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ORIGINAL ARTICLE **OPEN ACCESS**

In-Vitro and *In-Vivo* Evaluation of Antigen-Encapsulated Dextran Nanoparticles

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ABSTRACT
Dextran nanoparticles have garnered increased interest as vehicles for drug delivery due to their enhanced stability, minimal toxicity, straightforward and gentle preparation process, and ability to facilitate diverse routes of administration. The sub-micron size of the particles is not only appropriate for parenteral administration, but also suitable for mucosal routes of administration such as oral, nasal, and ocular mucosa. These routes are considered non-invasive. The dextran absorption enhancing effect also aids in facilitating the application of mucosal delivery. Moreover, dextran nanoparticles have demonstrated efficacy as a promising adjuvant in the context of vaccines. Hence, the primary goals of this review are to provide a comprehensive overview of the various preparation techniques employed for dextran nanoparticles, examine the diverse applications of these nanoparticles, and elucidate the mechanism by which they enter cells.
Keywords: Antigen, Dextran, Mucosal, Nanoparticles, Stability, Vaccines.

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INTRODUCTION

The effectiveness of numerous pharmaceuticals is frequently constrained by their capacity to access the specific location of therapeutic intervention. In the majority of cases involving conventional dosage forms, a significant portion of the administered dose does not reach the intended target site. Instead, the drug disperses throughout the rest of the body based on its physical and chemical characteristics. Hence, the task of creating a drug delivery system that enhances the pharmacological efficacy of a drug while minimizing its in vivo toxicity presents a significant challenge [1].

One strategy involves the utilization of colloidal drug carriers, which have the ability to deliver drugs to specific sites or targets while also ensuring optimal release profiles of the drugs. The concept of employing submicron drug delivery systems for drug targeting emerged and progressed subsequent to Paul Ehrlich's initial proposition of utilizing minuscule drug-loaded projectiles more than a century ago. Among these carriers, liposomes and micro/nanoparticles have been the subject of extensive investigation. Liposomes exhibit certain technological constraints, such as inadequate reproducibility and stability, as well as suboptimal drug entrapment efficiency. However, there are currently several commercially available drugs with low molecular weight that utilize this technology. Polymeric nanoparticles, known for their enhanced reproducibility and stability compared to liposomes, have been suggested as potential drug carriers to address various challenges in drug delivery [2].

Nanoparticles are discrete, solid particles dispersed in a colloidal medium, characterized by their size falling within the range of 1-1000 nm. These entities are composed of macromolecular substances and have the potential to be employed therapeutically as adjuvants in vaccines or as carriers for drugs. In this context, the active ingredient can be dissolved, entrapped, encapsulated, adsorbed, or chemically bonded. Nanoparticles can be composed of both synthetic and natural polymers. Nanoparticles can be classified into two distinct categories based on the method of preparation: nanospheres and nanocapsules. Nanospheres possess a monolithic structure, referred to as a matrix, within which drugs are either dispersed or adsorbed onto their surfaces. Nanocapsules possess a membrane-wall configuration, with drugs being either encapsulated within the core or adsorbed onto their outer surface.The term "nanoparticles" is used due to the inherent challenge in definitively determining whether these particles belong to a matrix or a membrane category [3].

MATERIAL AND METHODS

Dextran and Glutaraldehyde were procured from SD Fine Chemicals, Mumbai. The Typhoid (Ty21a) antigen was obtained from Merck, Mumbai. The Bovine Serum Albumin (BSA) was acquired from Qualigens Pharma Private Limited, located in Mumbai. The dialysis membrane was obtained from Himedia, a supplier based in Mumbai, India. All the other chemicals utilized were of analytical grade quality. The study utilized double distilled water as necessary.

Formulation preparation

Preparation of plain dextran nanoparticles entraping Ty21A antigen

All the nanoparticles were synthesized using a natural polymer as it was a necessary component for controlling the release of entrapped dextran and enhancing the structural integrity of the nanoparticles. An aqueous solution containing 8% v/v of glutaraldehyde was introduced to induce cross-linking and stabilize the nanoparticles in their current location. Polymer (dextran) with varying compositions was utilized to fabricate nanoparticles, which were subsequently assessed for a range of in vitro parameters [4].

The dextran nanoparticles (DNPs) were synthesized using a two-step desolvation technique with appropriate adjustments. In a laboratory setting, a quantity of dextran weighing 0.5 grams was dissolved in 10 milliliters of water that had been distilled twice. This process took place at a temperature range of 40 to 45 degrees Celsius. Subsequently, the dextran solution was subjected to desolvation by adding 15 milliliters of acetone. The liquid portion was removed, and the solid particles were dissolved again in 10 ml of distilled water while being agitated. Subsequently, a 500 µL aqueous solution containing the antigen was introduced into the aforementioned dextran solution. The resulting mixture was agitated at a speed of 600 revolutions per minute for a duration of 1 hour at a temperature of 40°C. The pH of the solution was modified to 7.4 by utilizing a 0.1 M sodium hydroxide (NaOH) solution. The temperature was set to a constant value of 40°C and the solution was agitated at a speed of 600 revolutions per minute (rpm). Subsequently, a volume of 25 milliliters of acetone was incrementally introduced into the polymer solution while stirring, in order to facilitate the re-dissolution of the polymer and consequently induce the creation of nanoparticles. During the process of stirring, a volume of 50 µl of an aqueous solution containing 8% glutaraldehyde (v/v) was introduced to facilitate the crosslinking and stabilization of nanoparticles that were present in the same location. Cysteine was utilized to neutralize the surplus of glutaraldehyde. The nanoparticles underwent purification through centrifugation at a force of 1000g for a duration of 40 minutes. Subsequently, they were reconstituted in double distilled water. Subsequently, the nanoparticulate suspensions underwent filtration using a 0.45 micrometer membrane filter manufactured by Milipore. The chemical compositions of different formulations [5].

The developed dextran nanoparticulate systems in the present study were subjected to the stability testing and the intactness of the antigen during preparation of systems and effects of exposure to different storage conditions were determined.

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, 37^0C 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 100^oC). 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].

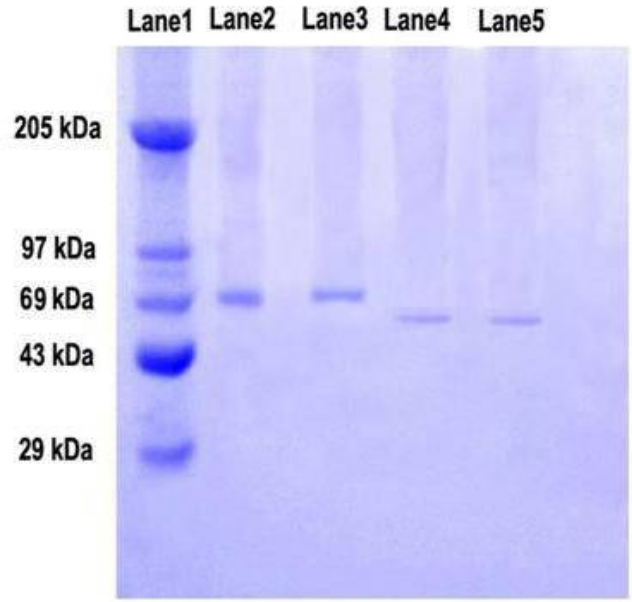

Figure 1: Photograph SDS-PAGE

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 of prepared formulation (loaded with Ty21a Ag) were carried out after storing the formulations at 4±1°C and 28±1°C for 42 days in screw capped amber coloured glass bottles. After every 7 days the formulations were evaluated for changes in size and percent residual antigen. The % residual Ty21a Ag was determined by Bradford Protein estimation method. The initial antigen content was considered as 100%.

In-vitro study

The ex-vivo study is performed in order to get the useful information about the possible in-vivo performance and effectivenss of the prepared nanoparticulate delivery system. In the present investigation NDDS vaccine formulation was designed to induce the best possible immune response against the antigen after intranasal administration. The uptake and transcytosis of antigen through mucosa and its subsequent delivery to macrophages (antigen pr and antigen presenting cells) is a requirement for successful mucosal intranasal immunization. Additionally it was also required that the developed vaccine will be nontoxic in nature [7].

The nanoparticulate vaccine formulations entrapping Ag85 (antigen for tuberculosis) were prepared, optimized and evaluated for various in vitro characteristics and found to be stable enough. The selective optimized formulations were evaluated by ex-vivo study to ascertain the cytotoxicity of the carrier system and cellular uptake of antigen loaded nanoparticles by the macrophages cell lines. The following formulations were used for ex-vivo study for comparison of mannosylated nanoparticles with unconjugated nanoparticles:-

Control (normal saline), plain antigen (Pl-Ag85), gelatin nanoparticulate formulations (GNP (Ag85) and MNGNP (Ag85)] and PLGA nanoparticulate formulations [PNP (Ag85), MNPNP (Ag85)]

Cytotoxicity study

Every new formulation develops should not be toxic to both the normal cells as well as the macrophageg. In the present case the vaccine was prepared to produce the antibodies, hence the carrier system(nanoparticles) should not be toxic to antigen presenting cells i.e. macrophages. Therefore the cytotoxicity study was performed by incubation of macrophages cell lines for 24h with various antigen

loaded formulations. After incubation the cells were double stained with annexin PE (BD Bioscience, USA), which stains only dead cells [8]. The stained dead cells were counted by FACS. Observations are recorded in table 2 and graphically shown in figure 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 (n=6) with free access of water and food. They were withdrawn of any food intake 3h before immunization. The study protocol as approved by Institutional Animal Ethical Committee of Dr. Hari Singh Gour University, Sagar was followed. To evoke an immune response, 1mg/kg body weight of antigen was given orally in small drops. Oral dosing was performed by inserting canulla to non-anesthetized animal. Care was taken that a new drop was only given when the former had been entirely inspired. Secondary immunization was done after 4 week with the same dose of formulation. The control group received a dose of plain nanoparticles [9].

The formulations were administered regarding their groups. Nanoparticles were resuspended in the saline so as to obtain the required concentration of Ty Ag. Thirty microtitre formulation was given orally of mice. After 15 minutes thirty microtitre of formulation was again given orally to the mice, in order to complete the dose of 10 µg of Ty Ag. Marketed formulation (Ty Ag) was administered through the I.M. route and a booster after 21 days of primary immunization. Control group was administered orally with normal saline buffer solution [10].

Table 3: Different formulations administered in mice

S. No.	Groups	Formulation administered	Dosing schedule	Route
1	I	Control	0	ORAL
2	II	Plain antigen	0, 7, 21,	ORAL
3	III	Marked formulation	0, 7, 21,	I.M.
4	IV	Dextran Nanoparticles	0, 7, 21,	ORAL
5	V	ED Dextran Nanoparticles	0, 7, 21,	ORAL

Sample Collection

Blood was collected by retro-orbital puncture (under mild ether anesthetized) after 0, 7, 21, and 28 days and serum was separated from blood and stored at -4 ºC until tested by ELISA for antibody [11].

Determination of IgG and IgA by Elisa Assay

Microtiterstrips coated with Typhoid Antigen are incubated with diluted standard sera and samples. During this incubation, specific antibodies are bound to the immobilized antigen. After removal of the unbound material by a washing procedure, the antigen-antibody complex in each well is detected with peroxidase conjugated anti-human IgG antibody [12]. After the removal of unbound conjugate, the micro titer strips are incubated with a substrate solution containing hydrogen peroxide and a tetramethylbenzidine buffer solution. A blue colour develops in proportion to the amount of Typhoid-specific IgG bound to the wells of the microtiterstrips. The enzymatic reaction is stopped by the addition of 2N H_2SO_4 and the absorbance values at 450 nm are determined. A standard curve is obtained by plotting each absorbance value versus the corresponding standard value (IU/ml). The concentration of IgG antibody to Tetanus Toxoid in patient samples is determined by interpolation from this standard curve [13].

Day One

- Wells microtiterplate were coated with 100 microliters/well Typhoid coating antigen at 0.4 mcg/ml in coating buffer
- Plates were covered with adhesive cover, incubated for 30 minutes at 37 degrees centigrade and then overnight at 4 degrees centigrade [14].

Day Two

- Wells were washed three times with wash solution, by hand or with automatic plate washer
- Blocking buffer was added, 100 microliters/well

- Plate was covered and incubated for one hour at 37 degrees centigrade
- Than plate was washed three times with wash solution, by hand or with automatic plate washer
- Samples were added (patient and control sera, blank and non-specific antibody) 100 microliters/well in triplicate
- Plate was covered and incubated for one hour at 37 degrees centigrade
- Then plate was washed three times with wash solution, by hand or with automatic plate washer
- Then HRP anti-human IgG conjugate was added, 100 microliters/well
- Plate was covered and incubated for one hour at 37 degrees centigrade
- Washed three times with wash solution, by hand or with automatic plate washer
- OPD substrate solution, 100 microliters/well was added
- Incubated at room temperature in the dark
- Readings (O.D.) were taken at 450 nm, at 5, 10, 15 and 30 minutes time points
- Sample concentration was calculated [15].

RESULTS AND DISCUSSION

The developed formulation should have ability to withstand environmental stresses as well as retain stability associated with antigen was assessed by above mentioned studies. The stability of antigen was checked in terms of its integrity following the preparation of formulation. A stable formulation must exhibit a constant particle size and a constant level of associated active antigen during the storage. Also the storage stability at different shelf temperatures was determined. Though immunological preparations are recommended to be stored under refrigerated conditions, the storage stability at room temperature was also performed to investigate the protective effect offered by the developed nanoparticulate systems to the associated antigen.

In process stability and integrity of the attached antigen was assessed using SDS-PAGE. The PAGE was run with spots of pure antigen and antigen extracted from formulations. Clearly visible bands for pure as well as extracted antigens are shown in figure no. 2.4 for Ty at around 49 kDa location. This reveals that the preparation conditions did not cause an irreversible aggregation or cleavage of the protein.

The selected Ty Ag loaded formulations and plain Ty Ag solution were stored in tightly closed amber colored bottles at 4±1°C and elevated temperature 28±1°C for 42 days and analyzed weekly. For dextran based nanoparticulate system, the results did not show significant difference on mean size up to 28 days of storage. On increasing temperature effect observed for size after 28 and 42 days was 359±26nm and 412±40nm respectively.

By considering initial antigen content as 100%, observations for dextran nanoparticulate system represented that after 28 days at 28±1°C around 71% protein and at 4±1°C more than 85% protein remained. Thus from these results, it can be concluded that for better stability, the formulation should be stored only at refrigerated conditions (Table 4-5 and Figure 3-4).

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

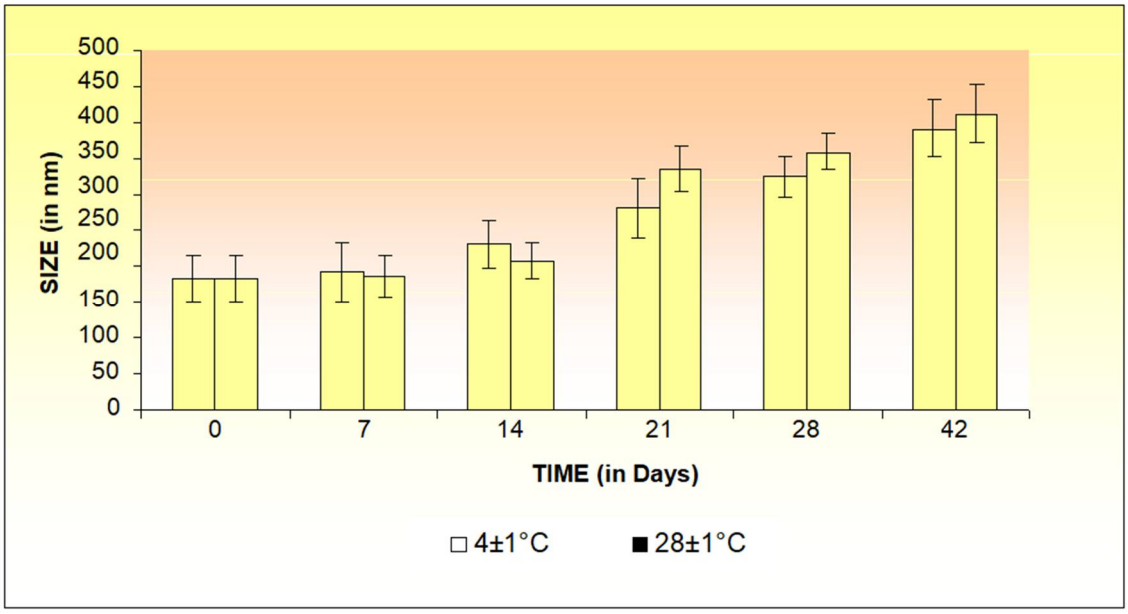

Figure 3: Effect of Storage on Particle Size

Table 5: Effect of Storage on Antigen Content

S.NO.	TIME (IN DAYS)	% RESIDUAL ANTIGEN			
		AT 4±1°C		AT 28±1°C	
		Ty	FORMULATION	Ty	FORMULATION
1	0	100	100	100	100
2	7	98±1.2	97±0.92	97±0.9	97±0.8
3	14	95±0.87	94±0.84	94±1.1	89±1.4
4	21	91±0.89	89±1.3	86±1.8	81±1.2
5	28	87±1.08	85±1.2	74±1.6	71±1.08
6	42	85±1.5	82±2.1	69±1.4	67±1.5
7	56	82±1.7	77±1.8	64±1.7	61±1.07

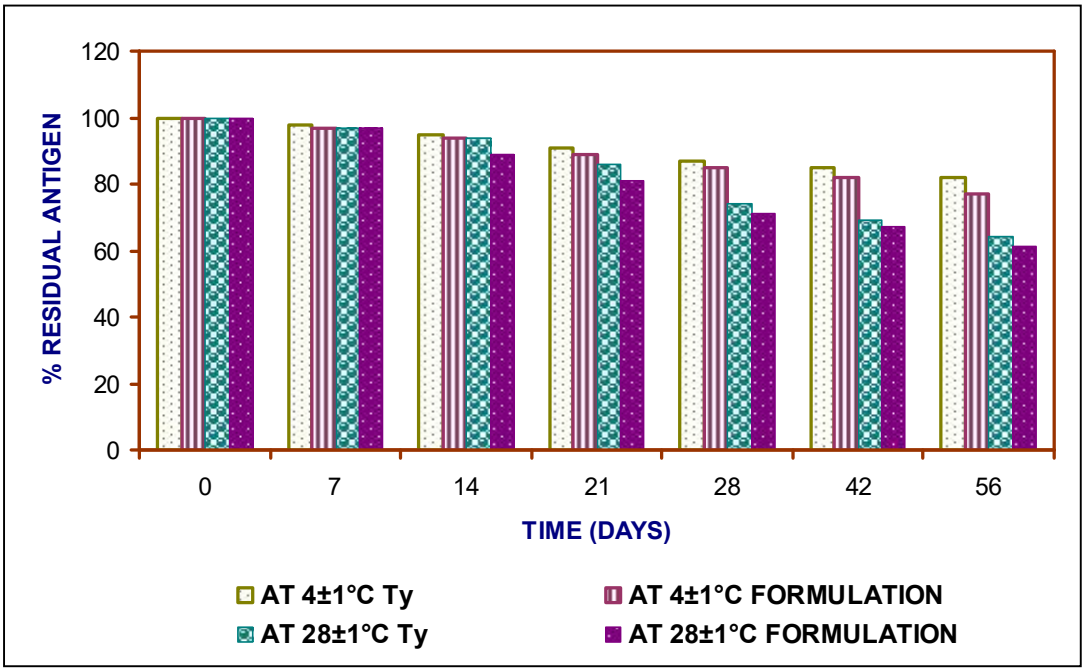

Figure 4: Effect of Storage on Antigen Content

The in-vivo study result showed that, after administration of formulation 10µg/ oral doses in the mice of 6-7 week old, gives positive responses for production of antibodies against the Typhoid antigen. In the

collected serum IgG was estimated by ELISA (Enzyme Linked Immuno Sorbent assay). And the IgA was estimated in salivary secretion by giving pilocarpine 0.2 ml (10 mg/ml) to mice. After orally administration of formulation, the immune responses were measured in serum and salivary secretions. In comparison intramuscular typhoid injection, the nanoparticulate formulations gave significant IgA response in salivary secretion, and also give IgG responses in blood serum.

Upon administering Ty in PBS and in formulation orally and in injection I.M. samples were collected in different time intervals. The results obtained after ELISA assays show on days 21 and 28 gives IgG and IgA production is higher. All the results suggest good immune response of the developed formulations (Dextran nanoparticles encapsulating Ty Ag) upon oral immunization as both systemic and oral immune responses were observed in significant magnitude (Table 6-7 and Figure 5-6).

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 ±0.0110
Plain antigen	Ty Ag/10µg / oral	0.0210 ±0.0101	0.194 ±0.0110	0.0241± 0.0130	0.542± 0.0101
Ty inj.	Ty inj./ 10µg / I.M.	0.0315 ±0.0120	0.198 ±0.0110	0.268± 0.0102	0.573± 0.0121
DXN NPs	Dextran nanoparticles TyAg/10µg / oral	0.0256 ±0.0101	0.108 ±0.0110	0.204± 0.0103	0.646 ± 0.0110
ED Ty-NPs	ED Dextran nanoparticles/10µg TyAg/ oral	0.0216 ±0.0123	0.166 ±0.0129	0.275± 0.0103	0.662 ±0.0120

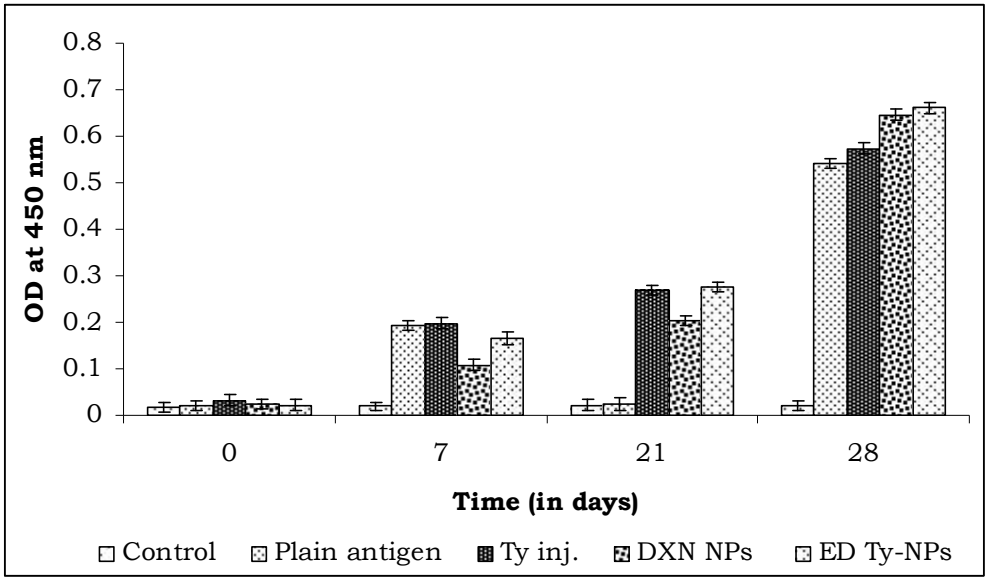

Figure 5: Anti- TyAg IgG levels with formulation (OD at 450nm)

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±0.0110	0.0231±0.0120
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±0.0117	0.0779±0.0118

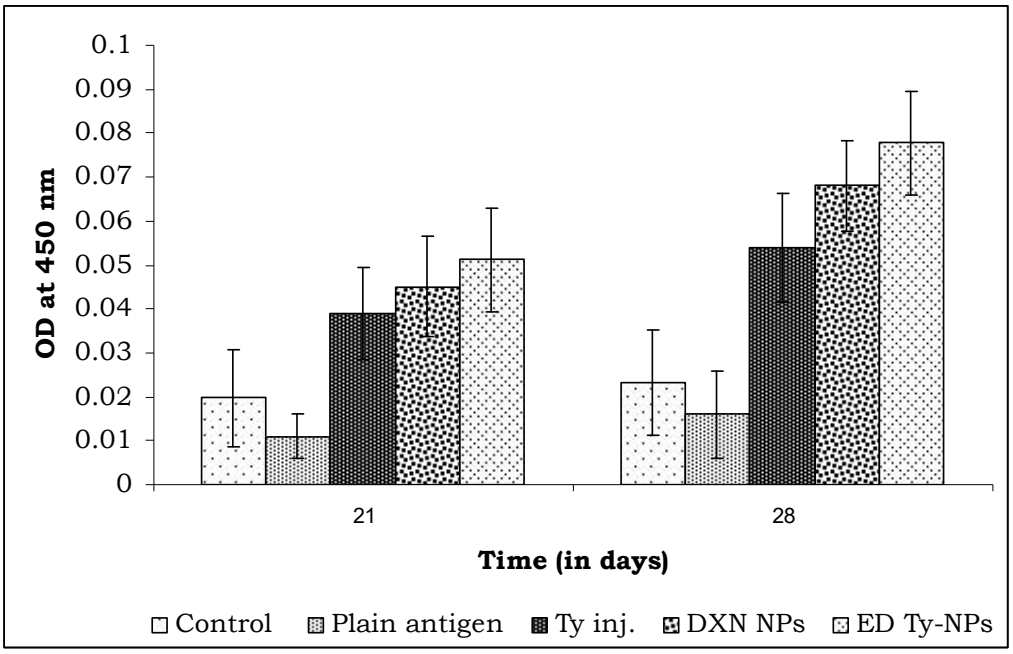

Figure 6: Anti- Ty Ag IgA levels with formulation (OD at 450 nm)

CONCLUSION

The antigen Ag TY can be successfully entrapped in nanosize carriers without affecting its immunogenicity (Structural integrity without fragmentation etc.). The polymeric nanoparticles entrapping antigen can be administered orally avoiding the painful parenteral route of vaccination. Nanoparticles prepared with dextran and antigen loaded were satisfactory stable if stored under refrigerated condition. In vivo studies performed in balb/c mice, the following conclusion could be drawn. (i) The dextran conjugation on the surface of nanoparticles entrapping AgTY, is the most successful approach for the development of Typhoid vaccine for oral route (ii) These nanoparticulate formulations act as antigen to produce cellular as well humoral immunity against Typhoid (iii) Mannose acts as ligand to mediate binding of nanoparticles to receptors followed by macrophage uptake and internalization via M cells (iv) The action of antigen entrapping of nanoparticles as fusogenic peptide causes endosomal escape which help in cytosolic processing followed by generation of cell mediated immunity which helps in the elimination of intracellular pathogen.

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.

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