Original Article

Preparation and characterization of PLGA Nanoparticles containing recombinant core-NS3 Fusion protein of hepatitis C virus as a nano-vaccine candidate

Hekmat S[1], Aslani MM[2, 5*], Shafiee Ardestani M[3#], Aghasadeghi MR[1], Siadat SD[2, 4], Sadat SM[1], Mahdavi M[6], Shahbazi S[1], Asgarhalvaee F[7], Ghahari SMM[1, 8], Tohidi F[9]

[1]Department of hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran.
[2]Microbiology Research Center (MRC) Pasteur Institute of Iran, Tehran, Iran.
[3]Department of Radiopharmacy, Faculty of Pharmacy, Tehran University of Medical Sciences. Tehran, Iran.
[4]Department of Mycobacteriology and pulmonary research, Pasteur Institute of Iran, Tehran, Iran.
[5]Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran.
[6]Department of Immunology, Pasteur Institute of Iran, Tehran, Iran.
[7]Department of Microbiology, Pharmacotical Science Branch, Islamic Azad University, Tehran, Iran.
[8]Department of Immunology, School of Medicine, Mazandaran University of Medical sciences, Sari, Iran.
[9]Department of Microbiology, Science and Research Branch, Islamic Azad University, Fars, Iran.

ABSTRACT

Introduction: Hepatitis C virus (HCV) is one of the most serious causes of cirrhosis, liver cancer and ultimately death, worldwide. The new direct-acting drugs are not accessible for many patients around the world and progress toward new therapeutic and anaphylactic vaccines design has not been fast enough. This study was aimed to prepare and assess a recombinant fusion protein core-NS3 (rC-N) of HCV with the accompaniment of d,l-polylactide-co-glycolide nanoparticles (PLGA NPs) as a nano-conjugated vaccine candidate. **Methods:** The rC-N protein containing the first domain of core and middle region of NS3 (residues 1095-1384) was loaded into PLGA NPs (rC-N/PLGA NPs) by NHS and DDC (equimolar: 0.5 mM) as conjugating agents. The morphology and average of surface roughness (Ra) of PLGA NPs and rC-N/PLGA NPs were demonstrated by atomic force microscope (AFM). The particle sizes, polydispersity index (PDI) and zeta potential were measured by Zetasizer. **Results:** The morphology of the nanoparticles was spherical and their surface Ra was measured to be 7.630 nm for PLGA NPs and 15.72 nm for rC-N/PLGA NPs. The average size (160.4 nm), zeta potential (-37.6 mV) and PDI (0.227) were also obtained for rC-N/ PLGA NPs. **Conclusion:** The surface Ra value of rC-N/PLGA NPs (15.72 nm) which was twice more than PLGA NPs (7.630 nm) confirmed a successful conjugation. The stability of nanoparticles behavior in the colloid was confirmed by the absolute value of zeta potential (|-37.6|= 37.6 mV) of rC-N/PLGA NPs. The spherical morphology, average size < 200, an absolute value of zeta potential > 30 mV, PDI < 0.5 were confirmatory indications that rC-N/PLGA NPs could be considered as vaccine candidate. The rC-N/PLGA NPs construct should be further evaluated via *in-vivo* challenges and demonstration of targeted delivery to the antigen presenting cells.

KEYWORDS: PLGA Nanoparticle, HCV vaccine, Core, NS3, fusion protein.

INTRODUCTION

High tendency of hepatitis C virus (HCV) to chronicity is a causative factor in over 75 % of HCV-infected patients causative factor in over 75 % of HCV-infected patients succumbing to cirrhosis, hepatocellular carcinoma or even death [1, 2].Newly-developed oral multiple direct-acting antivirals which offer 90-95% cure rate, low adverse effects and high tolerability have created a revolution in HCV treatment. Although this was an obvious step for HCV elimination, the very high-cost of the drugs has been a major obstacle to wide accessibility of such treatments by HCV-infected individuals especially in low-and middle-income countries. Therefore, developments of efficacious preventive or therapeutic vaccines as well as new accessible and effective drugs are the main goals

***Corresponding Author:** Mohammad Mehdi Aslani, Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran.
Email: mmaslani@yahoo.com
Tel/Fax: (+98) 2166969291
#Co-Corresponding Author: Mehdi ShafieeArdestani, Department of Radiopharmacy, Faculty of Pharmacy, Tehran University of Medical Sciences. Tehran, Iran.
Email: ri.ca.smut@inatsedraeeifahs
Tel/Fax: (+98) 2166959098

[DOI: 10.29252/vacres.3.8.9.48]

for the infection control and possibly eradication of this virus [3, 4].

Although more than 25 years have passed since the discovery of HCV, no effective vaccine against this virus is currently available. Between 15-25% of the infected patients are capable of inducing an immune response to clear the infection from their bodies and in the re-infected cases the rate of clearance remains high. These evidences indicate that the development of anaphylactic or therapeutic vaccines against HCV could be contemplated [5]. The classical vaccines are produced by whole live attenuated, inactivated or some extracted parts of the microorganisms which beside their effectiveness, may have some disadvantages. For instance, they may revert to the virulent forms and then trigger inflammation or autoimmune responses. Moreover, due to some biohazard concerns for microorganisms such as HIV and HCV, little success has been achieved in this regard. As a result, recombinant synthetic peptides or proteins which are based on conserved amino acid sequences of the viral proteins have attracted more attention in recent years. Such recombinant vaccines are more preferable due to their safety, ease of production and precise definition of their components. However, such vaccines do not induce sufficient immune responses; therefore, they need to be used with one or more effective and safe immune stimulatory and delivery components [6].

HCV is a positive sense, single-stranded RNA virus. The HCV genome is translated into a single protein in hepatocytes where they are then cleaved by cellular and viral proteases into structural (C, E1, E2, p7) and nonstructural (NS2, NS3, NS5A and NS5B) proteins [7]. Several studies have shown that the core and NS3 as two conserved proteins of HCV play the key role in stimulation of the cytotoxic T lymphocytes, T helper cells(Th cells), the humoral immune activity and eventually, the virus clearance [8-10]. As the first step, a bipartite recombinant core-NS3 (rC-N) fusion protein, made of the first domain of core (amino acid residues 1-118) and a truncated middle region of NS3 (amino acid residues 1094-1385)were evaluated by the computational analyses . The recombinant protein was then expressed and purified successfully, as described previously [11]. The chimeric antigen in the virtual environment showed an ability to introduce CD4+ and CD8+ T-cell responses [11].However, according to the previous experiments on protein vaccines, these responses remain weak and the presence of an adjuvant ora delivery system to induce targeted and boosted immunity are expected [12].

Nanoparticles can penetrate across biological barriers and contribute to enhanced bio-availability, controlled release of bioactive compounds, targeted delivery of vaccine antigens to the antigen-presenting cells (APCs), optimization of the immune responses and reduction in the number of boosting immunization [13, 14]. Synthetic polymers such as d,l-polylactide-co-glycolide (PLGA) have been previously approved by the U.S. food and drug administration (FDA).Such polymers contained encapsulated, surface-immobilized or surface-adsorbed antigens, capable of delivering peptides or protein antigens to the dendritic cells (DCs) [12, 14]. Due to advantages such as biocompatibility, biodegradability and good mechanical strength, PLGA has been under consideration as a suitable antigen delivery system. Moreover, PLGA is able to protect the peptides and protein antigens and can present them to target cells rapidly and over a long period while evading the endolysosomes [14-18].

Some properties of nanoparticles such as particle size, polydispersity, morphology, surface modification and potential of electrical charge are known to have impact on their penetration into the target cells and to improve their ability to induce enhanced and prolonged immune responses. The PLGA particles are able to create both cellular and humoral immune responses. The particle size, play a vital role in stimulating one of the two types of the immune responses. However, dendritic DCs are able to uptake the nanoparticles within the sizes of 20 to 250 nm. Phagocytosis is mainly used for the ingestion of the larger particles (0.5– 5 μm) by the macrophages. In general, smaller particles (< 500 nm), especially 40-50 nm, can stimulate CD8+ cytotoxic type1T cell responses while the larger particles (> 500 nm) promote CD4+ helper type 2 T cell and finally, the antibody responses [19].

The average particle size and the polydispersity index can be measured by photon correlation spectroscopy also called "dynamic light scattering" (DLS). This technique is based on the dispersion of the light caused by the Brownian motion of the particles [20-22].Imaging techniques such as scanning or transmission electron microscopy (SEM or TEM) and atomic force microscopy (AFM) provide information on the morphology and the size of the particles [21, 23].

Measuring the surface charge is an indicator for the efficiency of surface modification. Mobility of the charged particles is determined by zeta potential (ζ) analysis of the nanoparticles. Actually, zeta potential is an analysis for setting the surface charge of nanoparticles in colloids. Thus, the properties of solvent and the surface of nanoparticles have important impact on the zeta potential. Zetasizer is an instrument that detects the mobility of the charged particles by measuring their electrical potential. Zeta potential, also known as electrokinetic potential, is measured in millivolt (mV). The zeta potential value may be positive, neutral or negative; however, high absolute value is an important factor in this parameter. This value can be utilized to predict long-term stability of the colloid suspension [24, 25]. The correlation between the stability of the suspension or tendency toward flocculation of the nanoparticles and their zeta potential rates are shown in Table 1.

The existing studies show that PLGA is one of the most accepted polymers, capable of delivering antigens to specified sites in a controlled condition [15].Therefore, PLGA nano- or micro-particles have been used to produce vaccines against diseases caused by intracellular microorganisms such as hepatitis B [26], malaria [27], bacterial diarrhea [28], leishmaniasis [29], tuberculosis [30], toxoplasmosis [31] and influenza [32]. PLGA enhances the immunogenicity of the antigens by increasing their absorption by the lymphoid tissues and controlling their release [33].

Here, we conjugated a recombinant rC-N fusion protein with PLGA NPs, in order to induce a strong and targeted immune response against HCV which could potentially carry antigens towards APCs in a controlled manner. We then characterized this potential nano-particulate vaccine in terms of its physical attributes.

Table 1. Stability behavior of the colloids at various zeta-potential [25].

Zeta potential	Stability behavior of the colloids
0 to + - 5	Propensity of aggregation and flocculation
+ - 10 to + - 30	Weakly stable
+ - 30 to + - 60	Stable
> + - 61	Highly stable

[DOI: 10.29252/vacres.3.8.9.48]

MATERIALS and METHODS

The rC/N fusion protein production

The rC-N fusion protein was prepared as described previously. Briefly, the first domain of core (amino acid residues 1-118), an AAY linker and the middle region of NS3 (aa 1095-1387) were respectively cloned into pET 24a (+) vector, expressed in *Escherichia coli* BL21-DE3, purified by affinity chromatography and finally analyzed by SDS-PAGE and Western Blotting using anti-6xHis tag monoclonal antibody [11].

The rC/N fusion protein and PLGA nanoparticles conjugation

The biodegradable poly (D, L-lactic-co-glycolic acid) (PLGA) nanoparticles were conjugated to rC/N fusion protein by a modified method used by Guo and Lee [34]. Approximately, 500 mg of PLGA particles were activated by conjugating agents, namely, dicyclohexylcarbodiimide (DCC) (0.5 mM) and N-hydroxysuccinimide (NHS) (0.5 mM) in dimethyl sulfoxide DMSO (10 ml) as solvent at room temperature over 1 h. Conjugation reaction went onby adding 3 ml solution of rC-N fusion protein (1.6 mg/ml) to the activated PLGA solution in a drop-wise manner over 30 min. The mixture was placed on a magnetic stirrer for 24 h in dark. In order to remove impurities from the conjugated HCV rC-N fusion protein and PLGA nanoparticles (rC-N/PLGA NPs), the solution was centrifuged at 80'000 rpm for 30 min. The supernatant was then disposed and the sediment was washed by PBS for several times. The last sediment was dispersed with distilled water and was lyophilized.

Physical characteristics of PLGA NPs and rC-N/PLGA NPs

Preparation of diluted PLGA NPs and rC-N/PLGA NPs

Before measurements, dilution was done by adding 0.02 mg PLGA NPs or rC-N/PLGA NPs in two drops of chloroform and then in 3 ml deionized distilled water. All samples were dispersed by vortex and sonicated for 20 min (60 seconds, 30% amplitude). In order to evaporate chloroform, the tubes were warmed in water at 50-60°C. To prevent particles aggregation, all diluted samples were filtered by 0.20 µm RC-membrane (Sartorius) filters.

Morphology of PLGA NPs and rC-N/PLGA NPs by atomic force microscopy

Particle size, PDI and zeta potential

An atomic force microscope (AFM, model: Nano wizard II nano science AFM, JPK Instruments INC Germany) was used for surface morphology observation of the nanoparticles.

To prepare the slides for AFM, briefly, the microscope glass slides were washed by ethanol, then by distilled water and were dried carefully. One or two drops of diluted PLGA NPs or rC-N/PLGA NPs solution were added on the slides separately and dried at room temperature. Finally, the morphology, size and average surface roughness (Ra) of PLGA NPs alone and conjugated rC-N/PLGA NPs were evaluated by AFM instrument and JPK software (JPK's Nano Wizard® AFM, Germany).Mean size distribution, polydispersity index (PDI) and zeta potentials of the nanoparticles (i.e. PLGA NPs or loaded rC-N/ PLGA NPs) were determined by dynamic light scattering (DLS) (Zetasizer Nano ZS; Malvern Instruments, Malvern, UK) at 25°C. The nanoparticles were dissolved and dispersed in double-distilled water at a concentration of 1 mg/ml. In order to raise measurement accuracy, every sample was measured thrice.

RESULTS

AFM images of PLGA NPs and rC/N-PLGA NPs

Two-dimensional and three-dimensional (2D, 3D)images of PLGA NPs and rC/N-PLGA NPs were obtained by atomic force microscope (AFM) shown in Fig. 1 and Fig.2. The error signal (Fig.1A, Fig.2A) and height mode images (Fig. 1B and Fig. 2B) showed the spherical morphology of the nanoparticles. The 3D images of PLGA NPs and rC/N-PLGA NPs are shown in Fig. 1C and Fig. 2C.The 2D height mode images were used to obtain surface Ra while the 2D error signal mode and 3D images were used to obtain the best quality of the shape. The average size randomly obtained by AFM for these spherical forms of PLGA NPs and rC-N/PLGA NPs were mostly 90 nm and 100 nm, respectively. The AFM surface Ra characteristics of PLGA NPs showed an average roughness (Ra) of about 7.63 nm, while the surface Ra for rC/N-PLGA NPs was 15.72 nm. The surface Ra for PLGA NPs was approximately two times less than rC-N-PLGANPs.

Particle size distribution and zeta potential

The size distribution of PLGA and rC-N/PLGA NPs were calculated and shown in Fig. 3 and Fig. 4. The zeta size average of PLGA NPs was 130.0 nm, with narrow distribution of polydispersity index (PDI) of 0.103 (Fig.3),and the zeta size average of rC-N loaded with PLGA NPs was 160.4 nm with PDI = 0.227 (Fig.4). Average of zeta potentials belonging to PLGA NPs and rC-N/PLGA NPs are shown in Fig. 5 and Fig. 6.These values were-1.24 mV and -37.6 mV for PLGA NPs and rC-N/PLGA NPs, respectively. The absolute value of zeta potential of rC-N/PLGA NPs was | -37.6 | = 37.6 mV.

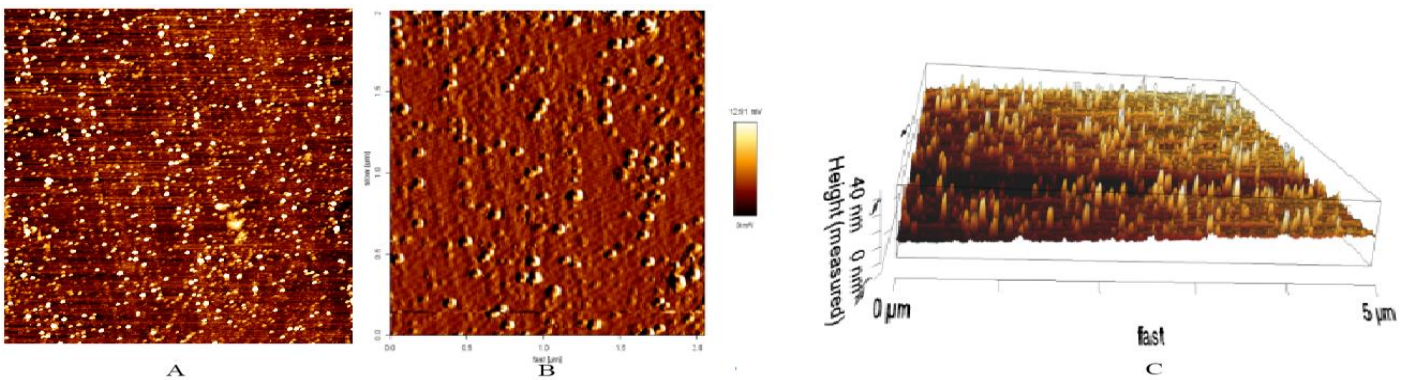

Fig. 1. Atomic force microscopic (AFM) presentation of PLGA NPs. A) Selected channel to process was height mode (fast 2.000 x slow 2.000 µm) 2000 nm^2. B) Selected channel to process was error signal (fast 2.000 x slow 2.000 µm) 2000 nm^2. C) Three dimension (3D) image obtained in height mode (fast 5.000 x slow 5.000 µm) 5000 nm^2.

[DOI: 10.29252/vacres.3.8.9.48]

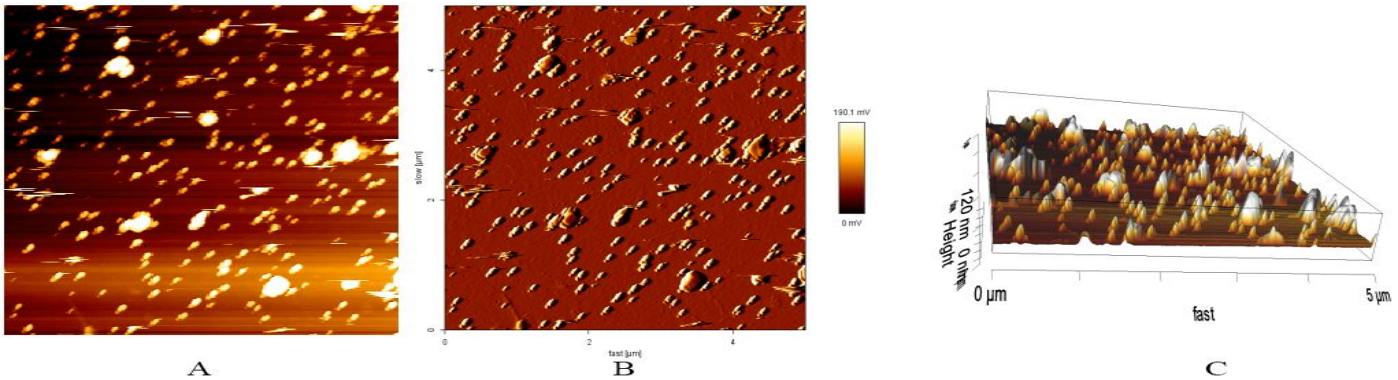

Fig. 2. Atomic force microscopic (AFM) presentation of rC-N/PLGA NPs. A) Selected channel to process was height mode (fast 5.000 x slow 5.000 µm) 5000 nm^2. B) Selected channel to process was error signal (fast 5.000 x slow 5.000 µm) 5000 nm^2. C) Three dimension image obtained in height mode (fast 5.000 x slow 5.000 µm) 5000 nm^2.

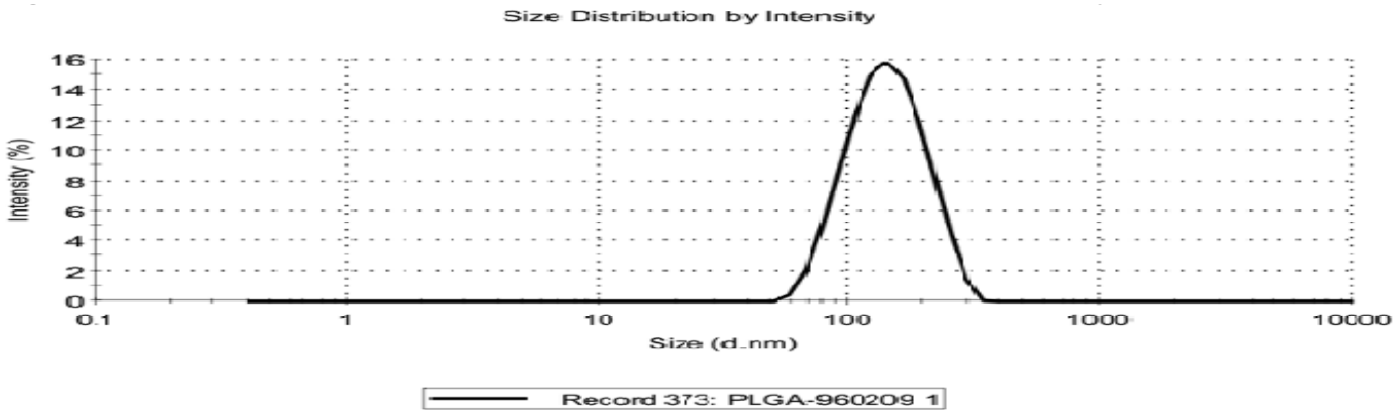

Fig. 3. Size distribution of PLGA NPs.

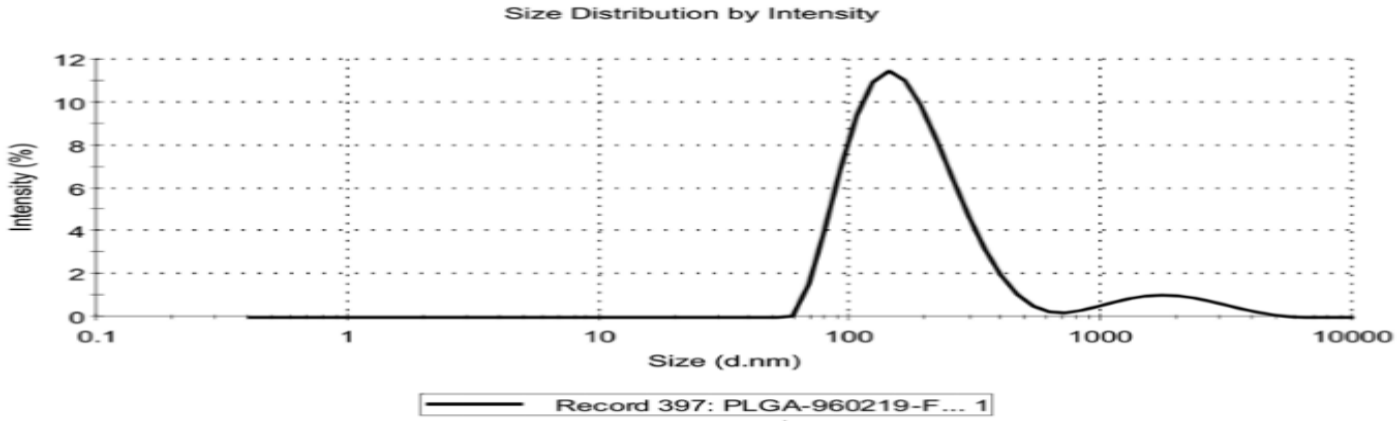

Fig. 4. Size distribution of rC-N/ PLGA NPs.

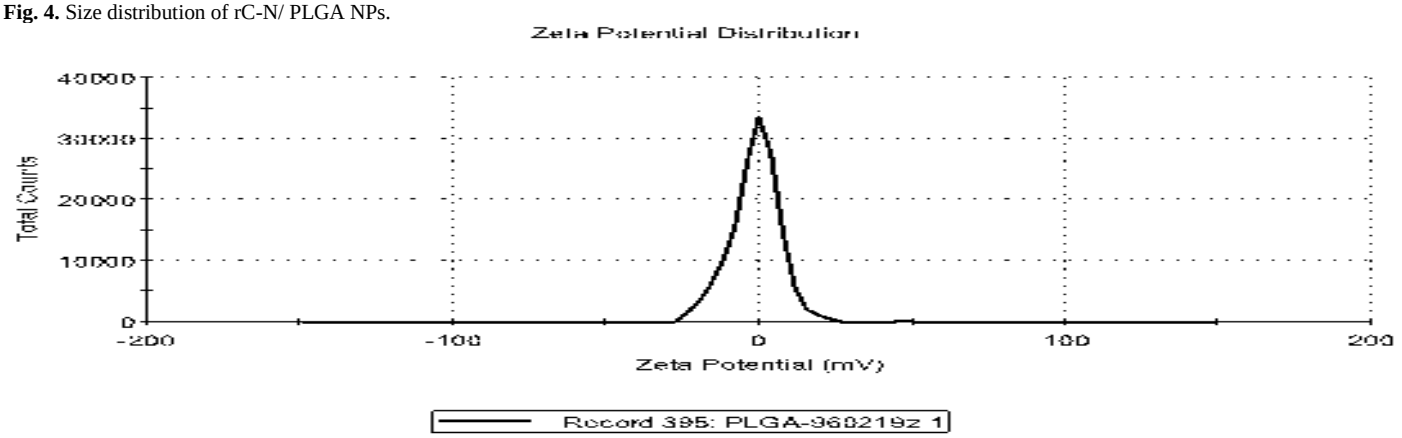

Fig.5. Zeta potential distribution of PLGA NPs.

[DOI: 10.29252/vacres.3.8.9.48]

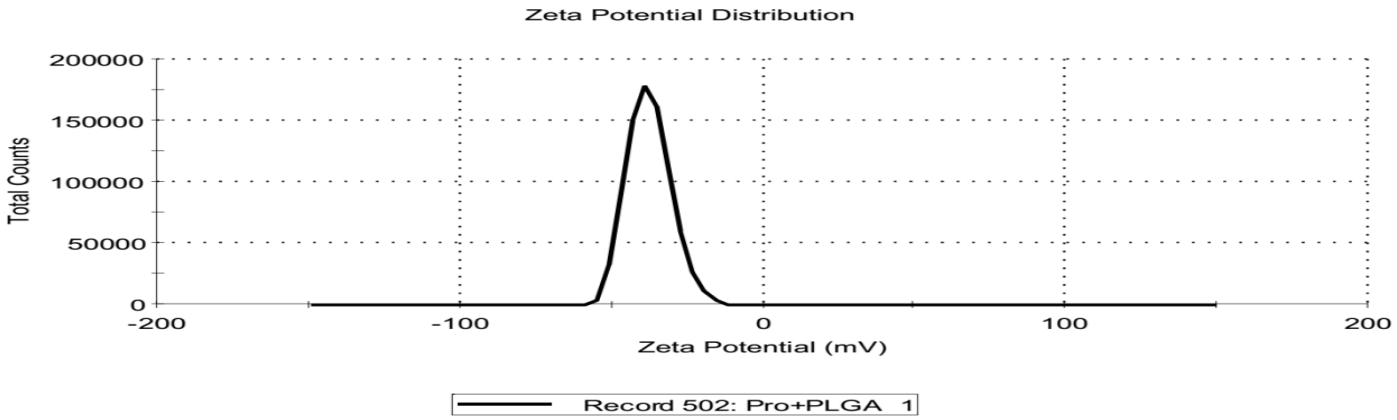

Fig.6. Zeta potential distribution of rC-N/ PLGA NPs.

DISCUSSION

Despite extensive advances in vaccine development, there is not yet a potent vaccine to eradicate or reduce the problem of HCV infection [35]. Therefore, new strategies and endeavors to design and prepare anaphylactic or therapeutic vaccines against HCV are deemed urgent. In this regard, we designed and produced a vaccine based on the conserved immune system stimulant parts of HCV, together with PLGA NPs as both an adjuvant and a delivery system. In our previous study, a rC-N fusion protein of HCV was produced as an antigen which its *in-silico* predictions showed it can potentially induce T-cell-mediated immune responses [11].

In this study, the rC-N fusion protein was connected to PLGA nanoparticles as a hydrophilic protein by a junction between the primary amin of rC-N fusion protein and the terminal carboxylic acid of PLGA NPs. An evidence for the occurrence of an accurate conjugation was the increase in average of the surface Ra of the nanoparticles [36].The AFM showed surface Ra for the conjugated rC-N/PLGA NPs (15.72 nm) was about two times more than PLGA NPs alone (7.63 nm). Moreover, the indication of a successful loading was obtained by the difference between the conjugated (rC-N/PLGA NPs) and non-conjugated (PLGA NPs) samples where the average surface Ra increased after the conjugation.

However, the penetration of nanoparticles into the cell cytoplasm depends on the particles' size, shape, surface physicochemical properties and their electrical charge potential [19]. Zhao et al.have demonstrated that spherical forms have a higher penetration rate into the cells [37]. This study provided spherical s 2D and 3D images of PLGA NPs or rC-N/PLGA NPs by AFM.

As a matter of fact, the microparticles have a tendency to promote humoral responses whereas the nanoparticles are able to penetrate into the infected cells and eventually induce specific cellular responses [19]. Typically, the size of the nanoparticles in clinical application must be less than 250 nm [38].According to our Zetasizer and AFM results, the size of rC-N/PLGA NPs showed to be in an appropriate range of less than 200 nm and the zeta average wasobtained to be about 160.4 nm. In a suitable condition, the PDI or heterogenicity index have to be less than 0.5 [20-22]. Accordingly, the PDI of rC-N/PLGA NPs was about 0.227. The size distribution of all the prepared nanoparticles was homogenous and appropriate for both *in-vitro* and *in-vivo* studies in the future.

The mobility of the charged particles is monitored by Zetasizer instrument by measuring the electrical potential [23, 24]. Zeta potential for rC-N/PLGA was -37.6 mV, and the absolute number| -37.6 | = 37.6mV was more than 30 mV. Therefore, propensity of particles flocculation was weak and our suspension had stable formation, with little tendency to aggregate [25]. The spherical morphology, average size 160.4 nm < 200 nm, absolute value of zeta potential37.6mV > 30 mV and PDI 0.27 < 0.5 are confirmatory evidences for forming suitable rC-N/PLGA NPs as a nano-vaccine. These data also showed that rC-N/PLGANPs were capable to disperse in colloids and can potentially penetrate into target cells and APCs. Altogether, our data strongly suggest these nanoparticles have the appropriate attributes of a nano-vaccine candidate to be further evaluated by immunological *in-vivo* experiments.

ACKNOWLEDGEMENT

S.Hekmat was supported by Education office of Pasteur Institute of Iran to pursue her Ph.D. study program.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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[DOI: 10.29252/vacres.3.8.9.48]

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[DOI: 10.29252/vacres.3.8.9.48]