International Journal of Newgen Research in Pharmacy & Healthcare
Volume-1, Issue-2, December 2023
www.ijnrph.com
ISSN 2584-0096 https://doi.org/10.61554/ijnrph.v1i2.2023.53

In-Vitro And In-Vivo Assessment of Antigen-Encapsulated Dextran Nanoparticles

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Received 11 Dec 2023, Accepted for publication 22 Dec 2023, Published 30 Dec 2023

ABSTRACT

Dextran nanoparticles have become increasingly popular for drug delivery purposes. They are known for their improved stability, low toxicity, simple and gentle preparation process, and versatility in terms of administration methods. The small size of the particles is not only suitable for parenteral administration, but also appropriate for mucosal routes of administration like oral, nasal, and ocular mucosa. These routes are typically less intrusive. The dextran absorption enhancing effect also helps in making the application of mucosal delivery easier. In addition, dextran nanoparticles have shown great potential as an effective adjuvant in the field of vaccines. Therefore, the main objectives of this review are to offer a thorough summary of the different methods used to prepare dextran nanoparticles, explore the wide range of applications for these nanoparticles, and clarify how they are able to enter cells.

Keywords: Nanoparticles, Stability, Vaccines, Antigen, Dextran, Mucosal.

INTRODUCTION

The efficacy of many pharmaceuticals is often limited by their ability to reach the precise site of therapeutic intervention. In most cases with conventional dosage forms, a considerable amount of the administered dose fails to reach the intended target site. On the contrary, the drug spreads throughout the rest of the body according to its physical and chemical properties. Therefore, the creation of a drug delivery system that improves the effectiveness of a drug while reducing its toxicity in the body is a major challenge [1].

A potential approach involves the use of colloidal drug carriers, which can effectively transport drugs to specific locations or targets and control the release of the drugs for optimal results. The idea of using tiny drug delivery systems for targeted drug delivery has developed since Paul Ehrlich first suggested using small drug-loaded projectiles over a hundred years ago. Liposomes and micro/nanoparticles have been extensively studied among these carriers. Liposomes have some limitations in terms of consistency and stability, as well as less than ideal efficiency in trapping drugs. Nevertheless, there are currently multiple drugs on the market that employ this technology and have a low molecular weight. Polymeric nanoparticles, renowned for their superior reliability and durability in comparison to liposomes, have been proposed as promising vehicles for drug delivery to tackle a range of challenges [2].

Nanoparticles are small solid particles that are dispersed in a colloidal medium. They have a size ranging from 1-1000 nm. These entities consist of large molecular substances and have the potential to be used therapeutically as adjuvants in vaccines or as carriers for drugs. Within this framework, the active ingredient has the potential to undergo dissolution, entrapment, encapsulation, adsorption, or chemical bonding. Nanoparticles can be made from a variety of synthetic and natural polymers. There are two main categories of nanoparticles: nanospheres and nanocapsules, which are differentiated by their method of preparation. Nanospheres have a solid structure called a matrix, where drugs are either spread out or attached to their surfaces. Nanocapsules have a unique structure, where drugs are either enclosed within

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the core or attached to the outer surface of the membrane-wall.The term "nanoparticles" is used because it is difficult to determine whether these particles belong to a matrix or a membrane category [3].

MATERIALS AND METHODS

From SD Fine Chemicals in Mumbai, we obtained dextran and glutaraldehyde. A typhoid antigen (Ty21a) was procured from Merck in Mumbai. The Mumbai-based Qualigens Pharma Private Limited supplied the bovine serum albumin (BSA). Himedia, an Indian company located in Mumbai, was sourced the dialysis membrane. The additional compounds that were used were all of analytical grade. When called for, the research made use of doubly distilled water.

Preparation of plain dextran nanoparticles entraping Ty21A antigen

The natural polymer was an essential component in the synthesis of all the nanoparticles because it controlled the release of the entrapped dextran and improved the nanoparticles' structural integrity. The nanoparticles were stabilized in their current location and cross-linked by adding an aqueous solution containing 8% v/v glutaraldehyde. The nanoparticles were made using different compositions of polymer (dextran) and then tested for various in vitro parameters [4].

A two-step desolvation method, with the necessary modifications, was used to produce the dextran nanoparticles (DNPs). In a controlled laboratory environment, 0.5 grams of dextran was dissolved in 10 milliliters of water that had undergone two distillations. Temperatures between 40 and 45 degrees Celsius were used for this procedure. Then, 15 mL of acetone was added to the dextran solution for desolvation. After removing the liquid, the solid particles were redissolved in 10 ml of distilled water with the use of an agitator. The antigen was then added to the previously described dextran solution using a 500 µL aqueous solution. After that, the mixture was heated to 40 degrees Celsius and stirred for one hour at 600 revolutions per minute. This was accomplished by adding a 0.1 M sodium hydroxide (NaOH) solution, which raised the solution's pH to 7.4. With the temperature maintained at 40°C and the agitation speed set at 600 rpm, the solution was prepared. The next step was to gradually add 25 milliliters of acetone to the polymer solution while stirring. This would help the polymer re-dissolve and trigger the formation of nanoparticles. An aqueous solution containing 8% glutaraldehyde (v/v) was added to 50 µl of nanoparticles in the same place to help them crosslink and stabilize while stirring. The excess glutaraldehyde was neutralized by using cysteine. We centrifuged the nanoparticles at 1000 g for 40 minutes to remove any impurities. They were then reconstituted in water that had been doubly distilled. Afterwards, a Milipore 0.45 micrometer membrane filter was used to filter the nanoparticulate suspensions. [5]

The stability testing, antigen intactness during system construction, and impacts of exposure to varied storage conditions were all addressed in this work by analyzing the dextran nanoparticulate systems that were generated.

Stability Study During Formulation Development by SDS-PAGE

Integrity of Ty antigen was checked by SDS-PAGE analysis of the Ty release overnight from the dextran nanoparticles (pH 1.2, 7.4, 370C at 100rpm). The sample was centrifuge at 4000 rpm to separate the antigen from nanoparticles an aliquot was then solubilised with the loading buffer and treated (5 min at 100oC). The SDS-PAGE was performed in accordance with standard protocols with 12% resolving gel, cast and run in tris glycine buffer at 25 mA and finally stained with Coomassie Brilliant Blue [6].

Table 1: Protein Marker

Proteins Used	Molecular Weight (Da)
Myosin, Rabbit Muscle	205,000
Phosphorylase b	97,400
Bovine Serum Albumin	69,000
Ovalbumin	43,000
Carbonic Anhydrase	29,000

Stability Study During Storage

Stability studies were conducted on the prepared formulation, which was loaded with Ty21a Ag. The formulations were stored in screw capped amber colored glass bottles and subjected to temperatures of 41oC and 281oC for a duration of 42 days. The formulations were assessed for any alterations in size and the percentage of remaining antigen every 7 days. The residual Ty21a Ag was determined using the Bradford Protein estimation method. The initial antigen content was regarded as 100%.

In-vitro study

The ex-vivo study is conducted to gather valuable insights into the potential performance and effectiveness of the prepared nanoparticulate delivery system in a real-life setting. In this study, the NDDS vaccine formulation was developed to maximize the immune response against the antigen when administered intranasally. Successful mucosal intranasal immunization relies on the uptake and transcytosis of antigen through mucosa, as well as its delivery to macrophages (antigen pr and antigen presenting cells). In addition, it was necessary for the vaccine to be non-toxic nature[7].

The vaccine formulations containing Ag85, an antigen for tuberculosis, were meticulously developed, fine-tuned, and thoroughly assessed for their in vitro properties. The results indicate that these formulations exhibit remarkable stability. We conducted an ex-vivo study to assess the cytotoxicity of the carrier system and the uptake of antigen-loaded nanoparticles by macrophage cell lines. The selective optimized formulations were evaluated for this purpose. For the ex-vivo study, the following formulations were utilized to compare mannosylated nanoparticles with unconjugated nanoparticles: - Control (normal saline) - Plain antigen (Pl-Ag85) - Gelatin nanoparticulate formulations (GNP (Ag85) and MNGNP (Ag85)] and formulations of PLGA nanoparticles [PNP (Ag85), MNPNP (Ag85)].

Cytotoxicity study

It is crucial to ensure that every new formulation is safe for both normal cells and macrophages. In this particular scenario, it is crucial for the vaccine to be formulated in a way that does not harm the antigen presenting cells, specifically macrophages, as they play a vital role in producing antibodies. Therefore, the carrier system, which in this case is nanoparticles, must be non-toxic. Thus, the cytotoxicity study involved incubating macrophage cell lines with different antigen loaded formulations for 24 hours. Following incubation, the cells were stained with annexin PE (BD Bioscience, USA), a dye that specifically targets deceased cells[8]. We used FACS to count the stained dead cells. Data is documented in table 2.

Table 2: In-vitro cell cytotoxicity of nanoparticulate formulations on macrophage cell line after incubation for 24 h

Formulation Used	% Cytotoxicity
Control	2.32±0.25
Pl-Ag (Ty)	3.20±0.62
DNP	3.42±0.43
MNDNP	5.71±0.38
PNP	3.30±0.63

In-vivo Study

Animals were housed in groups of six, with unrestricted access to water and food. They refrained from eating for 3 hours prior to immunization. The study protocol followed the approved guidelines set by the Institutional Animal Ethical Committee of Dr. Hari Singh Gour University, Sagar. In order to elicit an immune response, a dosage of 1mg/kg body weight of antigen was administered orally in small droplets. Oral dosing was carried out by inserting a cannula into a non-anesthetized animal. Great attention was given to ensuring that a new drop was only administered once the previous one had been completely depleted. The secondary immunization was administered four weeks later using the same dosage of the formulation. The control group was administered a dose of regular nanoparticles [9].

The formulations were administered based on their respective groups. The nanoparticles were suspended in saline solution in order to achieve the

desired concentration of Ty Ag. Oral administration of thirty microtitre formulations was provided to mice. After 15 minutes, an additional dose of 30 microtitre of formulation was administered orally to the mice to reach a total dose of 10 µg of Ty Ag. The administered formulation (Ty Ag) was given via the I.M. route, with a booster administered 21 days after the initial immunization. The control group received an oral administration of a PBS solution [10].

Table 3: Different formulations administered in mice

Groups	Formulation administered	Dosing schedule	Route
I	Control	0	ORAL
II	Plain antigen	0, 7, 21,	ORAL
III	Marked formulation	0, 7, 21,	I.M.
IV	Dextran Nanoparticles	0, 7, 21,	ORAL
V	ED Dextran Nanoparticles	0, 7, 21,	ORAL

Sample Collection

For the analysis, blood samples were obtained through retro-orbital puncture while the animals were under mild ether anesthesia. The samples were collected at four different time points: 0, 7, 21, and 28 days. Serum was then separated from the blood and stored at -4 ℃ until it was ready to be tested using the ELISA method to detect the presence of antibodies[11].

Determination of IgG and IgA by Elisa Assay

Microtiterstrips are coated with Typhoid Antigen and then incubated with diluted standard sera and samples. During this process, specialized antibodies are attached to the fixed antigen. Following the removal of any unbound material through a washing procedure, the presence of the antigen-antibody complex in each well is determined using peroxidase conjugated anti-human IgG antibody[12]. Following the elimination of unbound conjugate, the micro titer strips undergo incubation with a substrate solution that consists of hydrogen peroxide and a tetramethylbenzidine buffer solution. A blue hue emerges as the Typhoid-specific IgG binds to the wells of the microtiterstrips. The enzymatic reaction is halted by adding 2N H_2SO_4, and the absorbance values at 450 nm are measured. A standard curve is generated by graphing the absorbance values against their corresponding standard values (IU/ml). The concentration of IgG antibody to Tetanus Toxoid in patient samples is determined by interpolating from this standard curve [13].

Day One

Wells of the microtiterplate were coated with 100 microliters per well of Typhoid coating antigen at a concentration of 0.4 mcg/ml in a coating buffer. The plates were coated with an adhesive cover and left to incubate for 30 minutes at 37 degrees centigrade, followed by overnight incubation at 4 degrees centigrade [14].

Day Two

Wells were thoroughly cleaned three times using a wash solution, either manually or with an automatic plate washer. Blocking buffer was added, 100 microliters per well. The plate was covered and incubated for one hour at 37 degrees Celsius. The plate was washed three times with the wash solution, either by hand or using an automatic plate washer.

Various samples were included, such as patient and control sera, blank, and non-specific antibody. 100 microliters were added to each well in triplicate. The plate was then covered and incubated at 37 degrees centigrade for one hour. The plate was washed three times with a wash solution, either by hand or using an automatic plate washer. Next, the HRP anti-human IgG conjugate was added, 100 microliters per well.

The plate was carefully covered and placed in an incubator for an hour at 37 degrees centigrade. After that, it was washed three times using a wash solution, either manually or with the help of an automatic plate washer. 100 microliters of OPD substrate solution was added to each well. The solution was then incubated at room temperature in

the dark. Readings were taken at 450 nm at 5, 10, 15, and 30-minute time points. The concentration of the sample was calculated [15].

RESULTS AND DISCUSSION

The formulation's ability to withstand environmental stresses and retain stability associated with the antigen was assessed in the mentioned studies. We assessed the integrity of the antigen after formulating it to ensure its stability. A reliable formulation should maintain a consistent particle size and level of active antigen throughout storage. In addition, the study assessed the storage stability under various shelf temperatures. Although it is recommended to store immunological preparations under refrigerated conditions, we also conducted storage stability tests at room temperature to examine the protective effect provided by the developed nanoparticulate systems for the associated antigen.

The stability and integrity of the attached antigen were evaluated using SDS-PAGE to ensure the technical aspects of the process. The PAGE was conducted using spots of pure antigen and antigen extracted from different formulations. Figure no. 2.4 displays distinct bands representing both pure and extracted antigens, with Ty located at approximately 49 kDa. This indicates that the preparation conditions did not result in any permanent aggregation or cleavage of the protein.

The chosen Ty Ag loaded formulations and plain Ty Ag solution were kept in securely sealed amber colored bottles at 41°C and elevated temperature 281°C for 42 days and examined on a weekly basis. The results of the study did not indicate any significant variation in mean size over a 28-day storage period for the dextran based nanoparticulate system. The size effect observed after 28 and 42 days was 359±26nm and 412±40nm, respectively, as the temperature increased.

After 28 days at 281°C, approximately 71% of the protein content remained in the dextran nanoparticulate system. At 41°C, more than 85% of the protein was still present. Based on the results obtained, it is evident that in order to achieve optimal stability, it is recommended to store the formulation under refrigerated conditions (Table 4-5).

Table 4: Effect of Storage on Particle Size

S.NO.	TIME (in Days)	SIZE (in nm)	
		4±1°C	28±1°C
1	0	183±33	183±33
2	7	192±41	186±30
3	14	231±32	208±26
4	21	281±42	335±31
5	28	324±27	359±26
6	42	392±40	412±40

Table 5: Effect of Storage on Antigen Content

Days	% Residual Antigen			
	AT 4±1°C		AT 28±1°C	
	Ty	Formulation	Ty	Formulation
0	100	100	100	100
7	98±1.2	97±0.92	97±0.9	97±0.8
14	95±0.87	94±0.84	94±1.1	89±1.4
21	91±0.89	89±1.3	86±1.8	81±1.2
28	87±1.08	85±1.2	74±1.6	71±1.08
42	85±1.5	82±2.1	69±1.4	67±1.5
56	82±1.7	77±1.8	64±1.7	61±1.07

Based on the findings of the in-vivo study, it was observed that the administration of a 10µg/oral dose of the formulation to mice aged 6-7 weeks resulted in the production of antibodies against the Typhoid antigen. The ELISA method was used to estimate the IgG levels in the collected serum. Salivary secretion was assessed by administering pilocarpine 0.2 ml (10 mg/ml) to mice, allowing for estimation of IgA levels. Following the administration of the formulation, the immune responses were assessed in both serum and salivary secretions. When comparing the intramuscular typhoid injection to the nanoparticulate formulations, it was observed that the latter resulted in a noteworthy IgA response in

salivary secretion, as well as IgG responses in blood serum.

After administering Ty in PBS and in formulation orally and in injection I.M., samples were collected at various time intervals. The ELISA assay results indicate that there is a higher production of IgG and IgA on days 21 and 28. The results indicate a strong immune response to the developed formulations (Dextran nanoparticles encapsulating Ty Ag) after oral immunization. Both systemic and oral immune responses were observed at a significant level (Table 6-7).

Table 6: Anti- TyAg IgG levels with formulation (OD at 450nm)

Formulation/Dose / Route		IgG levels in serum			
		0 days	7th days	21th days	28th day
Control	Plane PBS solution	0.0180 ±0.0101	0.0191 ±0.010	0.0221 ± 0.0120	0.0197 ±0.011 0
Plain antigen	Ty Ag/ 10µg/ oral	0.0210 ±0.0101	0.194 ±0.011 0	0.0241 ± 0.0130	0.542± 0.0101
Ty inj.	Ty inj./ 10µg / I.M.	0.0315 ±0.0120	0.198 ±0.011 0	0.268± 0.0102	0.573± 0.0121
DXN NPs	Dextran nanoparticles TyAg/10µg / oral	0.0256 ±0.0101	0.108 ±0.011 0	0.204± 0.0103	0.646 ± 0.0110
ED Ty-NPs	ED Dextran nanoparticles/10µg TyAg/ oral	0.0216 ±0.0123	0.166 ±0.012 9	0.275± 0.0103	0.662 ±0.012 0

Table 7: Anti- Ty Ag IgA levels with formulation (OD at 450 nm)

Formulation/Dose/Route		IgA levels in salivary secretion after	
		21 days	28 days
Control Plane PBS solution		0.0197±0.011 0	0.0231±0.012 0
Plain antigen	Ty Ag/10µg / oral	0.011±0.0134	0.016±0.0112
Ty inj.	Ty inj./ 10µg / I.M.	0.039±0.0104	0.054±0.0124
DXN NPs	Dextran nanoparticles TyAg/10µg / oral	0.045±0.0114	0.068±0.0304
ED Ty-NPs	ED Dextran nanoparticles/10µg TyAg/ oral	0.0512±0.011 7	0.0779±0.011 8

CONCLUSION

The antigen Ag TY can be effectively encapsulated in nanosize carriers while preserving its immunogenicity. The structural integrity of the antigen remains intact without any fragmentation. By utilizing polymeric nanoparticles, the antigen can be conveniently administered orally, eliminating the need for the uncomfortable parenteral vaccination method. The nanoparticles prepared with dextran and loaded with antigen remained stable when stored in refrigerated conditions. Based on the in vivo studies conducted in balb/c mice, a conclusion can be drawn. The surface of nanoparticles entrapping AgTY has been modified with dextran conjugation, which has proven to be a highly effective method for developing an oral Typhoid vaccine. (ii) These nanoparticulate formulations stimulate the immune system to generate both cellular and humoral immunity against Typhoid. (iii) Mannose acts as a ligand that facilitates the binding of nanoparticles to receptors, which then leads to the uptake and internalization of the nanoparticles by macrophages through M cells. (iv) The process of antigen entrapping of nanoparticles through fusogenic peptide triggers endosomal escape,

facilitating cytosolic processing. This leads to the generation of cell-mediated immunity, aiding in the elimination of intracellular pathogens.

Conflict of Interest- No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

Acknowledgement: We would like thank to School of pharmacy, SunRise University, Alwar for providing all facilities to conduct the research work.

Funding Resources: None

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