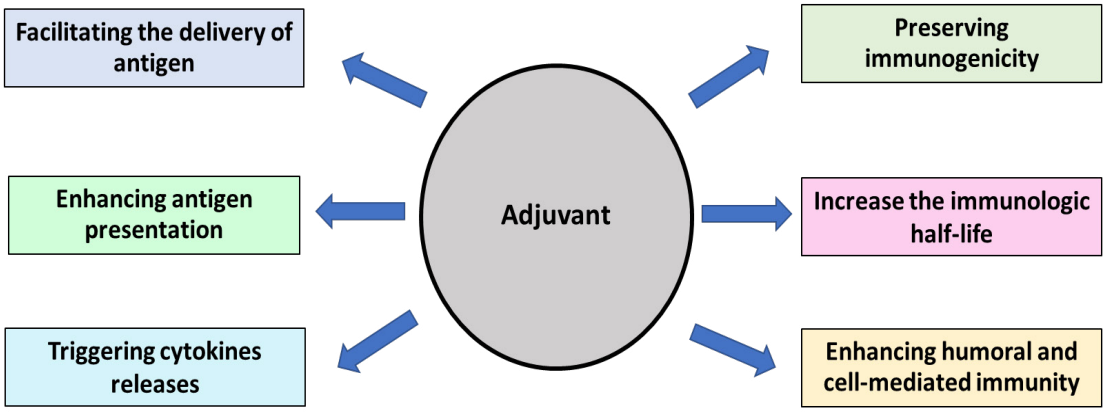

Fig 3. Several Mechanisms Improving Immunity. First, adjuvant is able to extend immunized antigens' immunological half-life; second, adjuvant is able to maintain immunogenicity and enhance delivery of antigens to antigen-presenting cells (APCs); finally, antigen is able to be processed and presented by APCs, and immunomodulatory cytokines are effectively induced

Through bypassing the antigens to cross the membranes of the endosome into the cytosol after digestion of the antigens in the surrounding adjuvant in the APCs, the adjuvants are also able to deliver the antigens to the cytosol and induce antigen presentation via the MHC class I molecules [32]. Targeting the antigens to macrophages or dendritic cells could be facilitated by particulate adjuvants such as liposomes. Therefore, these adjuvants are also able to instigate cytotoxic T-cell responses through the direct delivery of the antigens to the cytosol for antigen presentation via the MHC class I molecules [33]. The adjuvants that are membrane-active could perform the delivery of the cytosolic antigens by conducting antigen presentation, mimicking and appearing as viral infections or immunization using a live attenuated vaccine. The antigen that is delivered alone or in combination with the alum is only able to induce a humoral immune response, while the antigen that is delivered in the cytosol could avoid the process of the endosomal antigen and be presented by the MHC class II molecules [34]. Adjuvants could also trigger the secretion of immunomodulatory cytokines by the APCs. Lymphocytes induce the Th1 or Th2 responses, thereby resulting in the release of some cytokines after they are acted upon by the adjuvants [34].

The release of IFN-γ enables the adjuvants to stimulate Th1 responses. Furthermore, the antibody immunoglobulin G, which binds to the complement and has high affinity to the Fc part of IgG, could be enhanced by the delayed-type (type IV) hypersensitivity.

This mechanism could also improve the function in the complement-mediated lysis, while playing antibody-dependent and cell-mediated cytotoxicity effector roles [35, 36]. The investigation of vaccine adjuvants has been focused on several cytokines, such as the granulocyte-macrophage colony-stimulating factor, interleukin 2 (IL-2), IFN-γ, and IL-12 [37]. IL-12 is a cytokine that has recently been identified as a potential, significant contributor to the immunomodulatory activities of several immunological adjuvants. In this regard, Jankovic et al. conducted a study on mice, reporting that the supplementary IL-12 combined with alum and gp120 of the HIV-1 vaccine could increase the responses of the Th1 cytokines, as well as the IgG2 and IgG3 antibodies [38]. Toxoid bacterial vaccines have demonstrated the activity of adjuvants; such examples are cholera and pertussis toxin, which have been able to enhance the production of the IgA and IgE antibodies, while also inducing Th2-like responses. These adjuvants have also been able to trigger Th2-like immune responses through increasing the production of IgA, which assists in improving the prevention of viral transmission to the mucosal membrane [39, 40-42].

Nano adjuvants

Currently, research on nanovaccine development are underway, especially regarding the biodegradable

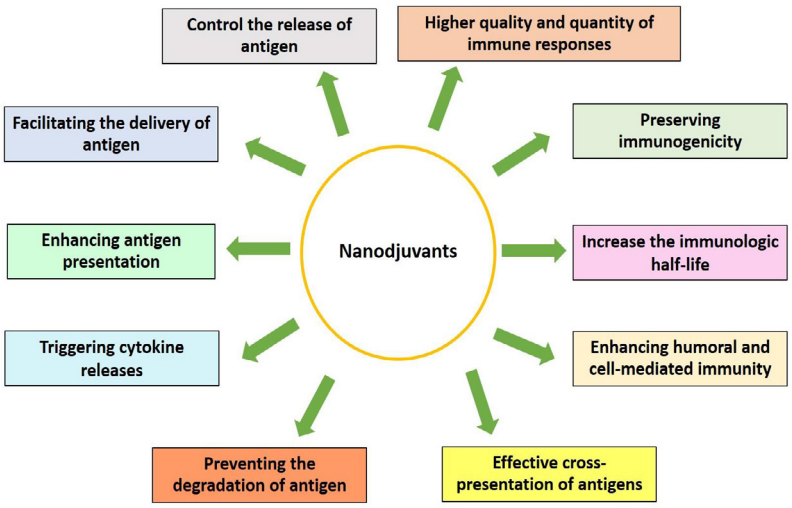

Fig 4. Advantages of Nano Adjuvants (Controlling release of antigens, superior quality, and increased immune responses; They prevent antigen degradation until uptake into immune cells and are involved in CD8+ T cells against viral infections through effective cross-presentation of antigens in MHC class I and MHC class II pathways; Nanoparticles are also potential vaccine delivery vehicles serving as adjuvants and providing effective antigen delivery)

and biocompatible nanopolymers that are used as the delivery systems to trigger both humoral and cellular immune responses [43]. It has been confirmed that these nanoparticles are potent immunomodulators in various types of vaccines, controlling the release of antigens and providing the better quality and higher quantity of immune responses (Fig 4).

It is also notable that preventing the degradation of antigens up to the point where it is accepted by the immune cells could be achieved [43].

Nanoparticle-based vaccines may play a key role in the $CD8^+$ T-cells in the prevention of viral infections through the effective cross-presentation of antigens in the MHC class I and MHC class II pathways [43]. On the other hand, nanoparticles are considered to be potential vaccine delivery vehicles that could serve as adjuvants for the effective delivery of antigens [44].

The safety and effectiveness of several nanoparticles and liposomes have been confirmed for use in modern vaccinology. For instance, degradable polymers such as polyesters poluylactic acid and the copolymers, polyorthoesters, polyanhydrides, and polycarbonates have extensive usage owing to their effective properties in combination or loading to prevent the degradation of delivered antigens *in-vivo*. In addition, nanoparticle-based vaccine delivery systems could effectively encapsulate antigens, thereby enhancing and/or facilitating their uptake by APCs, such as in dendritic cells (DCs) and macrophages *in-vitro* and *in-vivo* [45, 46]. The systems providing antigen delivery targeting DCs as professionally as APCs are mainly assessed as an optional strategy for vaccines.

The nanopolymer particles that target antigens to the DCs represent a novel approach to effectively deliver immunomodulators. The intracellular processing of antigens and antigen presentation by the MHC class I and II pathways, which induce both $CD4^+$ and $CD8^+$ T-cell responses, facilitates the uptake of nanoparticles by DCs [47]. An effective DC-targeting system could result from the development of polymer nanomaterials in the field of molecular vaccinology, representing a beneficial strategy for potential vaccines [47].

Polymer-based nano adjuvants for the HCV

Nanoparticles could potentially serve as vaccine delivery vehicles, functioning as adjuvants and providing effective antigen delivery [44]. Studies have confirmed that various nanoparticles and liposomes are safe and could be used effectively in modern vaccinology. For instance, an anionic poly amino acid-based block copolymer with galactose P41, which is an amphipathic alpha-helical cationic peptide derived from the HCV NS5A membrane anchor domain C5A, has demonstrated antiviral activity against the HCV [48] and HIV [49], while its application has been restricted due to its sudden removal from circulation and toxicity.

In a study in this regard, Zhang et al. reported a significant reduction in the cytotoxicity hemolytic effects and proteolytic degradation by its incorporation into an ionic nanocomplex, which could also preserve its function in the antiviral treatment of HIV and the HCV [50]. In the mentioned study, an antiviral peptide nanocomplex using P41 electrostatically enjoined with an anionic poly amino acid-based block copolymer with galactose was also formulated as an active targeting moiety, and the obtained results indicated that the galactosylated antiviral peptide nanocomplexes provided the glycoprotein receptor-mediated uptake in the hepatoma cell lines, while potently inhibiting the HCV core and NS5A proteins that prevented binding to lipid droplets, which was considered essential to the viral assembly and release stage [51].

For several decades**,** there has been intense focus on PLGA in the field of nanotechnology owing to its beneficial properties as a biodegradable synthetic polymer. The American Food and Drug Administration (FDA) has approved PLGA for human and veterinary use [28], and its particles have been shown to enhance the efficacy of delivery, while also improving the strength of vaccine formulation [52, 53]. Recently, PLGA has become widely recognized in vaccinology as a potential vaccine delivery system due to its gradual degradation rate before internalization by APCs [54, 55]. In general, APCs are greatly preferred in the particulate form rather than the soluble form in terms of the uptake of antigens and immunostimulators [55]. In addition, the particles could prevent the degradation of the vaccine antigen that is caused by the protease enzyme [56], while facilitating and improving the deposition following injection or oral delivery [57, 58]. Adjuvants or immunomodulators have been developed to enhance the effectiveness of vaccines and immune responses of the cells. The efficacy of

PLGA particles in the production of viral vaccines has been confirmed. Improvement of the cytosolic delivery of antigens significantly increases the antigen presentation by the MHC class I to T cells, including IL-2 secretion, especially compared to antigen-coated latex beads and soluble antigens [59, 60]. Regarding therapeutic viral vaccines, a study conducted on the combination of HPV16-E6 and E7 peptides with PLGA nanoparticles indicated that the efficacy improved in mice models compared to the use of peptides alone [61]. Therefore, it could be concluded that PLGA particles may have strong potential to function as protein carriers and adjuvants for the HCV subtype 1b therapeutic vaccine.

In another research, Roopngam et al. [62] observed that the HCV1b-E2 protein was effectively encapsulated in PLGA nanoparticles, and their use as a delivery system with adjuvant properties resulted in effective immunomodulatory function, which was particularly practical in therapeutic vaccines. According to the mentioned study, the PLGA nanoparticles had adjuvant function to trigger cell-mediated immune responses through increasing the expression of cytotoxic T cells, which is an essential function of therapeutic vaccines. Furthermore, the other findings of the study indicated that in a murine model, the HCV1b-E2-PLGA nanoparticles enhanced cellular immunity (Fig 5).

As a truncated form of an insoluble protein, the HCV1b-E2 protein could be efficiently delivered *in-vivo*, and APC uptake was also reported to induce the response of the cell-mediated immune system. Another investigation in this regard demonstrated the antigenicity of the HCV1b-E2 and HCV1b-E2-PLGA in the antibody response of mice. The adaptive immune response of cellular immunity was particularly assessed to determine the efficacy of the viral vaccine.

The investigation of the preventive antibody for the HCV1b-E2 has also been performed through the vaccination of mice, and the results indicated that the HCV1b-E2-PLGA could instigate specific humoral immunity although the antibody level of the HCV1b-E2-PLGA, which was significantly lower compared to treatment with the HCV1b-E2 peptide alone. This was due to the fact that the PLGA nanoparticles were able to gradually release the HCV peptide, which is an important characteristic of an adjuvant to increase long-lived immunity [63].

Therefore, the HCV1b-E2-PLGA effectively induced humoral- and cell-mediated immune responses and is recommended for use as a preventive HCV vaccine.

Furthermore, the group of lymphocytes treated with the HCV1b-E2-PLGA nanoparticles produced higher levels of IFN-γ compared to the group treated with the peptide alone.

A previous report on PLGA nanoparticles demonstrated higher IFN-γ in a murine model [61], and IFN-γ was reported to encourage secretions, thereby allowing the critical cytokines to eradicate viral infections, which was released by the cytotoxic T cells in normal conditions, as well as type I and type II helper T cells, which are natural killer cells (NK and NK T-cells) [64, 65].

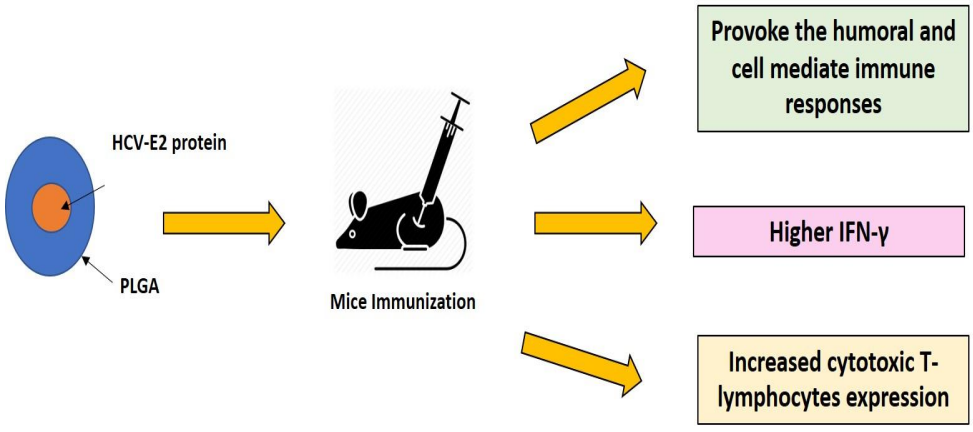

Fig 5. HCV1b-E2 Protein Encapsulated in PLGA Nanoparticles and Their Ability to Act as a Delivery System to Provide Adjuvant Properties for Use as an Effective Immunomodulator (PLGA nanoparticles provided adjuvant properties to induce humoral- and cell-mediated immune responses, such as increased IFN-γ and cytotoxic T cell expression)

Table 1. Polymer-based Adjuvants for Hepatitis C Vaccines

Adjuvants	HCV Materials	References
Anionic Polyamino Acid Copolymer Nanoparticles	HCV NS5A	47
PLGA Nanoparticles	HCV1b-E2	61
PLGA Nanoparticles	rC–N Fusion Protein of HCV	67
Gelatin Nanoparticles	*NS2* Gene	69
Micelles of Triblock Copolymer	DNA Encoding Multiple-epitope Antigen Gene of HCV	70
Polyanionic Carbosilane Dendrimer	None	72

In addition, IFN-γ was reported to indirectly increase the activity of the cytotoxic T cells [65] and stimulate the NK cells as, while inducing the differentiation of the helper T cells (especially type I helper T cells) and facilitate the differentiation of cytotoxic T cells [66]. As a result, the increased IFN-γ was able to raise the activity level of the cytotoxic T cells to destroy cancer cells, and the effective cellular immune responses could also improve. The mentioned report was the first study to indicate that the HCV1b-E2 truncated form, which is an insoluble protein, could be encapsulated into PLGA nanoparticles as a successful strategy for the delivery of insoluble proteins for vaccine immunization (Fig 5). This novel strategy could potentially be used in the treatment of HCV1b-infected patients as a therapeutic vaccination. Core and NS3 proteins have also been applied as anti-HCV agents along with the nanoparticles as they play a critical role in the clearance and induction of immune response against the HCV [67]. The core-NS3 (rC-N) recombinant protein has also been reported to induce T cell-mediated immune responses [68]. Moreover, rC-N/PLGA nanoparticles have been prepared by the primary amine of the rC-N conjugation with a carboxylic group of polymer nanoparticles [67], penetrating the target cells [67]. NS2 is another candidate for the HCV vaccine, and the *NS2* gene has successfully been conjugated to gelatin nanoparticles [69]. In the mentioned investigation, NS2/gelatin nanoparticles were produced through expressing the *NS2* gene into the bacterial cell (*E. coli*), and this platform was observed to be as safe as a non-viral vaccine against the HCV.

Recently, poly(d,l-lactic acid)-co-poly(ethylene glycol)-co-poly(d,l- lactic acid [PLA–PEG–PLA]) and poly(d,l-lactic-co-glycolic acid)-b-poly(ethylene glycol)-poly(d,l-lactic-co-glycolic acid [PLGA-PEG-PLGA]) have been applied as triblock copolymers and potent carriers in gene delivery [70]. The structure of PEG helps improve the copolymer hydrophilicity [71]. Some parts of the genes of the HCV genotype 1b are DNA-encoding, which were encapsulated into PLGA-PEG-PLGA and PLA-PEG-PLA. These copolymers could effectively deliver the gene with multiple epitopes of antigens and enhance the humoral and cellular responses through the sustained release of multiple-epitope antigen genes from the micellar structural system. Another polymer-based nano-system is polyanionic dendrimers, which is able to act as a prophylactic vaccine against the HCV to inhibit the binding of the HCV to target cells [72].

In summary, the development of potential adjuvants for the HCV and a system for the successful delivery of the vaccine could be facilitated by the advancements in polymer chemistry and molecular immunology (Table 1), thereby resulting in the further improvement of vaccines. Owing to their beneficial properties, biodegradable polymers (e.g., polyesters, polylactic acid and their copolymers, polyorthoesters, polyanhydrides, and polycarbonates) are considered appropriate in terms of combination or loading to prevent the degradation of delivered antigens *in-vivo* and have been used frequently (Table 1).

Perspectives

The development of effective prophylactic and therapeutic vaccines for the HCV is required for the prevention of a global epidemic. Most importantly, it would be more beneficial to produce a therapeutic vaccine for the treatment of HCC rather than a vaccine for its prevention. On the other hand, several drawbacks have been attributed to the peptide vaccine, including the low immunogenicity of the protein, high

instability, delivery complications, and lack of the efficient presentation of the antigens. Currently, researchers are particularly focused on nanomaterials for the optimization of antigen delivery and adjuvant activity as potential immunomodulators.

According to the findings of the current research, both the prophylactic and therapeutic vaccines for the HCV could be developed using polymer-based nanoparticles. The potent efficacy of the biodegradable adjuvants of the vaccine in combination with the viral peptide antigen for the HCV vaccine was observed to be highly beneficial. Therefore, the development of an effective vaccine delivery system to facilitate the future improvement of vaccines could be achieved through the advancement of polymer chemistry and molecular immunology. A potential therapeutic vaccine for patients infected with the HCV could be obtained by this novel strategy. However, the testing of these adjuvants for human use and human models based on clinical trials is required as the murine model has proven insufficient for establishing a conclusion regarding the safety and efficacy of the vaccine. Therefore, advanced research studies are recommended on the effective vaccines with potential adjuvant formulations, as well as the more frequent licensing of the vaccines that could provide new methods to fulfill clinical needs. However, factors such as the poor stability and safety of vaccines (i.e., lack of efficacy) have caused numerous adjuvants to fail in the development stage of production.

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