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Preparation and characterization of PLGA nanospheres loaded with inactivated influenza virus, CpG-ODN and Quillaja saponin

Maryam Mohajer [1], Bahman Khameneh [2], Mohsen Tafaghodi [3*]

[1] Student Research Committee, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[2] Department of Food and Drug Control, Students Research Committee, Mashhad University of Medical Sciences, Mashhad, Iran
[3] Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

Objective(s): The purpose of this study was preparation and evaluation of PLGA nanospheres containing the influenza virus and different adjuvants, Quillaja saponin (QS) and CpG-ODN.
Materials and Methods: Nanospheres were prepared using the double emulsion-solvent evaporation method. The morphological and physicochemical properties were studied by scanning electron microscopy (SEM), determination of zeta potential, encapsulation efficiency and release profile.
Results: The particle size of formulations was less than 1000 nm, except for formulations containing antigen. The results were confirmed with SEM images. Encapsulation efficiency of antigen, QS and CpG ODN were 80%, 62% and 31%, respectively. The zeta potential of nanospheres was about -30 mV. The burst release was observed for all encapsulates and reached to about 48%, 44% and 35% within 90 min for antigen, CpG-ODN and Qs content, respectively.
Conclusion: The formulations showed proper physicochemical properties. These nanospheres have good potential to be used as delivery systems/adjuvants for immunization against influenza.

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Introduction

Influenza viruses contain different types of hemagglutinin (H) and neuraminidase (N) glycoproteins; all treat H1, H2, H3, N1, and N2 as pathogens for human beings (1). Vaccination against influenza is the most cost-effective strategy among all treatments or prevention approaches (2).

Novel approaches to vaccine development have been proposed during the last decades. They are generally safe but show poorly immunogenic properties when administered solely. Currently, the only approved vaccine adjuvants for human use is aluminum salts (generally referred to as alum) and MF59. Consequently, an urgent need exists for developing new, safe, biocompatible, and potent immunostimulatory adjuvants. Several new adjuvants are presently under clinical trials; however, most of them have been proven to be toxic for routine clinical applications (3). The safety considerations, biocompatibility and stability properties are the main restrictions in adjuvant development. In previous studies the adjuvant properties of saponin derived components such as Quillaja saponins (QS), extracted from *Quillaja saponaria* bark, were demonstrated (4). It was shown that inducing both cellular and humoral immunity was due to the immunoadjuvant properties of QS. This component could induce Th1 cells, CD8+ cytotoxic T lymphocytes (CTL), IgG2a, and IgG2b antibody responses (5).

Application of nanotechnology is studied as an alternative approach for enhancing immune responses (6). The engineering of nanostructured particles, such as polymeric nanospheres, holds great promise for the development of novel immunomodulatory agents (7).

These nanostructures can be applied to more effectively manipulate or deliver immunologically

*Corresponding author: Mohsen Tafaghodi. Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. Tel: +98- 513 8823255; Fax: +98- 513- 8823251; email: tafaghodim@mums.ac.ir

active substances to desirable sites. In the field of immunology, successful applications of nanotechnology will lead to new generations of adjuvants with a broad spectrum of immunological activities (8).

Polymeric nanoparticles have been extensively used for antigen delivery in the nasal cavity. The efficacy of nanoparticles following nasal delivery is, at least in part, attributed to their uptake into the nasal-associated lymphoid tissue (NALT). PLGA is an FDA-approved polymer which is frequently used for mucosal delivery of drugs and antigens (5).

The purpose of this study was preparation and evaluation of PLGA nanospheres containing influenza virus, and Quillaja saponin and CpG ODN as adjuvants for nasal immunization.

Materials and Methods

Materials

PLGA (50:50) polymers, Resomer® RG503H (MW 28, 032) and RG502H (MW 8631) were obtained from Boehringer Ingelheim (Ingelheim, Germany). QS was purchased from Sigma (USA). CpG oligodeoxynucleotide was purchased from Microsynth (Switzerland). Influenza vaccines were purchased from Vaxigrip Company. Polyvinyl alcohol (PVA) (87–89% hydrolyzed, MW 31000 – 50000 g/mol) was obtained from Fluka (Buchs, Switzerland). All other used chemicals were analytical reagent grade.

Methods

Preparation of PLGA nanospheres

PLGA nanospheres were prepared using a W/O/W emulsion and solvent evaporation technique (8). For preparation of different nanospheres, 1 mg influenza virus and 150 of QS or 600 µg of CpG-ODN were dissolved in 150 µL distilled water (W1 solution). W1 solution was mixed and emulsified in PLGA solution (200 mg PLGA in 600 µL dichloromethane) by probe sonication (80 sec) (Soniprep-150, MSE, UK). The resulting W/O emulsion was then emulsified in PVA solution (8 mL, 1, 2.5 or 5% w/v). This secondary emulsion was then added to 32 mL of 5% w/v PVA solution and stirred for 3 hr to evaporate the organic solvent. Nanospheres were collected by centrifugation (4000 g for 20 min), washed twice with distilled water, and after flash-freezing in a dry-ice/acetone mixture, was lyophilized overnight (Heto Drywinner, DW3, Denmark).

Characterization of PLGA nanospheres

Morphology and particle size

The morphology and size of nanospheres were evaluated by scanning electron microscopy (SEM) (Leo, 1450 vp, Germany). Particle size and distribution was also studied by dynamic light scattering (DLS) method (Zetasizer nanoseries, Malvern, UK).

Encapsulation efficiency of influenza antigen and QS in PLGA nanospheres

The amounts of antigen and QS content in PLGA nanospheres were assayed using a 'two-step' extraction method. Briefly, 10 mg of freeze-dried nanospheres were dissolved in dichloromethane (DCM, 1 mL). The suspension of dissolved polymer and precipitated antigen and QS was centrifuged at 10,000 g for 10 min and the polymer containing supernatant was discarded. The pellet consisting of the precipitated encapsulates was re-dispersed in 250 µL NaOH, 0.1 N and the amounts of antigen and QS were determined by the Bicinchoninic acid (BCA) protein assay method. Briefly, a stock solution of antigen was prepared in water for injection (WFI). Working-standard solutions were prepared in WFI from the stock solution ranging from 0.5 to 200 µg/ml. 150 µl of each standard solution was added to a microplate (five replicates) followed by 150 µl of BCA standard working reagent (SWR) (prepared by mixing 50 parts of BCA reagent A and 1 part of reagent B). The microplate was covered and incubated at 37^{o}C for 2 hr. The plate was then cooled to room temperature and the absorbances were read at 562 nm.

Encapsulation efficiency of CpG ODN in PLGA nanospheres

After the extraction as mentioned above, a spectrophotometric method was used to evaluate the encapsulation efficiency of CpG-ODN in PLGA nanospheres. The absorbance of CpG-ODN at 260 nm was used to determine the amount of CpG-ODN in nanospheres. As Influenza antigen has absorbance at 260 nm, it was not possible to quantify them simultaneously. Therefore, nanospheres encapsulated with ALM or CpG-ODN were prepared under similar conditions and were used to estimate the encapsulation efficiencies separately. For each batch of nanospheres the encapsulation efficiency was determined in triplicates.

In vitro release study

The release profile of antigen, QS and CpG-ODN from PLGA nanospheres was studied according to the previous studies with some modifications (9). Briefly, 30 mg of nanospheres were added to tubes containing 9.6 mM phosphate buffer saline (pH 7.4, 10 mL) and stirred at 25 rpm in shaker incubator at 37±1°C. During 4 hr, every 30 min, each test tube was centrifuged and 500 µL of supernatant was aspirated and replaced by fresh medium. Amount of the released antigen and QS was determined by

Table 1. Size, zeta potential and encapsulation efficacy of different nanospheres

Formulation	Z-average (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency
Antigen-containing	1003±16	0.278±0.02	-31±4	%80.3±6.08
QS-containing	643 ±4	0.397 ±0.00	ND	%62.67 ±2.57
CpG-ODN-containing	582	0.651	ND	%31.55± 1.38

ND: Not determined

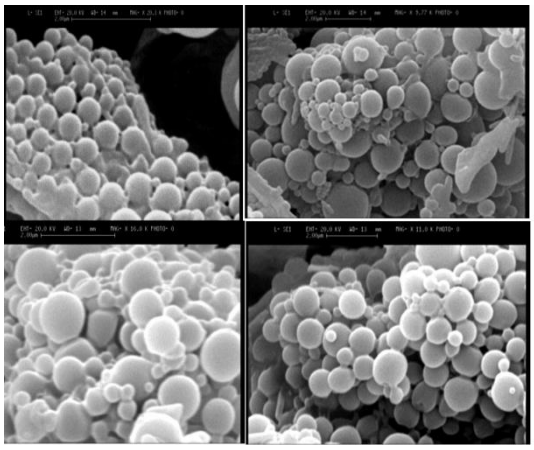

Figure 1. Scanning electron micrographs of the nanospheres

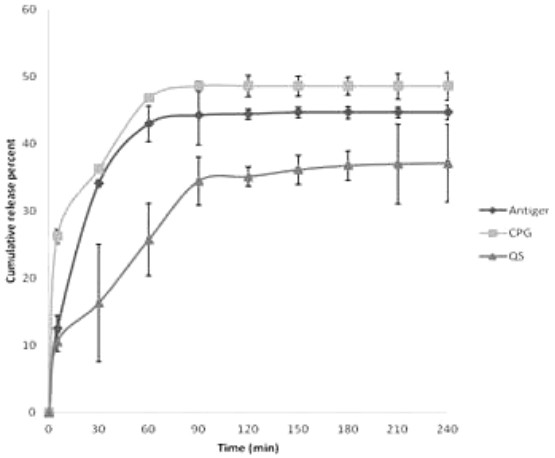

Figure 2. Cumulative releasing profiles of antigen, CpG and QS from the nanospheres in PBS solution at 37°C

Lowry protein assay method. The amount of protein in the sample was estimated using a standard curve of BSA solution ranging from 10 to 1000 ug/ml as standard protein. The release of CpG-ODN in the collected buffer was quantified by UV spectrophotometry at 260 nm. Each experiment was performed in triplicate.

Statistical analysis

Statistical analysis was carried out by one-way ANOVA and unpaired Student's t-test. *P*-values less than 0.05 were regarded as significant.

Results

Characteristics of nanospheres

Based on the microscopic observations, PLGA nanospheres were spherical, discrete and had smooth surfaces (Figure 1). SEM images exhibited that morphological features of particles showed clear dependency on the PVA percentages (data not shown). By increasing the PVA percentages, smaller and smoother particles were obtained; the best results were observed for nanospheres prepared with 5% PVA solution.

The Z-average, PDI, zeta potential, and encapsulation efficiency of nanospheres were summarized in Table 1. These data indicated that the antigen encapsulation was sufficiently enough for lead formulations. The results showed that the particles containing CpG-ODN and QS were nano-sized. However, aggregation was observed for the series of nanospheres which were prepared with less than 5% PVA. The zeta potential values of nanospheres were -30 mV.

In vitro release study

Release profile of antigen, QS and CpG-ODN from nanospheres were illustrated in Figure 2.

Statistical analysis revealed that there were significant differences between releasing profiles of three compounds. While the release rate of QS from nanospheres was the lowest, CpG-ODN exhibited a burst releasing feature.

According to the statistical analysis, there were no significant differences among cumulitive release percentages after 90 min.

Discussion

Influenza vaccine has been widely used for prevention against morbidity and mortality from influenza exposure. It is estimated that it is more effective in adult and healthy adolescents but less effective in very young or old people. The other disadvantage of routine influenza vaccines is attributed to negligible induction of sIgA antibodies, which is important for establishing efficient immune responses. These disadvantages of conventional influenza vaccines have led to investigation of new vaccine types.

Novel dosage forms hold a promising position in the field of new vaccine development. Nanosphere formulations have been considered as efficient adjuvants in the recent years. Intranasal administration of antigens is used to induce potent mucosal, humoral and cellular immune responses. Combinations of the above mentioned approaches are predicted to enhance immunoregulatory responses. In this study, the nanosphere formulations containing various adjuvants and

antigens were prepared and characterized. These particles were intended to develop an intranasal vaccine.

The morphological properties of prepared formulations showed the effect of PVA concentration on particle size (10–11).

The physicochemical properties of formulations were illustrated in Table 1. As shown, the zeta potential was approximately -30 mV. This value indicated that formulation was stable for long periods of storage.

The size of nanoparticles plays an important role in immunoadjuvant properties of the particles. Due to this fact, increasing the PVA concentration might be lead to decrease size of nanospheres. The nano-sized particles are more potent for inducing mucosal immune responses. These capabilities of the nanoparticles might be attributed to their long residence time in the nasal cavity (12).

The encapsulation efficiency of the three compounds was studied separately. The data indicated that the encapsulation efficiency of CpG-ODN was the lowest; a result that was related to the low molecular weight of this compound. The high encapsulation efficiency of the antigen is an important factor. In this study, the antigen was efficiently encapsulated in nano-formulations. These data were consistent with the previous findings (12).

The release profile of encapsulates from the nanospheres showed two steps. An initial burst release was followed by a slower release step, as shown in Figure 2. Approximately 48%, 44% and 35% of the antigen, CpG-ODN and QS content, respectively, were released in the initial burst release within 90 min. This burst release could be attributed to the release from surface of nanoparticles. The second step could be related to the diffusion controlled release. These findings were supported by previous results (13). There were no significant differences between releasing behavior after 90 min for all formulations. This could be due to matrix cleavage after 90 min. In previous studies, the burst release was attributed to dissolution of drug adsorbed or located close to the surface of the nanoparticles (13).

The differences between release patterns of different compounds could be attributed to the molecular properties of them as mentioned earlier. The correlation between molecular weight of drug and release profile from nanoparticles was previously investigated (14). It was demonstrated that the molecular weight of drug plays an important role in release profile and effective response of formulated nanomedicines. CpG-ODN with the lowest molecular weight showed the highest release rate. Due to the higher molecular weight of antigen and QS in comparison with CpG-ODN, the lower release rate could be predicted.

The other important factor in release profile is molecular weight of PLGA polymer. By choosing a higher molecular weight polymer, the release rate could be limited and controlled (15).

Conclusion

Most of the antigen remained in encapsulated form in nanoparticles and could be better detected by the immune system. Considering the acceptable particle size, loading and release profile of nanospheres loaded with antigen and adjuvants merit further *in vivo* studies.

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