Tacrolimus monohydrate loaded lipid polymer hybrid nanoparticles Formulation and stability study

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Abstracts

The major goals of this work were to study the influence of formulation factors on the design of Tacrolimus monohydrate loaded lipid polymer hybrid nanoparticles (TAC-CS-LPHNs). Formulation factors like 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000 (DESPE-PEG2000)\lecithin ratio shows a significant increase in particle size with increase mass ratio and insignificant change in zeta potential. Using DSPE instead of DSPE-PEG2000 and increase polymer concentration cause a significant ($p<0.05$) increase in particle size. Also, data reveals that the solvent acetonitrile produces good quality and low particle size than acetone. In addition, TAC-CS-LPHNs were exposed to various harsh environments to assessing the stability of nanoparticles against various triggering external stimuli like change ionic strength, pH, storage temperature and time. Short term stability study shows that TAC-CS-LPHNs was stable for 2 weeks after lyophilization at 25˚C while stable up to 8 months when storing at 4˚C as a lyophilized powder. The aqueous dispersion was only stable for up to 3 weeks at 4˚C storage conditions. An increase in the ionic strength shows a marked increase in particle size and decrease in the magnitude of zeta potential with inversion of sign of zeta potential to positive. The effect of pH indicates a marked increase in the magnitude of zeta potential with an increase in pH and a significant change in particle size. The effect of urine shows an insignificant change in particle size while the plasma effect demonstrates a significant increase in particle size in non-PEGylated TAC-CS-LPHNs.

Keywords: Polymer hybrid ،Tacrolimus, Stability, Formulation, Lipid polymer hybrid.

جسيمات التاكرولمس النانوية الهجينة والمكونة من الدهون والبوليمر

العوامل المؤثرة على التصنيع وتقيم الاستقرارية

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الخلاصة

ان الهدف الرئيسي لهذا البحث هو دراسة تاثير العوامل التصنيعية على تصميم جسيمات التاكرولمس النانوية الهجينة والمكونة من الدهون والبوليمر. بالاضافة الى تقيم مدى تحمل الجسيمات للمؤثرات الخارجية كالتغير بالحرارة والحموضية والتاثير الايوني بالاضافة الى مدة الخزن. عوامل كثيرة قد تم دراستها ككمية البوليمر والدواء ونسبة الدهون للبوليمر المستخدمة. بالاضافه الى تقييم المحلول وقد وجد ان الاسيتونتريل هو افضل للتصنيع.

ان دراسة الاستقرارية على المدى القصير قد بينت ان الجسيمات تبقى مستقرة لمدة ثلاثة اسابيع عند خزنها كمحلول بدرجة حرارة 4 مئوي بينما تبقى مستقرة لثلاثة ايام فقط بدرجة 25 مئوي.

ان العامل المؤثر الكبير على الاستقرارية هو التاثير الايوني حيث اثبتت الدراسة ان الجسيمات تتاثر سلبا بزيادة التركيز الايوني في المحلول.

الكلمات المفتاحيه: البوليمر والدهون الهجين، التاكروليمس

Conflict of interest

The authors declare no conflict of interest.

Introduction

Most of the active pharmaceutical ingredients are hydrophobic, which result in low dissolution and may diminish absorption through the intestinal tract and results in low bioavailability. Several methods have been invented to improve the water solubility of API, like using a nanocarrier system like liposome, nano-emulsion, polymeric nanoparticles and others [1].The development of core-shell lipid polymer hybrid nanoparticle (CS-LPHNs) as a drug delivery system to improving solubility and permeability to the cell and thus enhancing uptake. CS-LPHNs combine the advantages of liposome and polymeric nanoparticles and eliminate the limitations of liposome like instability and low entrapment efficiency[3].In the present work, Tacrolimus monohydrate was formulated as CS-LPHNs by self-assembly nanoprecipitation methods. PLGA was used as a polymer to form the core, while lipid-like lecithin was used to form a monolayer around the core. Lipid-Polyethylene glycol was introduced as a stabilizer for the CS-LPHNs system via steric hindrance, reducing the need for large amounts of lipid to synthesis CS-LPHNs[4].CS-LPHNs were prepared by nanoprecipitation methods and using polymer, lipid and lipid-PEG. Different factors that may affect formulation have been evaluated thoroughly. In addition, nanoparticles have been exposed to different stability triggers like pH, ionic strength, temperature and storage time. The outcome response of the nanoparticles to be tested was the zeta potential, particle size and PDI.

Materials and Methods

Tacrolimus monohydrate was purchased from Hangzhou Hyper Chemicals Limited (China). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000 (DSPE-PEG$_{2000}$ and Soya bean phosphatidylcholine were purchased from Avanti Polar Lipids, USA. Poly (D, L-lactide-co-glycolide) lactide: glycolide (PLGA) (75:25), mol wt 66,000-107,000 was purchased from Sigma-Aldrich, Chemie GMBH, Germany. All the other chemicals were of analytical quality.

1. **Preparation of Tacrolimus monohydrate loaded lipid polymer hybrid nanoparticles (TAC-CS-LPHNs)**

The preparation of TAC-CS-LPHNs was described thoroughly in the previous study[22].Briefly, a self-assembly single-step nanoprecipitation method has been used. The organic phase containing TAC and PLGA was dropped using an insulin syringe onto the aqueous phase which dissolved lecithin and DSPE-PEG2000 under magnetic stirring with a stirring rate of 500 rpm. The resulting solution was sonicated for 3 min at a frequency of 42 kHz and a power of 50 W. The free drug, PLGA, lipids and organic solvent were removed by washing TAC-CS-LPHNs solution three times using an Amicon® Ultra-4 centrifugal filter unite (MWT 10000 Dalton) at a rate of 1000 rpm which may resuspend again in deionized water to obtain the desired concentration. The resulting TAC-CS-LPHNs were stored at 4˚C, or freeze-dried and lyophilized for storage at-20˚C [5].Different formulas were prepared as shown in (Table 1).

2. **Factors influencing TAC-CSLPHNs formulation**

The influence of different formulation factors on the particle size and zeta potential was investigated. The optimal results of each formulation factor for CS-LPHNs were used in subsequent experiments. Unless otherwise stated, the standard preparation method described earlier was employed.

3. **Influence of lipid/polymer ratio**

Different lipid/polymer ratios ranging from (42%-9%) have been used to show its effects on particle size, zeta potential and PDI.

4. **Influence of DSPE-PEG$_{2000}$\lecithin ratio**

Different DSPE-PEG$_{2000}$\lecithin ratios have been used to show its effect on particle size, zeta potential and PDI.

5. **Influence of polymer amount**

Different amounts of PLGA polymer have been used in the formulation to study its effect on particle size, zeta potential and PDI.

6. **Influence of solvent**

Two solvents (acetonitrile and acetone) have been used to dissolve TAC and PLGA in formulation to study its effect on particle size, zeta potential and PDI.

7. **Influence of drug loading amount**

A variety of TAC (1,2,3 mg) amounts were used in the formulation to investigate the effects on particle size, zeta potential, and PDI.

8. **Influence of DSPE-PEG2000**

Two types of stabilizers have been used (DSPE and DSPE-PEG_{2000}) in the formulation to study the effect of PEG on stability, particle size, zeta potential and PDI.

9. **Influence of organic phase volume**

The organic phase is used to solubilize TAC and PLGA in the volume of (1ml and 2ml) to show the effect of drug and polymer concentration on the particle size, zeta and PDI.

Table (1) Formulas of TAC-CS-LPHNs.

Formula code	TAC mg	PLGA mg	Lecithin mg	DSPE-PEG$_{2000}$ mg	DSPE mg	ACTN ml	Acetone ml	Water ml	Lipid\ polymer (%)	DSPE-PEG$_{2000}$\ Lecithin (%)
PTX-1	**1**	**5**	**1.4**	**0.7**	**-**	**2**	**-**	**20**	**42**	**50**
PTX-2	**1**	**5**	**1**	**0.5**	**-**	**2**	**-**	**20**	**30**	**50**
PTX-3	**1**	**5**	**0.8**	**0.4**	**-**	**2**	**-**	**20**	**24**	**50**
PTX-4	**1**	**5**	**0.6**	**0.3**	**-**	**2**	**-**	**20**	**18**	**50**
PTX-5	**1**	**5**	**0.5**	**0.25**	**-**	**2**	**-**	**20**	**15**	**50**
PTX-6	**1**	**5**	**0.3**	**0.15**	**-**	**2**	**-**	**20**	**9**	**50**
PTX-7	**2**	**5**	**0.6**	**0.3**	**-**	**2**	**-**	**20**	**18**	**50**
PTX-8	**3**	**5**	**0.8**	**0.4**	**-**	**2**	**-**	**20**	**24**	**50**
PTX-9	**1**	**5**	**-**	**-**	**-**	**2**	**-**	**20**	**0**	**0**
PTX-10	**-**	**-**	**0.8**	**0.4**	**-**	**2**	**-**	**20**	**0**	**50**
PTX-11	**1**	**7.5**	**1.2**	**0.6**	**-**	**3**	**-**	**30**	**24**	**50**
PTX-12	**1**	**5**	**0.6**	**0.6**	**-**	**2**	**-**	**20**	**24**	**100**
PTX-13	**1**	**5**	**0.4**	**0.8**	**-**	**2**	**-**	**20**	**24**	**200**
PTX-14	**1**	**10**	**1.6**	**0.8**	**-**	**4**	**-**	**40**	**24**	**50**
PTX-15	**1**	**5**	**0.75**	**0.4**	**-**	**1**	**-**	**20**	**23**	**53**
PTX-16	**1**	**5**	**0.75**	**0.4**	**-**	**-**	**2**	**20**	**23**	**53**
PTX-17	**1**	**5**	**0.75**	**-**	**0.4**	**2**	**-**	**20**	**23**	**53**
PTX-18	**-**	**5**	**0.75**	**0.4**	**-**	**2**	**-**	**20**	**23**	**53**
PTX-19	**1**	**5**	**0.75**	**0.4**	**-**	**2**	**-**	**20**	**23**	**53**
PTX-20	**2**	**5**	**0.8**	**0.4**	**-**	**2**	**-**	**20**	**24**	**50**
PTX-21	**2**	**5**	**0.5**	**0.3**	**-**	**2**	**-**	**20**	**16**	**60**
PTX-22	**1**	**5**	**0.7**	**0.5**	**-**	**2**	**-**	**20**	**24**	**71**
PTX-23	**2**	**5**	**0.5**	**0.6**	**-**	**2**	**-**	**20**	**22**	**120**
PTX-24	**2**	**5**	**0.6**	**0.6**	**-**	**2**	**-**	**20**	**24**	**100**
PTX-25	**1**	**5**	**1.15**	**-**	**-**	**2**	**-**	**20**	**23**	**53**

Characterization of CS-LPHNs

Particle size

Particle size was measured using dynamic light scattering, also called particle Size Analyzer (Brookhaven Instruments Corporation, USA). The CS-LPHNs were diluted by water at 0.1 mg\ml and evaluated for particle size and PDI [6]

Zeta potential

Electrophoretic light scattering is the most frequently used method for estimating zeta potential since it is used to measure particle velocity in an electric field. The zeta potential was measured at a temperature of 25°C using electrophoretic light scattering in a Zetasizer Nano ZS (Malvern, Worcestershire, UK) apparatus[6]

Stability study

It is of primary importance to know the colloidal stability of TAC-CS-LPHNs used in the solution for better optimization considering the final nanoparticle. To develop a well-defined nanocarrier for use in therapeutic relevance, adequate characterization of TAC-CS-LPHNs with dynamic light scattering (DLS) and zeta potential (ζ) should be performed after a predetermined time interval.

Short-term storage stability

Short-term stability was assessed for both liquid and lyophilized optimized formula of TAC-CS-LPHNs in different storage conditions (4˚C±2 and 25˚C±3). 5 ml volume of the optimized formula in an aqueous solution at pH 7.5 were stored in tightly closed glass containers for 3 months and tested every 2 weeks.[7**]**.The appearance homogeneity, average particle size and polydispersity index of the nanoparticles were determined and results were performed in triplicate[8].For lyophilized batches, powders were reconstituted with water and evaluated for physicochemical properties[9].

Long-term storage stability

The lyophilized optimized formula of TAC-CS-LPHNs stored in the closed vial at 25˚C for 8 months. The powder is then resuspended in 10 ml of deionized water (pH 7.5) at a concentration of 100µg\ml. The TAC-CS-LPHNs assayed for particle size, Zeta and free TAC leakage measured by HPLC.[13]

Factor influencing physical stability

The development of stable TAC-CS-LPHNs needs to be tested for external stimuli such as temperature, pH and electrolyte concentration of the surrounding fluid which may trigger the chemical and physical transformation resulting in changed particle size, PDI, particle shape, zeta potential, load or release profiles, and in vivo and in vitro activities[10.]Therefore, understanding the physicochemical stability of the lipid-polymer hybrid carriers, under various physiological conditions, is important to ensure the developed delivery systems will remain in a state of adequate quality throughout their shelf life as well as within the human body.A few mL of TAC-CSLPHNPs were freshly prepared as described above and passed through a 0.45 µm Millipore filter paper. The obtained suspensions were kept at 4°C as the stock solution. A variety of conditions were used to assess the physical stability of TAC-CS-LPHNs[9]

Effect of Urine

1. 5 mL of optimized TAC-CSLPHNs freshly prepared.
2. 2 mL of stock solution was diluted with urine up to 5 ml.
3. Store at 4˚C for 24 hrs.
4. Measure particle size, ζ and PDI by DLS.

Effect of pH

1. 20 mL of optimized TAC-CSLPHNs freshly prepared
2. 1 mL of stock suspension was adjusted to pH 1,3,5,6,7,9,12 using 0.1 M HCl and 0.1 M NaOH, and the volume was adjusted to 5 ml using water.
3. The samples were kept at 4˚C for 24 hrs.
4. Measure particle size, ζ and PDI by DLS.

Effect of Ionic strength

1. 20mL of optimized TAC-CSLPHNs freshly prepared
2. 5mL of stock solution was diluted with NaCl solution to obtain different ionic strengths (1.33,2,2.4,3.2 and 3.6 M) at pH 7.4
3. The samples were kept at 4˚C for 24 hrs.
4. Measure particle size, ζ and PDI by DLS.

Effect of Plasma

1. 5 mL of freshly prepared TAC-CS-LPHNs using DSPE-PEG_{2000} and DSPE.(PTX-17, PTX-19)
2. 2ml of each stock solution was diluted with 5 mL of freshly prepared plasma.
3. The samples were kept at 4˚C for 24 hrs.
4. Measure particle size, ζ and PDI by DLS.

Statistics study

All data was conducted in triplicate and provided as mean±S.D. Statistical significance was determined using two-way ANOVA in the Minitab program, with a significance level of $p<0.05$ considered important.

Results and discussion

The preparation process involves primarily using three materials, PLGA, lecithin and DSPE-PEG_{2000} which have been approved by the FDA for medical use. In addition to that, lecithin is a natural substance extracted from soybeans. We expect CS-LPHNs to be biocompatible, biodegradable and safe to use for medical applications. The speed of the stirring for further experiments was standardized using the visual examination of nanoparticle dispersion. The standardization of stirrer speed is tabulated in the table (2). At the moderate speed of the stirrer, there was uniform nanoparticle dispersion with no particle aggregation. However, at slow speed, the formation was inadequate and hence led to the deposition of the solids at the surface of the aqueous phase. At high stirrer speed, there was the aggregation of the nanoparticles. This may be due to the high shear causing insufficient stabilization of nanoparticles and causing particle aggregation. Therefore, all formulas were prepared at a moderate speed of stirring.

Table (2). Stirring rate optimization.

Stirring	Speed	Observation
Low	100 rpm	Aggregation of the polymer on the surface of the solution
Moderate	500 rpm	Uniform distribution with no aggregation at the surface
High	1000 rpm	Aggregation seen and collected on the surface of magnetic bar

Particle size, Zeta potential and PDI

The particle size and ζ were recorded in the table (3).

Table (3). Particle size, Zeta potential and PDI of TAC-CS-LPHNs.

Formula	Zeta potential (mv)	Particle size (nm)	PDI
PTX-1	**-15.5±0.19**	**152±1.2**	**0.139±0.067**
PTX-2	**-20±0.12**	**143±0.25**	**0.137±0.02**
PTX-3	**-28±0.14**	**137±2.5**	**0.088±0.09**
PTX-4	**-25±0.27**	**133±5.2**	**0.263±0.02**
PTX-5	**----**	**121±1.76**	**0.203±0.08**
PTX-6	**-31±0.22**	**120±1.09**	**0.161±0.06**
PTX-7	**-15±0.32**	**160±1.01**	**0.161±0.0193**
PTX-8	**-0.2±0.26**	**150.6 ±1.25**	**0.197±0.0123**
PTX-9	**Aggregation**		
PTX-10	**-28±0.14**	**117±1.27**	**0.254±0.016**
PTX-11	**-28±0.16**	**161±2.25**	**0.13±0.01**
PTX-12	**------**	**153±1.88**	**0.084±0.12**
PTX-13	**------**	**170±0.25**	**0.141±0.133**
PTX-14	**------**	**166±1.92**	**0.048±0.18**
PTX-15	**-21±0.28**	**151±3.09**	**0.122±0.22**
PTX-16	**-13.9±0.13**	**140±3.11**	**0.133±0.13**
PTX-17	**-37.6±0.11**	**195.5±5.25**	**0.381±0.018**
PTX-18	**-35.5±0.43**	**111.5±2.44**	**0.144±0.017**
PTX-19	**-27.5±0.102**	**124±1.01**	**0.174±0.2**
PTX-20	**-20.9±0.26**	**149±1.91**	**0.166±0.012**
PTX-21	**-17±0.32**	**193±3.56**	**0.326±0.22**
PTX-22	**-28.9±0.19**	**150±2.99**	**0.22±0.065**
PTX-23	**-14.9±0.23**	**178±1.03**	**0.315±0.034**
PTX-24	**-17.5±0.15**	**176±1.69**	**0.307±0.1**
PTX-25	**-36±0.12**	**159±2.04**	**0.42±0.09**

The lipid to polymer ratio, polymer amount and drug amount optimization is critical for stability and was discussed in a previous article[22]. The change in a mass ratio of DSPE-PEG_{2000}\lecithin may influence the TAC-CS-LPHNs properties. This effect can be detected by changing the mass ratio and keeping the L\P ratio and other parameters constant. The effect on particle size was observed in formulas PTX-3, PTX-12 and PTX-13 in which the mass ratio was 50%, 100% and 200% respectively, while the ratio of L\P ratio was kept constant at 24%. Figure (1, A) shows a significant ($p<0.05$) increase in particle size with an increase in mass ratio. PTX-22 and PTX-3 having ζ -28.6 and -28 mv with constant L\P ratio was 24% and DSPE-PEG_{2000}\lecithin mass ratio was 71% and 50% respectively, indicated nonsignificant changes in ζ were observed with changing mass ratio. After 2 days of storage in a refrigerator at 4 C, a decrease in lecithin less than 0.6 mg causes CS-LPHN aggregation. Organic solvents of increasing dielectric constants exhibit increasing polarity and water miscibility. Two organic solvents have been used to dissolve TAC and PLGA (acetone and acetonitrile) as shown in PTX-16 and PTX-19. Figure (1, B) predicted a significant change in particle size ($p<0.05$) when using acetone instead of acetonitrile and significant ($p<0.05$) changes in ζ. Acetone produces cloudy solutions and is of poor quality. For this reason, acetonitrile was used in our experiments.

The colloidal stability of CS-LPHNs can be enhanced with the presence of PEGylated lipid by sterically preventing particles from aggregating to gathering and thus enhancing stability. The effect of PEG can be demonstrated in formulas PTX-17 and PTX-19 in which DSPE and DSPE-PEG_{2000} are used respectively. A significant ($p<0.05$) increase in PDI and particle size when using DSPE instead of DSPE-PEG_{2000} as shown in figure (1, C). The same results were observed by Zheng et al, in which, a decrease in the CS-LPHNPs size (from 230 to 150 nm) with increasing PEG fraction.[11].The concentration of drug and polymer in the organic phase should be monitored and optimized due to its effects on particle size, ζ and PDI. These effects are observed when comparing formulas PTX-15 and PTX-19. The drug and polymer concentrations in formula PTX-15 were 1mg/ml and 5mg/ml, respectively, whereas the drug and polymer concentrations in formula PTX-19 were 0.5mg/ml and 2.5mg/ml, respectively, and the organic phase/aqueous phase ratio was 1:10 for PTX-19 and 1:20 for PTX-15. As shown in figure (1, C), a significant change ($p< 0.05$) in ζ and size were observed by decreasing the volume of the organic phase and increasing the concentration of polymer and drug. This finding matches with previous research studies, due to the high viscosity of the organic solvent resulting from the high amount of PLGA used. The droplet will be bigger from a highly viscous solution and thus large particles will result.

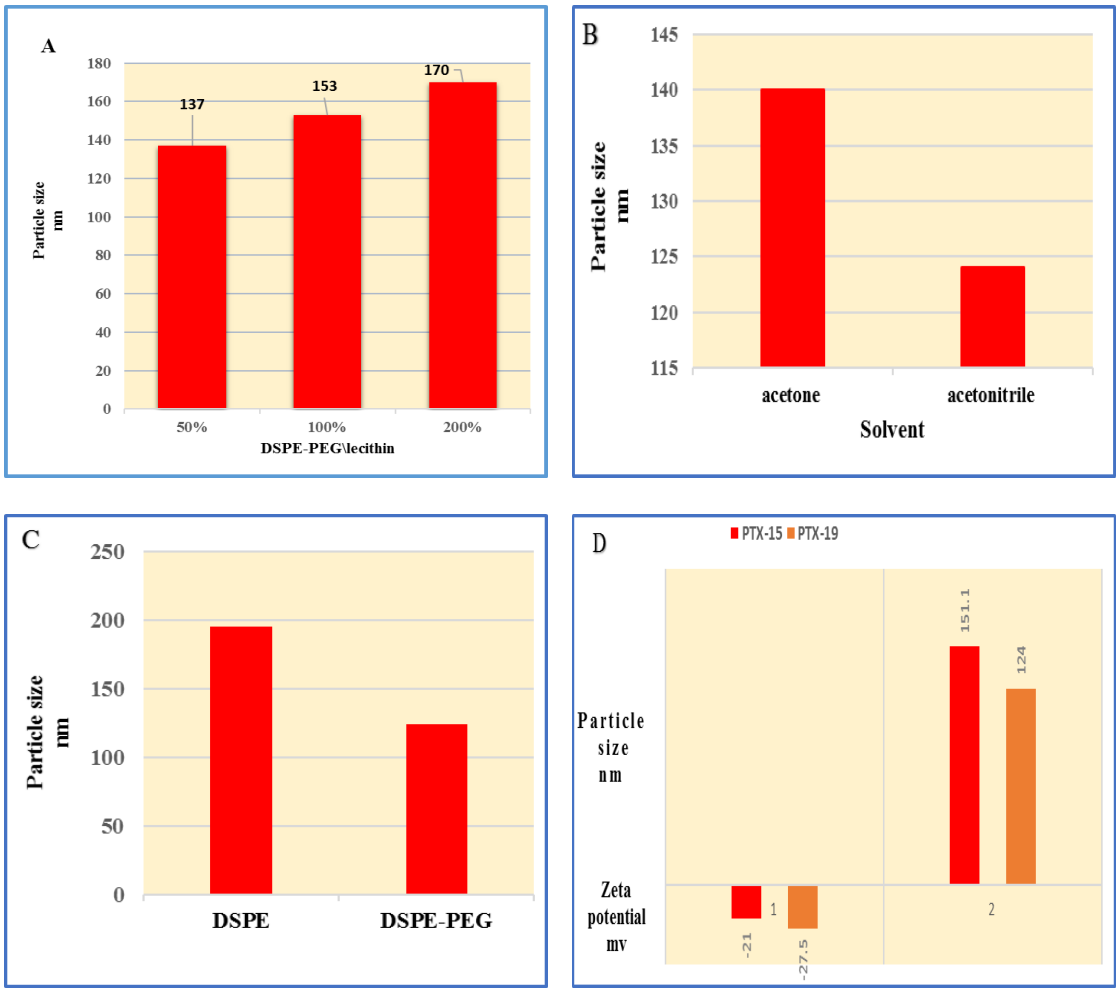


Figure (1). Different factors influencing the formulation of TAC-CS-LPHNs. (A). The effect of DSPE-PEG$_{2000}$\lecithin on particle size. (B) Effect of solvent type on particle size. (C) The effect of lipid-PEG on particle size. (D) The effect of organic phase volume on particle size and zeta.

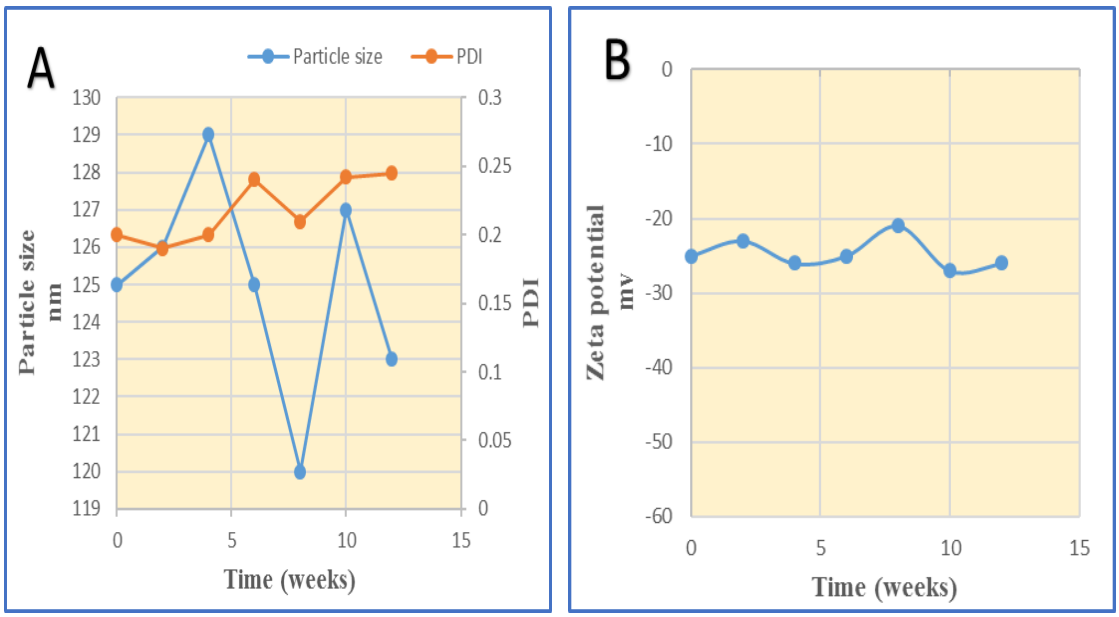
Stability study

Stability is defined as the capacity of a drug substance or drug product to remain within established specifications to maintain its identity, strength, quality, and purity throughout the retest or expiration dating periods.[12]

In our project for developing TAC-CS-LPHNs, we design a stable drug delivery system that has acceptable particle size, zeta and PDI. External stimuli such as temperature, pH, and ionic strength can cause physicochemical changes in the lipid and alter the surface charge, resulting in aggregation and particle size increase.[13]

Short term stability

The observation from storage study data at 25 ˚C of lyophilized powder shows shrinkage and little changes in colour and appearance of the cake after 2 weeks. The measured size and ζ show significant ($p< 0.05$) change as revealed by figures (2, C and D). This could be due to water molecule absorption from the vial stopper or an incorrect closer.[14].Data obtained from a dispersed aqueous solution of TAC-CS-LPHNs stored at 4˚C reveals stable nanoparticles that can be retained for up to 3 weeks with no or little changes in nanoparticle characteristics. Storage for more than one month shows significant ($p< 0.05$) changes in size, Zeta and PDI. The decrease in zeta potential in aqueous dispersed solution may be due to acid conditions produced by the degradation of PLGA into lactic acid and glycolic acid. The decrease in zeta potential may encourage aggregation and growth of particle size.[15] We can conclude from this data that TAC-CS-LPHNs can be stabilized by lyophilization to eliminate the water effect and stored in a well-closed container at 4±3˚C.



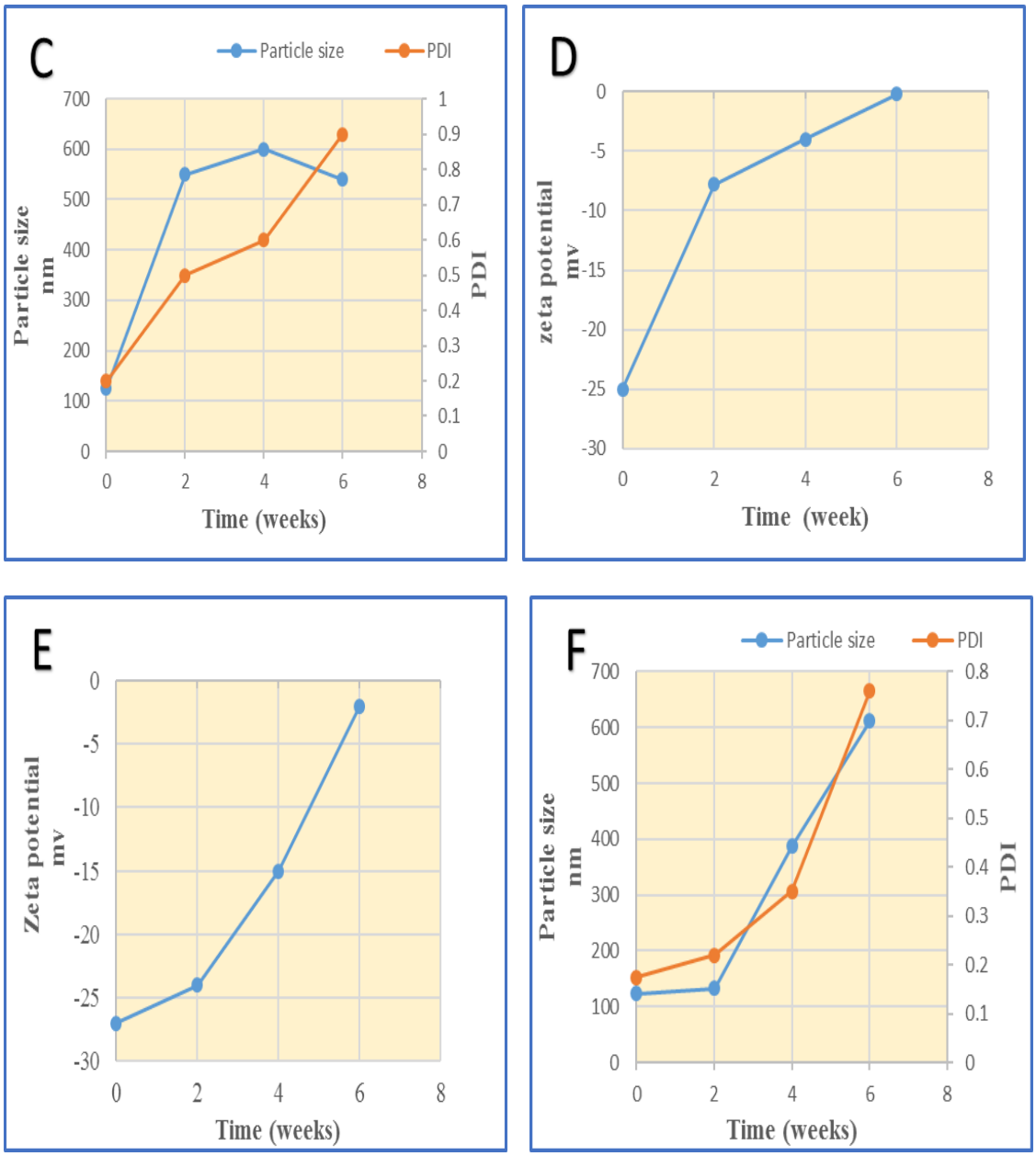


Figure (2). Graphic representation of short-time storage stability study on (A) the mean size and polydispersity index of the lyophilized TAC-CS-LPHNs at 4˚C. (B) the Zeta potential of the lyophilized TAC-CS-LPHNs at 4˚C. (C) the mean size and polydispersity index of the lyophilized TAC-CS-LPHNs at 25˚C. (D) Zeta potential of the lyophilized TAC-CS-LPHNs at 25˚C. (E) the Zeta potential of the aqueous dispersed TAC-CS-LPHNs at 4˚C. (F) the particle size and PDI of the aqueous dispersed TAC-CS-LPHNs at 4˚C.

Long term stability study

The lyophilized powder was stored for up to 8 months in the closed vial at 4±3˚C. The data was recorded after reconstituting in deionized water as shown in table (4) and figure (3).

Table (4). Characteristics of lyophilized TAC-CS-LPHNs after 8-month storage.

Lyophilized Optimized TAC-CS-LPHNs	Particle size	Zeta potential	PDI
Before storage	125.23±0.01	-25±0.2	0.2±0.33
After 8 months	119±0.1	-24.8±0.08	0.25±0.5

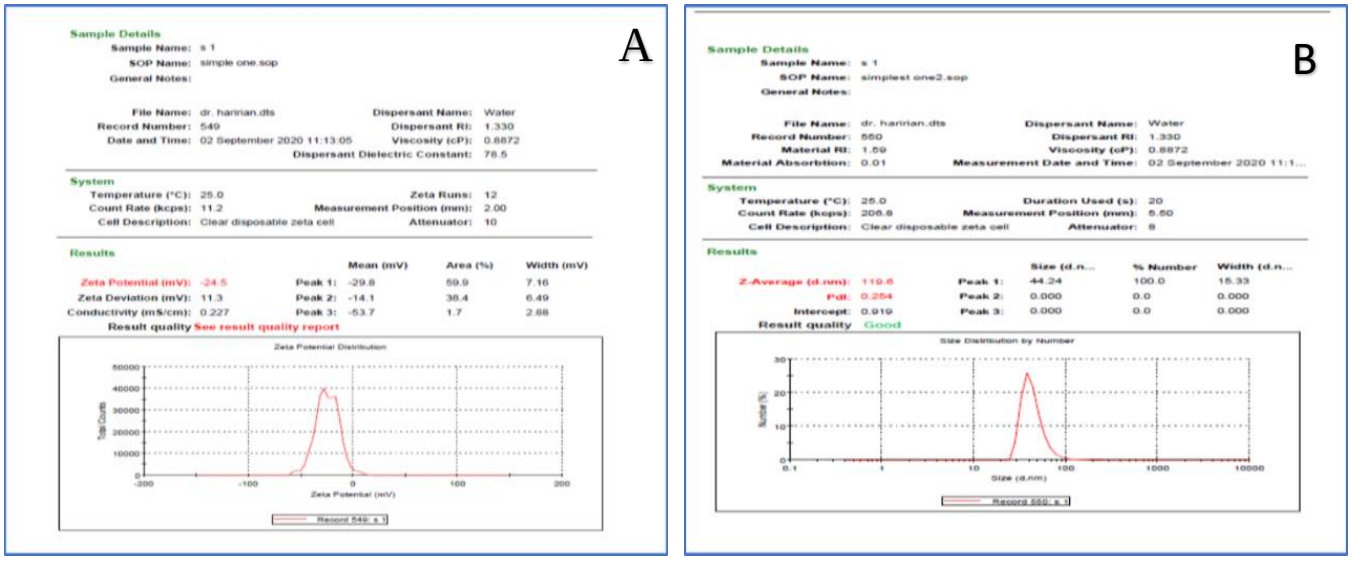


Figure (3). (A) Zeta potential of lyophilized TAC-CS-LPHNs after 8 months storage 4±3˚C. (B) Particle size and PDI of lyophilized TAC-CS-LPHNs after 8 months storage at 4±3˚C.

Factor influencing physical stability

As discussed previously, many factors can influence the physical stability of TAC-CS-LPHNs in different ways. These factors are:

Effect of urine

Particle size was detected using a zeta sizer in concentrated mode. Data show insignificant changes in particle size and PDI compared with water as shown in figure (4, A). The zeta sizer can't detect the zeta potential because the yellowish colour of urine interferes with the measurement.

Effect of pH

The physical stability of loaded TAC-CS-LPHNs was studied over a pH range of 1 to 12, progressing from acidic to basic pH.

The pH of the solution was controlled by adding drops of NaOH/HCl at constant TAC-CS-LPHNs concentration. The experimental data were recorded and observed in figures (4, B and C), which show the influence of pH on size, ζ and PDI. Data shows that the particle size was significantly ($p<0.05$) increased at pH 5 and 12 while showing a nonsignificant increase at acidic and neutral pH.

PDI shows fluctuation in value as a result of changing ζ and size, with the highest value was observed at pH 12 may be due to dropping in Zeta potential magnitude which indicated the hetero-dispersion. The zeta potential of nanoparticles increases significantly ($p< 0.05$) in magnitude with increasing pH. At low pH value (1) the ζ is positive due to conversion of carboxylic groups into unionized form while ζ becomes negative and increases in magnitude as pH becomes more basic, especially when pH is more than the isoelectric point of PLGA (6.12) [16], the increase in the magnitude of ζ may be attributed to ionization of carboxylic group and an increase in the number of potential determining ions. As a result, Nernst's potential increases, resulting in a higher value.[17].At pH 12, the DLS study shows the appearance of 3 peaks (peak1 having the size of 72.3 nm representing 55%, peak 2 with particle size 354 nm representing 41% and peak 3 having particle size 4972 nm representing 2.2%) and. The decrease in the magnitude of ζ may be attributed to the degradation of PLGA at basic conditions.[18]

Effect of ionic strength (IS)

In the absence of salt, neutral to slightly acidic pH and low temperature of 4˚C, TAC-CS-LPHNs were stable for 3 weeks. As shown in figures (3, D) and (4), TAC-CS-LPHNs may accommodate ionic strength of up to 1.33M. As IS increases, the ζ will decrease and converted to positive with an increase in particle size of more than 1 micron and PDI equal to 1. This indicated the instability of TAC-CS-LPHNs.

In an aqueous solution, TAC-CS-LPHNs are stabilized by electrostatic forces generated by (double layer) as a result of negative charge on the surface of TAC-CS-LPHNs that are generated from the ionization of carboxylic group from PLGA polymer. [19].This charge generates a potential that is termed Zeta potential. In addition to that, TAC-CS-LPHNs stabilized sterically by PEG from DSPE-PEG_{2000}. It has been proved that the presence of Na^+ ions will decrease the magnitude of ζ by compression of the double layer and adsorption at the surface that leads to neutralizing the surface charge. [17] As a rule, the colloidal stability of nanoparticles decreases as the absolute value of ζ is less than 30. [20]Increase in Na^+ ions addition leads to the inversion of Zeta potential charge with the creation of positive charge. The TAC-CS-LPHNs stabilized again as ζ reached +29 mV.

The effect of IS on the particle size and PDI are shown in figure (5) after 24 hrs. storage at 4˚C. The gradual increase in particle size and PDI to 152 nm at IS=1.33 M compared to 126 nm at IS=0, followed by a sharp increase in particle size (more than 1) at IS=2 M.

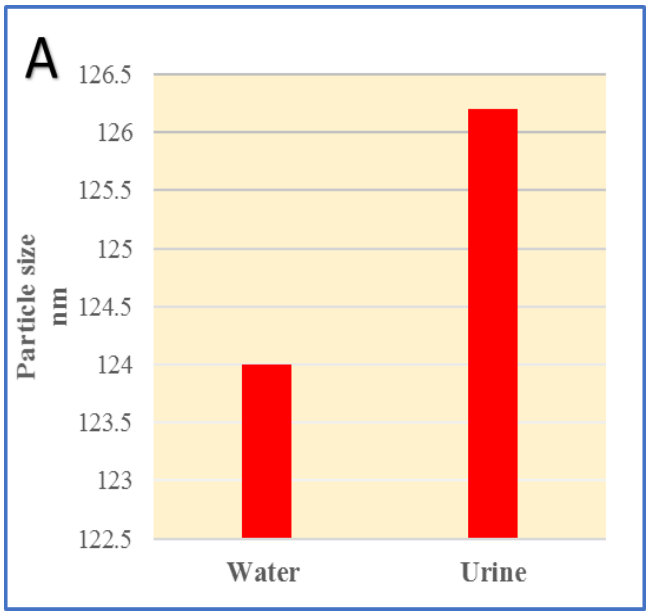
As more NaCl is added, Na^+ ions will be adsorbed on the surface of nanoparticles, leading to the creation of positive charge Zeta potential and compression of double-layer around TAC-CS-LPHNs. For this reason, the hydrodynamic size of high IS was low (66 nm with IS 3.6M). [21,17].The reality is more complex than that, but this study provided some evidence about the stability of TAC-CS-LPHNs in different conditions. Nanoparticles may be still stable despite having low ζ. This is because Zeta potential gives information about the electrostatic forces, but no other forces that may be involved in the stabilization of nanoparticles like Van der Waals attraction force. Also, there is steric stabilization by PEG which plays an important role in making the nanoparticle more resistant to deteriorating factors.

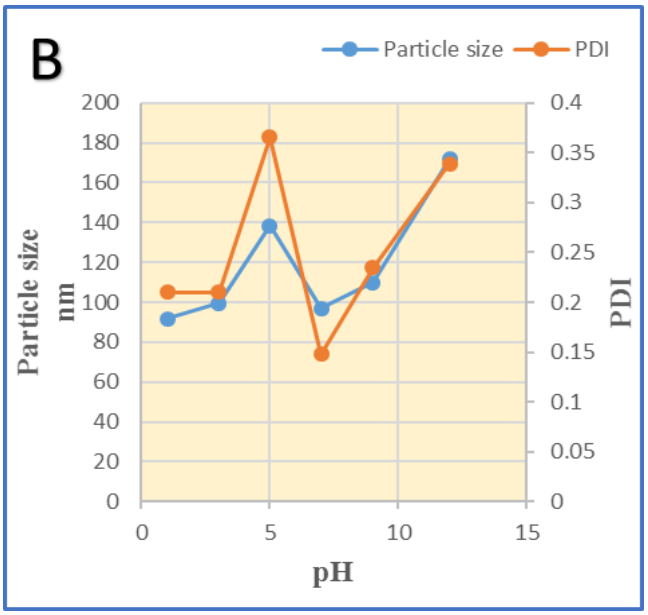
Effect of Plasma

Testing of plasma stability is important for predicting in vivo behaviour of nanoparticles. To examine the plasma effect, two different formulas have been tested; one contains DSPE and the other DSPE-PEG_{2000}. The effect on particles size, ζ and PDI was measured by DLS. Data is shown in table (5), a significant change in both zeta potential and particle size when TAC-CS-LPHNs were dispersed in human plasma. The difference between the formula formulated with DSPE-PEG_{2000} and the other reveals the protective effect of PEG.

Table (5). Characteristics of TAC-CS-LPHNs in water and plasma.

Dispersion media	Particle size nm	Zeta potential mV	PDI
Water (PTX-17)	195.5±0.09	-37.6±0.3	0.381±0.09
Water (PTX-19)	124±0.01	-27.5±0.102	0.174±0.2
Plasma (PTX-19)	142.5±0.22	-6.3±0.23	0.319±0.012
Plasma (PTX-17)	647.9±0.5	-8.18±0.54	0.642**±0.1**





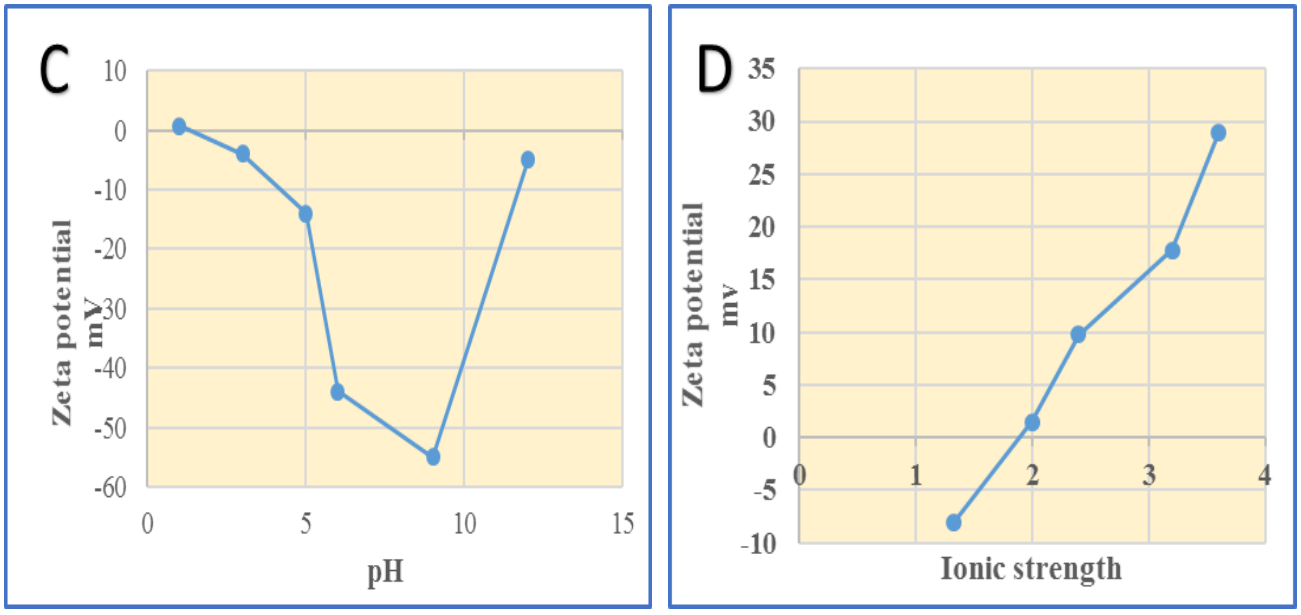


Figure (4). Trigger factors influencing the stability of TAC-CS-LPHNs. (A) Effect of urine on the particle size at 4˚C.(B) Effect of pH on the particle size and PDI at 4˚C. (C) Effect of pH on Zeta potential 4˚C. (D) Effect of ionic strength on Zeta potential at 4˚C.

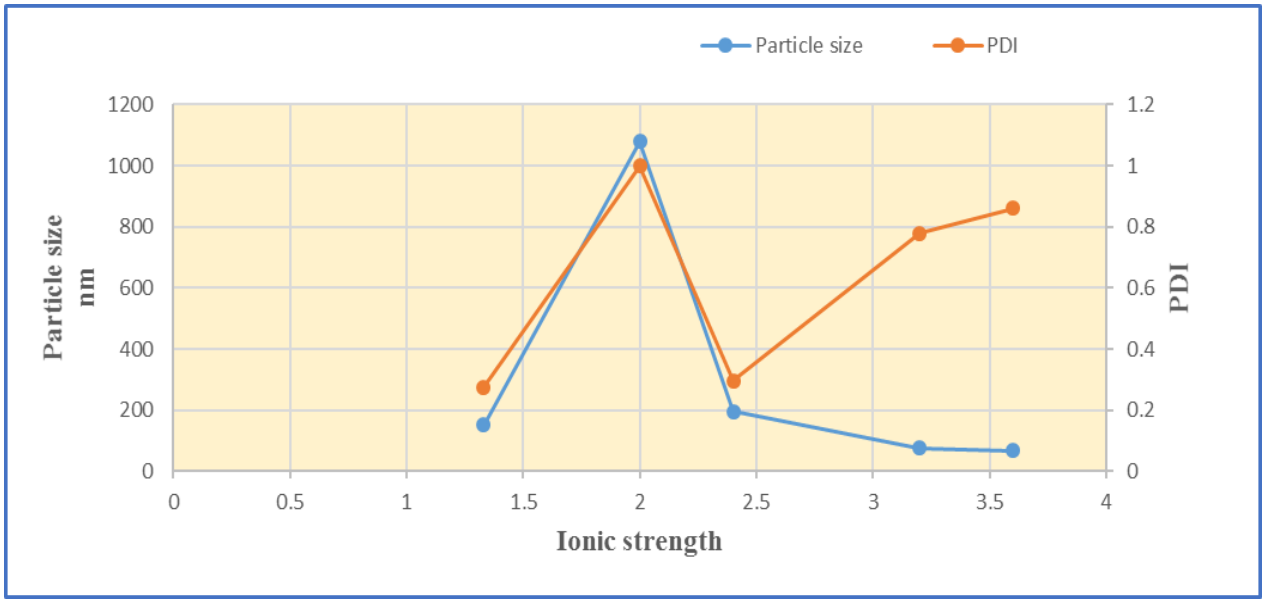


Figure (5). The effect of ionic strength on the particle size and PDI of the aqueous dispersed TAC-CS-LPHNs at 4˚C.

Conclusion

Data demonstrated that the mass ratio of DSPE-PEG_{2000}\lecithin, organic solvent and concentration of polymer in organic solution has a significant effect on the formulation of TAC-CS-LPHNs. In addition, factors like ionic strength, pH and storage temperature have the most significant effect on the stability of nanoparticles.

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