



Toxicological Analysis of Nanoparticles and Microparticles Used as Oral Vaccine Delivery Systems using Vero Cell

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10.18805/IJAR.B-5246

ABSTRACT

Background: The present study was carried out to evaluate *in-vitro* toxicity associated with chitosan nanoparticles, Gantrez® nanoparticles and poly-lactide co-glycolide (PLG) microparticle in Vero cell line. The cytotoxicity of all three micro/nano-particles was assessed using different concentration.

Methods: For each concentration of these delivery systems, the confluent monolayer of Vero cells was treated for a period of 48 hours and studied for morphological alteration and cell survivability after the treatment.

Results: It was observed that the different concentrations of chitosan nanoparticles and Gantrez® nanoparticles did not have significant effect on the cell viability as evident from the non-significant difference between the OD540 of formazan product formed from MTT in treated and untreated cells. The concentration of chitosan nanoparticles and Gantrez® nanoparticles up to 1000 µg/ml did not have any influence in cellular metabolic activities and viability. However, a slight reduction (statistically insignificant) in the cellular viability and metabolic activities were observed when PLG microparticles were used at 1000 µg/ml.

Key words: Poly(anhydride) nanoparticle, Poly-lactide co-glycolide (PLG) microparticle, Toxicity, Vero cell line

INTRODUCTION

In the realm of poultry vaccination, the advantages of oral administration over the parenteral route are manifest. This approach offers expedited and simplified mass application, obviating the need for highly specialized personnel. Notably, it eliminates the risks associated with needle stick injuries and cross-contamination, a crucial consideration in large-scale implementations (McCluskie *et al.*, 2000). Expanding the horizons of vaccine delivery, nanoparticles and microparticles have emerged as pivotal tools in the biological sciences, particularly in the domain of oral drug and vaccine delivery systems. Distinct for their versatility, nanoparticles crafted from natural or synthetic polymers, lipids, proteins, and phospholipids have garnered significant attention due to their heightened stability and amenability to surface modifications (Stark, 2011). This adaptability allows for precise tailoring to achieve controlled drug release and site-specific localization, achieved by fine-tuning material characteristics or modifying surface chemistry (Herrero-Vanrell *et al.*, 2005). In the process of nanoparticle development for oral vaccine delivery, rigorous safety evaluations are imperative. *In vitro* assays constitute the forefront of methods that discriminate between safe and potentially hazardous nanoparticles. These assays also provide insights into specific mechanistic pathways, encompassing oxidative stress, generation of inflammatory cytokines, and the internalization of nanoparticles within cells (Patravale *et al.*, 2012). While the literature is somewhat scant on *in vivo* and *in vitro* toxicological assessments of certain nanoparticle carriers, such as poly-lactide co-glycolide (PLG) microparticles, chitosan, and Gantrez®

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How to cite this article: Hazarika, D., Borah, D.P., Boruah, J.S., Begum, Y., Sultana, A., Tamuly, S., Devchoudhuri, K.B., Borah, P. and Kalita, D.J. (2024). Toxicological Analysis of Nanoparticles and Microparticles Used as Oral Vaccine Delivery Systems using Vero Cell. Indian Journal of Animal Research. doi: 10.18805/IJAR.B-5246

Submitted: 23-9-2023 **Accepted:** 23-4-2024 **Online:** 16-07-2024

nanoparticles for oral vaccine delivery, the current study embarks on a comprehensive exploration. The focus of this study is to evaluate the cytotoxic effects of these oral vaccine delivery systems within a suitable cell line through *in vitro* assessments.

MATERIALS AND METHODS

This study was conducted in 2021 at the laboratories of the Department of Veterinary Biochemistry and Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781022.

The PLG microparticle were prepared by double emulsion method as described by Sultana *et al.*, (2022).

Briefly, the poly-lactide co-glycolide microparticles were synthesized by dissolving 250 mg of PLG (50:50) in 1.5 ml of dichloromethane. To this solution, 150 μ l of ultrapure water was added, followed by sonication for 20 seconds. Subsequently, 30 ml of 2% polyvinyl alcohol (PVA) was added and the mixture was sonicated again for 5 minutes. The suspension was then stirred overnight. After stirring, the suspension was transferred into pre-weighed 1.5 ml microcentrifuge tubes and centrifuged at 10,000 $\times$ g for 5 minutes. Finally, the resultant pellet was lyophilized for future use.

The Gantrez® nanoparticles were prepared as per the method described by Camacho *et al.* (2011). Briefly, Gantrez® nanoparticles were prepared by initially dissolving 100 mg of polyvinyl/maleic anhydride (PV/MA) in 5 ml of acetone, followed by stirring for 5 minutes. Subsequently, 10 ml of antigen solution was added, and the mixture was stirred continuously for 15 minutes to form a white suspension. The suspension was then centrifuged at 27,000 $\times$ g for 20 minutes at 4°C. The resultant pellet was re dissolved in 200 μ l of ultrapure water and further centrifuged at 20,000 $\times$ g for 10 minutes at 4°C. This centrifugation step was repeated, and the final pellet was stored at 20°C overnight. Post-storage, the pellet was lyophilized, weighed, and stored at -20°C until further use.

The chitosan nanoparticle was generously provided by Material Nanochemistry Laboratory, Physical Science Division, Institute of Advanced Study in Science and Technology, Vigyan Path, Paschim Boragaon, Garchuk, Guwahati, Assam.

The chitosan nanoparticles, Gantrez® nanoparticles and PLG microparticles were characterized in terms of zeta size and zeta potential by dynamic light scattering (DLS) using Malvern Zetasizer Nano series. The cytotoxicity of all three micro/nanoparticles were assessed in Vero cell line using different concentration as described by earlier workers

(Basarkar *et al.*, 2007; Ojer *et al.*, 2013; Zaki *et al.*, 2015). For each concentration, the confluent monolayer was treated for a period of 48 hours and studied. At every 24 h interval, cells were observed for visible morphological changes under inverted microscope (Karl-Zeiss, Germany) and microparticle/nanoparticle treated cells were compared with the untreated cells. After 72 h, the cells were trypsinized and the live and dead cell counts were determined using 0.4% (w/v) trypan blue exclusion method using hemocytometer and the MTT assay was done as per the method described by (Bahuguna *et al.*, 2017).

All the values were expressed as mean $\pm$ standard error (SE). The normality of the data was determined by Shapiro Wilk test. The between group difference of mean was determined by one way ANOVA (normally distributed data) or Kruskal-Wallis test (non-normally distributed data). All of the statistical analysis was carried out in statistical software R (R Core Team, 2021).

RESULTS AND DISCUSSION

The zeta sizes of chitosan nanoparticles, Gantrez® nanoparticles and PLG microparticles were determined to be 247.4 nm, 262.8 nm and 3654 nm, respectively. Concurrently, the zeta potentials for these particles were measured at 12.8 mV, -52.6 mV and 8.65 mV, respectively (Fig 1). The evaluation of cytotoxicity for all three particles was conducted on Vero cell lines employing various concentrations. Subsequently, each concentration was applied to a confluent monolayer for a duration of 48 hours, followed by an examination of morphological changes and assessments of live and dead cell counts, among other parameters, post-treatment.

Morphological evaluation of treated cells

The morphological assessment of Vero cell-line treated with PLG microparticles, Gantrez® nanoparticles and chitosan

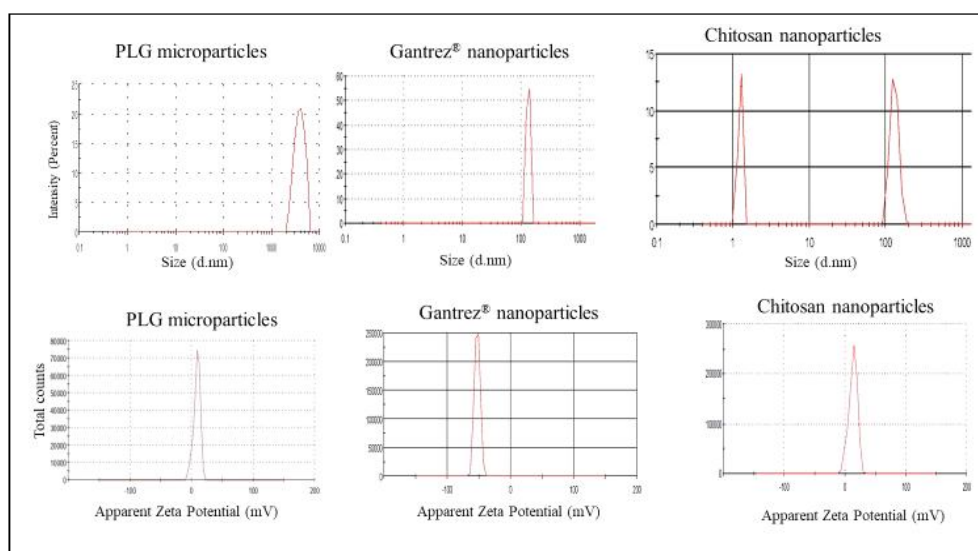


Fig 1: Zeta size and zeta potential of PLG microparticles, Gantrez® nanoparticles and chitosan nanoparticles.

nanoparticles involved comparing them to untreated control cells. Remarkably, all cells subjected to various concentrations of these particles exhibited a preserved cell monolayer structure with minimal to no compromise in cellular integrity or architecture, closely resembling the untreated cell counterparts (Fig 2). This finding suggests that the introduction of these micro/nano-particles did not induce significant morphological alterations, indicating a potential lack of cytotoxic effects and supporting their suitability for further biomedical applications.

Cell viability

Cell viability was assessed through careful observation of various cellular indicators, including changes in cell morphology such as rounding, granulation, loss of intracellular matrix, clumping, and eventual detachment from the culture surface. The comprehensive analysis of these cellular manifestations revealed compelling results: all three types of delivery systems and their respective concentrations employed in this study exhibited a notable absence of cytotoxicity in Vero cells. Quantitative assessments of live

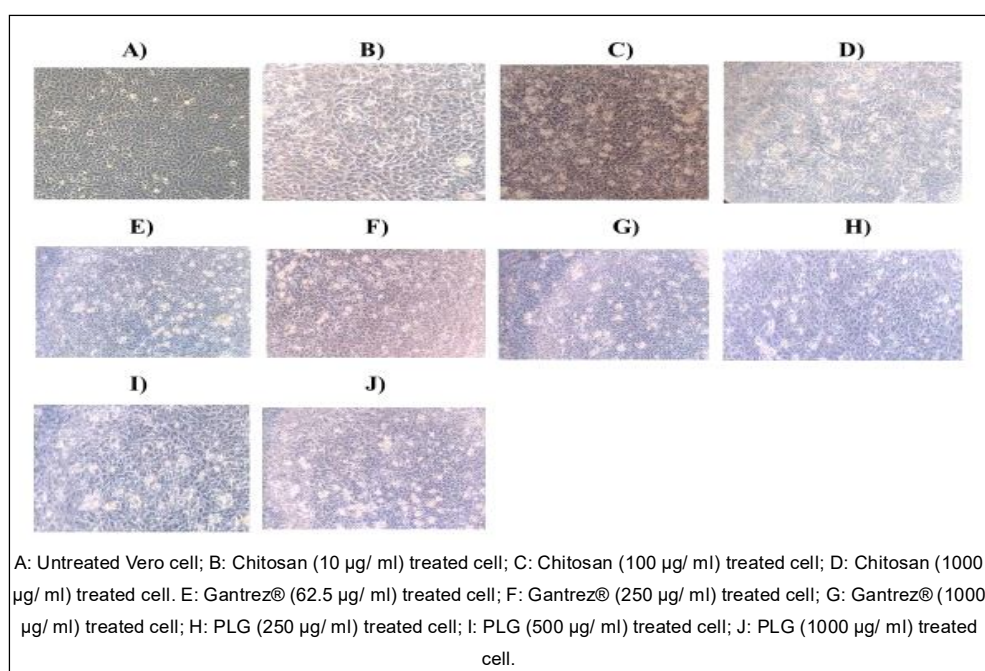


Fig 2: Morphological examination of treated and untreated Vero cell line.

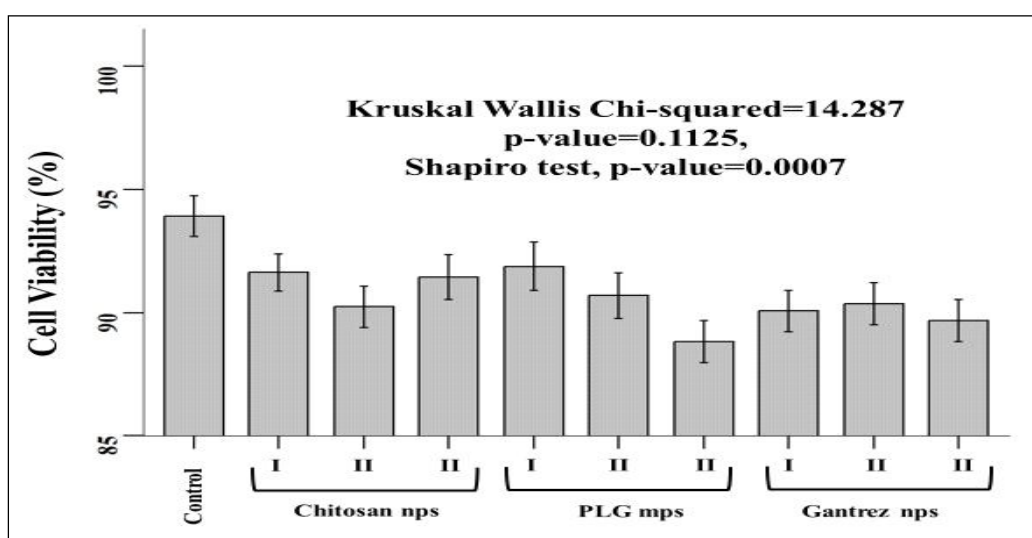


Fig 3: Cell viability of Vero cell lines treated with different nano-microparticles.

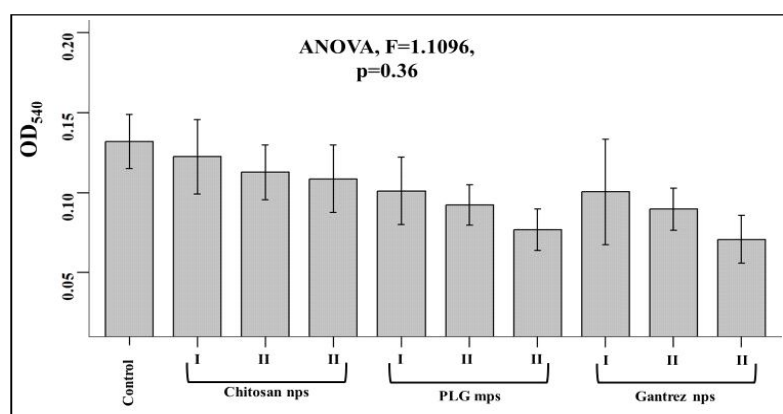


Fig 4: MTT assay of Vero cell treated with different concentration nano/microparticles.

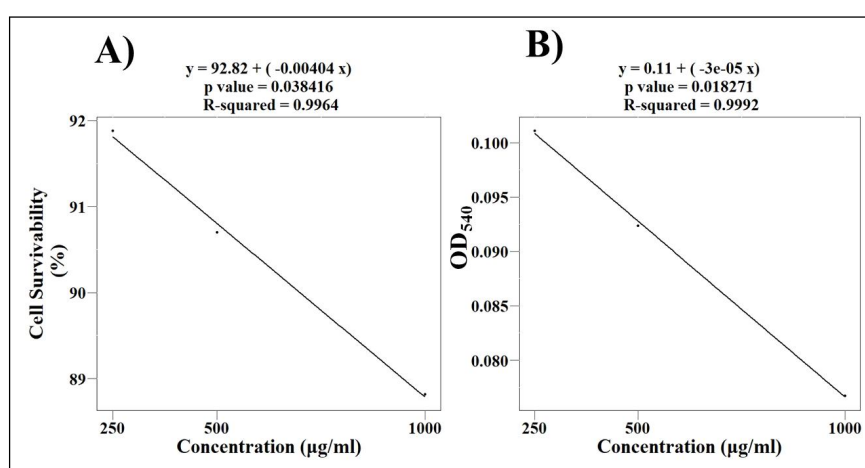


Fig 5: Effect of increasing concentration of PLG microparticles on cellular metabolism and survivability of Vero cells.

and dead cell counts in treated cultures were in line with those in the untreated control cultures. Although a slight reduction in the live cell count was observed in the treated cultures compared to the control group, statistical analysis indicated that this difference was not statistically significant. Specifically, in the control cultures, the live cell count was estimated at 2.3×10^4 cells/ml, while in cultures treated with nanoparticles, this count ranged from 1.8 – 2.06×10^4 cells/ml. These findings collectively highlight the negligible impact of different nanoparticle and microparticle concentrations on cell survivability (Fig 3). Furthermore, it is worth noting that the various concentrations of chitosan nanoparticles, Gantrez® nanoparticles and PLG microparticles did not exert a significant influence on cellular metabolic activity. This observation was evident from the absence of a statistically significant difference between the optical density at 540 nm, formazan product formed from MTT, in both treated and untreated cell cultures (Fig 4). This comprehensive analysis underscores the biocompatibility and non-cytotoxic nature of the investigated nanoparticles and microparticles towards Vero cells. Nonetheless, a noteworthy correlation was observed between the incremental concentration of PLG

microparticles and a substantial reduction in cellular viability (Fig 5).

The chitosan, a biopolymer, undergoes degradation within biological systems under the influence of enzymes such as lysozyme and a family of enzymes collectively known as chitinases (Kean and Thanou, 2010). Interestingly, previous research by Gao *et al.* (2011) indicated that chitosan nanoparticles falling within the size range of 200 to 300 nm exhibited some level of toxicity when tested in a zebrafish model. In the context of our current study, however, it is noteworthy that the concentrations of chitosan nanoparticles, Gantrez® nanoparticles and PLG microparticles employed did not induce cytotoxic effects on Vero cells up to the concentration of 1000 µg/ml. This absence of cytotoxicity may be attributed to the inherent biocompatibility and non-toxic nature of these particles, suggesting that they do not exert harmful effects on Vero cells. This aligns with similar observations reported by Essa *et al.* (2020) and Ojer *et al.* (2013) while assessing the cytotoxic effects of PLG microparticles and poly(anhydride) nanoparticles. Their study involved different concentrations and incubation times, revealing that a decrease in cell

viability was only observed at very high concentrations (1 and 2 mg/ml) and extended incubation periods (48 hours). Furthermore, the cytotoxicity of chitosan nanoparticles (CSNPs) has been shown to be relatively low, concentration-dependent and influenced by particle size. Zaki *et al.* (2015) reported that CSNPs exhibited relatively low toxicity, irrespective of particle size, at low concentrations (10 and 100 µg/ml). The lowest cell viability was observed at the highest CSNPs concentration (1000 µg/ml). Contrary to these findings, our study demonstrates a lack of statistically significant cytotoxic effects on Vero cells by the particles under investigation. Research spanning from 1998 to 2022 has consistently demonstrated that chitosan nanoparticles display minimal cytotoxic effects. This observation holds true across a diverse range of particle compositions, cytotoxicity assays and cell line evaluations, as reviewed by Frigaard *et al.* (2022). This body of evidence underscores the inherent biocompatibility of chitosan nanoparticles, highlighting their potential for a wide array of applications in the biomedical field. However, it is noteworthy that poly-lactide co-glycolide (PLG) microparticles did exhibit a slight increase in cell death and a decrease in metabolic activity in correlation with increasing microparticle concentration (Fig 5). This effect may be attributed to an elevated accumulation of lactic acid and glycolic acid, which in turn decreases the pH of the cellular media, as suggested by Chiu *et al.* (2021). In conclusion, while the non-cytotoxicity of chitosan, Gantrez® nanoparticles and PLG microparticles in our study aligns with previous research indicating their biocompatibility, the observed slight cytotoxic effects of PLG microparticles at higher concentrations may be related to alterations in the cellular microenvironment. This comprehensive assessment underscores the importance of understanding the complex interactions between nanoparticles, microparticles and biological systems, emphasizing the necessity for further investigations to elucidate the underlying mechanisms of these observed effects.

CONCLUSION

In an *in-vitro* study, various concentrations of nano/microparticles were applied to Vero cell cultures, reaching a maximum concentration of 1000 µg/ml. Morphological assessment revealed the preservation of the cell monolayer structure with minimal changes in cellular integrity and architecture. Notably, Gantrez® nanoparticles, chitosan nanoparticles and PLG microparticles did not significantly alter cellular architecture. However, as the concentration of PLG microparticles increased, a slight negative impact on cell viability and metabolic activity, as indicated by MTT assay results, was observed. In contrast, chitosan nanoparticles and Gantrez® nanoparticles exhibited no significant influence on cell viability or cellular metabolic activities. In summary, the study's findings indicate that chitosan nanoparticles and Gantrez® nanoparticles do not exert adverse effects on Vero cell viability or metabolic activities, even at concentrations as high as 1000 µg/ml.

Conversely, PLG microparticles slightly negatively affect both cell viability and metabolic activities within the Vero cell line.

ACKNOWLEDGEMENT

We hereby Acknowledge the DBT, Government of India, for financial support and the Dean, Faculty of Veterinary Science, along with CIF, IASST for facilitating resources.

Conflict of interest

I, provide this statement on behalf of all authors, declare that there are no conflicts of interest regarding the publication of the article. The research was financially supported by the Department of Bio-Technology, Ministry of Science and Technology, India. We also acknowledge the support provided by the Central Instrumentation Facility of the Institute of Advanced Studies in Science and Technology, Guwahati and the Director of Post-Graduate Studies, Assam Agricultural University. None of the supporting entities had any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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