Nano-engineering chitosan particles to sustain the release of promethazine from orodispersables

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Abstract

Orally dispersing tablets (ODTs), also known as orodispersibles, were first introduced into the market in 1980s to overcome dysphagia problems amongst paediatrics and geriatrics. Despite their abilities to avoid swallowing difficulties, frequency of dosing stood as a barrier for these formulations. The aim of the current study is to produce and optimize a sustained release orally disintegrating tablets (SR-ODT), with the aid of chitosan. A design of experiment (DoE) was first performed using Minitab to determine the effect of five independent variables on three dependent responses when producing the nanoparticles using ionotopic gelation. The variables studied were (tripolyphosphate concentration TPP, Chitosan concentration CS, acetic acid concentration, Chitosan: tripolyphosphate ratios and stirring time) and the responses were (particle size, surface charge and encapsulation efficiency). A formulation with optimum particle size, surface charge and encapsulation efficiency was prepared and further coated with polyvinylpyrolidine (PVP), polyethylene glycol (PEG) and polyethylene co-acrylic acid (PEAA). Minitab studies revealed that the nanoparticles' particle size was affected by most of the independent variables except stirring time and the ratios of CS to TPP. The optimized nanoparticles showed particle size of 153.8±14 nm, surface charge of 31.4±0.9 mV and encapsulation efficiency of 99.7±0.06%. The DSC showed that PMZ was solubilized within chitosan nanoparticle, whereas SEM images indicated that all the samples were spherical in shape with smooth surface and had similar size to that measured by DLS. After coating and dispersing into the tablets' matrices, the tablets were evaluated to determine the friability, disintegration time and tensile strength. All tablets were at an appropriate friability (less than 1%) and had tensile strength above 2.5 N/mm^2. Besides, all the tablets managed to disintegrate within 40 seconds while sustaining the drug release over 24 hours.

Keywords:- orally disintegrating tablets, chitosan, design of experiment, sustained release, polymers.

1.Introduction

Oral drug delivery is the most common route of drug administration and the last 10 years have witnessed significant developments in oral formulations as novel dosage forms and manufacture technologies have been introduced. A new dosage form known as orally dispersing tablets (ODTs) was introduced in 1980s to overcome a common clinical problem known as dysphagia among paediatric and geriatric populations. The clinical study conducted by Lindgren and Janzon (1991) showed that 35% of patients aged between 50 and 69 suffered from some degree of dysphagia. It has also been established that nearly 1 in 5 patients avoid taking oral medication due to swallowing difficulties (Lindgren & Janzon, 1991; Krause & Breitkreutz, 2008). Dysphagia is also associated with poor patient compliance, the latter is a foremost medical issue that costs more than $290 billion a year (Fulzele, Moe & Hamed, 2012.; Gryczke, Schminke, Maniruzzaman, Beck, & Douroumis, 2011).Therefore, the need for a viable oral disintegrating formulation is paramount. ODTs are also termed as orodispersible in the European Pharmacopoeia and defined as 'tablets that disperse or disintegrate in less than 3 mins in oral cavity before it is turned into a paste that can be easily swallowed (Hirani, Rathod, & Vadalia, 2009; Beckert, Lehmann, and Schmidt, 1996; Wagh, Kothawade, Salunkhe, Chavan, & Daga. 2011)

The first generation of ODTs achieved a lot of success, with various properties and characteristics of ODTs offered by the numerous preparation techniques. Nonetheless, the first generation of ODTs failed to overcome challenges such as delivering acid labile drugs, macromolecule and high doses. A lot of studies investigated new approaches to circumvent these technical issues. Further research into ODTs resulted in the production of sustained-release oral disintegrating tablet (SR-ODT) with the aim of improving the oral disintegrating drug delivery system. This is where the tablet disintegrates completely in the mouth but also

sustain the duration of action. This will reduce the frequency of dosing and will enhance patient adherence to ultimately improve the quality of lifestyle for patients (Abdul & Poddar. 2004). Many approaches such as microencapsulation (Sunitha, & Amareshwar. 2010; Shazly, Tawfeek, Ibrahim, Auda, & El-Mahdy, 2013), nanoparticles (Kondo, Ito, Niwa, & Danjo, 2013) ion exchange resins (Chen et al., 1992; Gokhale and Sundararajan., 2013) and stimuli-responsive polymers (Beckert, Lehmann, and Schmidt 1996; Abbaspour, Sadeghi & Garekani, 2008) have been adapted to control the drug release across ODTs.

Recently, chitosan (CS) has attracted great attention in pharmaceutical industry to produce sustained release delivery systems, due to its biodegradability and biocompatibility, in addition to, its nontoxicity (Jiang, Pan, Cao, Jiang, Hua, & Zhu, 2012). Chitosan is considered as one of the most abundant natural polysaccharide (Jiang, Pan, Cao, Hua, &Zhu, X.2012; Bugnicourt, Alcouffe, & Ladavière. 2014) which is chemically known as a β-(1,4)-2-acetamido-D-glucose and β-(1,4)-2-amino- D-glucose and comprises of of glucosamine copolymerised with N-acetyl glcosamine (Kaloti, & Bohidar 2010), the primary amino group and two free hydroxyl groups on carbon (C8) provides a positive charge on the surface (Fig 1A) .CS has a pka of 6.3-7 and is only soluble in aqueous media at low pH, which might lead to a premature release of the drug.

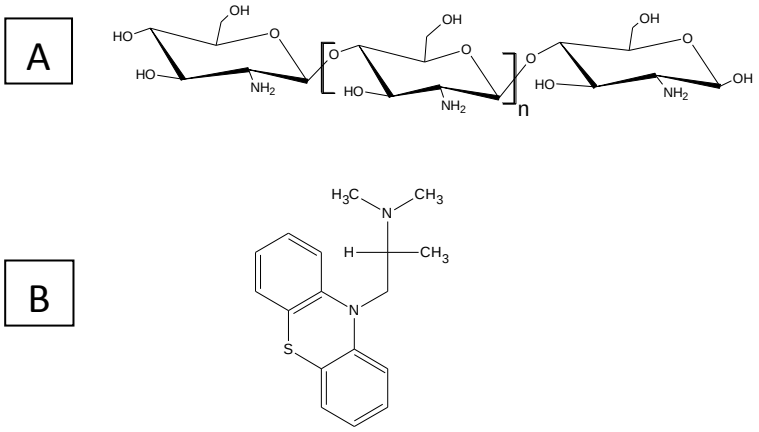


Fig 1:- Chemical structure of chitosan (A) and promethazine (B).

Chitosan is considered to be safe, as low molecular weight chitosans are eliminated easily by the kidney, while, the larger molecular weight polymers are degraded by chemical and enzymatic catalysis, furthermore the enzyme catalysis is dependent on the availability of chitosan amino group. The ability of CS to form nano-microparticulate systems depends on its ability to form covalent cross-linking between the chitosan chain and the functional cross-linking agent such as polyehtlene glycol (PEG), dicarboxlylic acid or tripolyphosphate. Patel et al (2013) utilised CS to develop a sustained delivery system of Rifampicin. Rifampicin nanoparticles were prepared by ionic gelation method in presence of tween-80 and tripolyphosphate to act as surfactant and cross-linker respectively. The prepared nanopartiuculate system had particle sizes of 181nm – 383nm and managed to sustain rifampicin release for 28-34 hours. It was further concluded that extensively cross-linked nanoparticles displayed decreased drug release rates (Patel, Parikh, & Aboti, 2013). Li-Q et al attempted a new microencapsulation technique to produce a SR ODT for scopolamine hydrobromide, where the nanoparticles are encapsulated to produce a sustained-release effect. The particles were produced using ionotropic gelation followed by spray drying, *in vitro* studies showed that tablets have disintegration time of <45s, particle size of 300 nm and managed to release 90% of the drug within 90min (Li, , et al., 2011). Other studies demonstrated that CS alone might not be able to

sustain the drug release. Abdelbary et al conducted *in vitro* and *in vivo* evaluation of microencapsulated glipizide for orally extended delivery. After preparing glipizide microcapsules by ionotropic gelation technique, the microcapsules were coated with alginate alone or combined with carbomer 934P. It was concluded that the extended release of drug depended on the composition of the outer coat. Microparticles coated with sodium alginate alone or in combination with low molecular weight (LMW) chitosan were found to be unsuccessful at retarding the drug release. However, when LMW chitosan was replaced by high molecular weight chitosan, approximately 80% of the drug was released after 8 hours. Other polymers were employed in preparing sustained release particulate systems across ODTs. The production of ketoprofen controlled release ODT was investigated using Eudragit RS-30D. The pellets were directly compressed and the *in vitro* studies revealed disintegration time of 30s. (Wei, Yang, & Luan, 2013).

Promethazine (PMZ) is the model drug used in this study (Figure 1B); pharmacologically PMZ is used as a H1 and alpha-adrenergic receptor antagonist, with a limited effect on dopaminergic receptor. PMZ is used widely to treat allergy symptoms such as itching, runny nose, sneezing, itchy or watery eyes, hives, and itchy skin rashes (Kavanagh, Grant et al. 2012). PMZ also prevents motion sickness and treats nausea, vomiting and pain after surgery. Furthermore, PMZ is used as a sedative or sleep aid (Ford, Rubinstein et al. 1985). Pfeil and colleagues have found that PMZ is considered as the mostly prescribed antiemetic in the US, as more than 90% of the prescriptions for antiemetic's are promethazine in comparison to other antiemetic on the market (Adolph, et al., 2012)

Due to the wide interest and promising results obtained when using chitosan to produce a sustained-release nanoparticles the aim of this study is to produce a sustained release nanoparticle system, to be integrated into an oral disintegrating tablet matrix. The study also aims to compare the effect of different coating polymers on the drug release profiles of PMZ and their toxicity on Caco-2 cells.

2. Material and method

2.1. Material

Promethazine hydrochloride (MW 320.88) was purchased from Tokyo chemical industry co, (Tokoyo,Japan). Chitosan (CS) of medium molecular weight (MW, 190,000-310,000 Da) and with degree of deacetylation (DD) of 75%, Sodium tripolyphosphate (TPP), Polyethylene glycol PEG (Mn 80,000 units), Polyvinylpyrrolidone (PVP), Poly ethylene co acrylic acid, Magnesium stearate fluka (analytical standard ≥99.5%) and D (+)-Lactose Monohydrate were all purchased from Sigma-Aldrich (Mo, USA), L-substituted hydroxypropylcellulose; LH-B1 -MW, 140,000 Da, 11% hydroxypropoxy content, degree of polymerization of 790 and 0.2 molar substitution- was a gift from Shin-Etsu Chemical co.td. (Tokyo, Japan).

The Caco-2 cell lines were obtained from Sigma Aldrich (Dorset, UK), while Essential Eagle's Medium (EMEM) L-glutamine, fetal bovine serum Penicillin Streptomycin were all purchased from Fisher Scientific (Loughborough, UK).

2.2 Methods

2.2.1. Design of experiment (DoE)

A factorial design of experiment was used to determine the effect of six dependent variables on three responses and to optimize the experiment conditions to achieve a nanoparticulate system with small particle size (100-300nm) with maximum drug loading. A fractional factorial design was generated where the

variables used in this design were CS concentration (0.1-0.5% w/v), TPP concentration (0.1-.05% w/v), acetic acid concentration(0.5-1% v/v), CS:TPP (5:1- 5:2) ratio and drug concentration(0.4-0.8 mg/mL) and stirring time (30- 90 mins) while the responses were particle size, surface charge and drug loading. A total of 16 experiments were performed in order to optimise the properties of nanoparticles produced (Table 1). In order to minimise the effect of extraneous factors on actual responses, the experimental runs were randomized. The response surface model was evaluated using equation (1) where Y is the response value predicted by the model of which α_0 is a constant whereas α_i , α_{ij}, α_{ijh} are linear, 2-way and 3-way interaction coefficient respectively. A response optimizer was used to obtain optimum conditions to produce nanoparticles in the size range of (100-250nm) and maximum drug load. The experimental design and data analysis were carried out using Minitab statistical package (Minitab® 17.1.0, Minitab inc., PA, USA)

$$Y= \alpha_0+\sum \alpha_i X_i+\sum \alpha_{ij} X_{ij}+\sum \alpha_{ijh} X_{ijh}$$ *Equation 1*

Table 1:- Matrix of 16 runs used to optimise chitosan nanoparticles

Number of runs	CS-conc (%w/v)	TPP-conc (%w/v)	CS:TPP ratio	Stirring time (min)	drug concentration (mg/ml)	acetic acid (%v/v)
1	0.5	0.1	5:2	30	0.4	0.5
2	0.1	0.1	5:1	30	0.4	1.0
3	0.1	0.5	5:2	30	0.4	1.0
4	0.1	0.1	5:2	30	0.8	0.5
5	0.1	0.5	5:2	90	0.4	0.5
6	0.1	0.1	5:1	90	0.4	0.5
7	0.1	0.5	5:1	90	0.8	1.0
8	0.1	0.1	5:2	90	0.8	1.0
9	0.5	0.5	5:1	30	0.4	0.5
10	0.5	0.1	5:1	30	0.8	1.0
11	0.1	0.5	5:1	90	0.8	0.5
12	0.5	0.1	5:1	90	0.8	0.5
13	0.5	0.5	5:2	90	0.8	0.5
14	0.5	0.5	5:2	30	0.8	0.1
15	0.5	0.5	5:1	90	0.4	0.1
16	0.5	0.1	5:2	90	0.8	0.1

2.2.2. Preparation of CS/TPP nanoparticles

CS/TPP nanoparticles, were prepared using ionotropic gelation method (Calvo, Remunan, Vila, & Alonso, ,1997) CS solution was prepared in concentrations of (0.1%-0.5%w/v) in acetic acid solution (0.5%-1% v/v). A Second solution of TPP was prepared at concentration of (0.1%-0.5%w/v) in deionized water. After filtration using 0.24µm syringe filters (Millex®-HA,Merck KGaA, Germany), TPP was added to CS solution dropwise until ratios of (5:1 and 5:2) were achieved. The obtained CS:TPP solutions were stirred under ambient conditions for (30-90 mins), which led to spontaneous formation of nanoparticles. The nanoparticles were obtained by centrifugation of the sample at 20.000 rmp for 30min at temperature of 4^{o}C using (SIGMA 3-30K, SciQuip, Germany) and the pellets obtained were washed by dispersing the pellets in distilled water and centrifugation for 15min. (Calvo, Remunan, Vila, & Alonso, ,1997; Najafabadi, Abdouss, & Faghihi, 2014; Makhija, & Vavia 2002)

PMZ nanoparticles were prepared using similar method. Where the drug (PMZ) was added in concentration of (0.4-0.8mg/mL) to the CS solution under magnetic stirring for 30min (Calvo, Remunan, Vila, & Alonso, ,1997; Najafabadi, Abdouss, & Faghihi, 2014; Makhija, & Vavia 2002) before adding TPP solution.

2.2.3. Preparation of CS and PMZ coated nanoparticles

After optimizing CS nanoparticles, the obtained particles were further coated using three polymers namely; PEG, PVP and PEAA to sustain the drug release across the nanoparticulate system. CS/TPP coated nanoparticles were prepared by adding the polymer in concentration of (10-20mg/ml) to the CS/PMZ solution prior to initiating the ionic gelation by adding TPP.

2.2.4. Tablet formulation

The prepared nanoparticles were embedded inside orally disintegrating tablet matrix made of 25% LH-B1, 1% lubricant (Magnesium stearate), nanoparticles contacting 5% drug (10mg or equivalent of PMZ) and 69% diluent (D (+)-Lactose Monohydrate), all ingredients were mixed using (WAB Turbula®,willy A,Bachofen AG, Switzerland) and compressed using uniaxial hydraulic press (Specac tablet presser, Slough, UK) and split die which prevents mechanical failure by allowing triaxial decompression. The prepared tablets were cylindrical with a diameter of 13 mm and weight of around 500 mg. Tablets were left in desiccators until characterisation studies were performed.

2.2.5. HPLC Analysis

PMZ analysis was performed using (Shimadzu, Shimadzu Corporation, Japan) HPLC system. RP-C18 column (250x4.6 mm, 5µm) was used to retain PMZ using mobile phase made of acetonitrile and 0.354%v/v triethylamine solution (pH of 2.5 adjusted with orthophosphoric acid), in a ratio of 41:59 (v/v) respectively. Mobile phase was pumped using a quaternary pump at a flow rate of 1ml/min. PMZ had retention time of 2.36±0.01 mins when analysed at λ_{max} of 250 nm. The analytical method was validated according to International Conference of Harmonization (ICH) guidelines. Calibration curve was established at concentrations ranges of 10-200µm with coefficient of variation (R^2=0.99) and curve equation (y = 56839x + 10^6).

2.2.6. Nanoparticles characterisation

2.2.6.1. Dynamic light scattering transmission:

Particle size distribution, polydispersion and zeta potential (ξ) of the nanoparticles were analysed through DLS, the analyses were performed using diluted suspension of nanoparticles at 1:10 v/v dilution using Malvern Zetasizer 300HSA (Malvern Instruments, UK) fitted with a detector at angle of 90° . All the analysis were carried out at room temperature and expressed as mean±SD of three readings. Zeta potential (ξ) was measured in triplicates by photon correlation spectroscopy (PCS) using Malvern Zetasizer 300HS_A (Malvern Instruments, UK).

2.2.6.2. Thermogravimetric analysis (TGA)

A thermogravimetric analyser (Toledo SDTA/TGA 851e, UK) was used in this study to measure the moisture content and decomposition temperature of PMZ and its prepared nanoparticles. 5- 10 mg of samples were loaded on to an open pan and were analysed between 20-500 ^{o}C at 10 ^{o}C/min scanning rate and under nitrogen stream. Software (STAR^{e}SW 10.00) was used to analyse the obtained thermograms.

2.2.6.3. Differential scanning calorimetry (DSC).

Differential scanning calorimeter (Mettler Toledo, DSC822^e, UK) was used to explore the physical transformation of PMZ and the prepared nanoparticles by determining the heat flow from and to the sample. Approximately 2-5 mg of the samples were weighted and transferred to an aluminum sample pan (50 µL capacity). Intra cooler 2P system was used to initially cool the samples to 25 ^oC and then sample heated to 250 ^oC at a rate of 10 ^oC/min. Nitrogen was used as a purge gas at a flow rate of 20 mL/min. The obtained thermograms were analysed using STAR^{e}SW 10.00 software. All experiments were performed in triplicate and an empty aluminum pan was used as a reference cell for all the measurements. Both sample and reference pans were covered by aluminum lids and pierced on the top.

2.2.6.4. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM, Zeiss Evo50- Oxford instrument, UK) was used to study the surface morphology of PMZ and the prepared nanoparticles. Samples were prepared by sprinkling PMZ or adding a drop of nanoparticles suspension onto specimen stubs. After drying the suspension, stubs were loaded onto a universal specimen holder. In order to enable electricity conduction, samples were coated with a fine layer of gold using a sputter coater (Polaron SC500, Polaron Equipment, Watford, UK) at 20 mA for three mins at low vacuum and in the presence of argon gas (Polaron Equipment, Watford, UK).

2.2.6.5. Determination of encapsulation efficiency of nanoparticles using HPLC.

HPLC method (section 2.2.5) was used to determine the percentage of encapsulation efficiency in the prepared nanoparticles. In this process, the supernatant of the nanoparticle that was collected during centrifugation was filtered and analysed using HPLC and equation (2) was used to calculate % PMZ encapsulation efficiency.

$$\%Drug\ encapsulation\ efficiency = \frac{total\ amount\ of\ drug - free\ amount\ of\ drug}{total\ amount\ of\ drug} x100 \quad Equation\ 2$$

2.2.6.6. Sulforhodamine B (SRB) cytotoxic assay.

2.2.6.6.1 Caco-2 cells culture

The Caco-2 cell line was grown in Minimum Essential Eagle's Medium (EMEM) that was supplemented with 200 mM L-glutamine, 10% fetal bovine serum, 10,000 U of Penicillin and 10 mg/mL of Streptomycin. Caco-2 cells were maintained in humidified atmosphere of 5% ±0.5 CO_2 and at a temperature of 37 ±0.5 ^oC. All experiments were preformed between passages 57-60.

2.2.6.6.2 Sulforhodamine B (SRB) cytotoxic assay

Cytotoxic effect of the prepared nanoparticles was evaluated using Sulforhodamine B (SRB). SRB protocol was adapted from Vichai and Kirtikara (2006). Briefly, Caco-2 cells were seeded in a 96 well plate at a density of 20,000 cell/well. The cells were incubated for 24 hours at a temperature of 37 ±0.5 ^oC and humidified atmosphere of 5% ±0.5 CO_2. The nanoparticles were centrifuged, and the supernatant discarded, the nanoparticles were re-suspended in the treatment media prior the test. The cytotoxic assay was evaluated for the following concentration of 40 mg/mL, 20 mg/mL and 10 mg/mL of nanoparticles suspension. After the 24 hours cultured period, the cell media was removed and 100 µL of the test materials were added. The test materials used were: nanoparticles (different concentration) suspension, the negative control

(treatment media only) and positive control (50µm trytona X). This followed by another 24 hours incubation time using the same condition above. After the second incubation, the cells were fixed by treatment with 100 µL of 10% Trichloroacetic acid (TCA) for 1 hour. Then, the TCA was washed out thoroughly with water and left to dry overnight. SRB dye was added to each well (100 µL of 0.4% SRB) for 30 mins then washed out using 1% acetic acid and the plate was kept for drying overnight. The SRB dye was de-stained using 100 µL tris buffer and the optical density was measured at 565 nm using Epoch Spectrophotometer (Bio TeK, VT, USA).

2.2.7. Tablet evaluation

2.2.7.1. Measurement of tablet tensile strength

The force required to crush the prepared tablets was measured using tablet hardness apparatus (Schleungier 4M, Thun, Switzerland). The measured force was used to determine the tablet tensile strength using equation (2) (Digital Vernier Dial Caliper Gauge Micro Meter 150mm(UK).

$$\sigma = \frac{2F_c}{\pi dt} \quad \textit{Equation 3}$$

Where σ is the tablet tensile strength, Fc is the crushing force required to break the tablet, d is the tablet diameter and t is the tablet thickness. All measurements were done in triplicate.

2.2.7.2. Measurement of tablet disintegration time

Disintegration time is the time required for tablets to disintegrate completely without leaving any solid residue. *In vitro* disintegration time was evaluated using US pharmacopoeia monograph (<701> disintegration). Erweka ZT3, Appartebau, GMBH ,Husenstamm, Germany) was used in this study as a disintegration apparatus and distilled water (800 ml) as disintegration medium; the disintegration medium temperature was maintained at 37±0.5 °C by thermostat. Six tablets were placed in the basket rack assembly and covered by transparent plastic disks. The disintegration time was taken as the time required for tablets to disintegrate completely without leaving any solid residue. All the measurements were carried out six times and presented as mean ± standard deviation.

2.2.7.3. Measurement of friability

The friability will be determined as a percentage of weight loss in a random sample of tablets. A random sample of tablets will be weighed on an analytical balance to achieve a total mass weight of (>5g), based on the British pharmacopoeia guidelines for friability testing. Then tablets were placed in a friabilator (Erweka AR 400 ,Germany) for 4 min at 25rmp, after that the tablets were dusted and reweighed. Percentage friability will be calculated using equation (3)

$$\%\ friability \frac{initial\ weight - final\ weight}{initial\ weight} * 100 \quad \textit{Equation 4}$$

2.2.7.4. Dissolution test (Drug release)

The dissolution of ODTs tablets containing 10 mg of PMZ or equivalent amount of PMZ nanoparticles was evaluated using USP II paddle method (Caleva 9ST, Germany). The prepared tablets were placed into dissolution vessels containing 900 mL of 0.01M HCL buffer (pH 1.2) and the dissolution media was maintained at 37^{o}C±0.5^{o}C and stirred at 50 rpm. 5mL of samples were collected at a predetermined time intervals (5min,10min, 15min, 20min, 30min, 60min, 90min, 120min, 6hr, 22hr, 24hr) then filtered through

0.45 µm Millipore filters. The dissolution media was replaced by 5mL of fresh dissolution media in order to maintain a constant volume. After proper dilution samples were analysed by HPLC method (section 2.2.5).

2.2.7.5. Statistical analysis

Formulation were prepared and analysed in triplicate and the results were expressed as ± mean standard deviation. Graph pad Prism® 6 (version 6.5) was used to analyse the date obtained, the results were analysed by two-way ANOVA (Tukey) *p<0.05* was considered to be statistically significant for this analysis.

3. Results and discussion

3.1. Design of experiment (DoE)

Two-level factorial design of experiment was performed, with the use of six parameters (CS concentration, TPP concentration, acetic acid concentration, stirring time and CS:TPP ratio), where Minitab generated 16 experiments, that were produced and evaluated based on the three variables; particle size, surface charge (ξ) (Fig 2) and encapsulation efficiency (EE). The DoE approach was employed in order to optimize the experiment conditions and produce a sample with the desired properties, a small particle size (100-300nm) and high encapsulation efficiency. High encapsulation efficiency means use of fewer amounts of nanoparticles, hence tablet characteristics would not be compromised especially disintegration time. In other words, poor entrapment efficiency would require high amounts of the polymeric nanoparticles which would bind strongly to other excipients in the tablet matrix and tablet disintegration would fail. After evaluation of all samples, the date was uploaded into Minitab, to statistically analyses the data obtained, Minitab generated a number of graphs to show the impact of each variable on the responses.

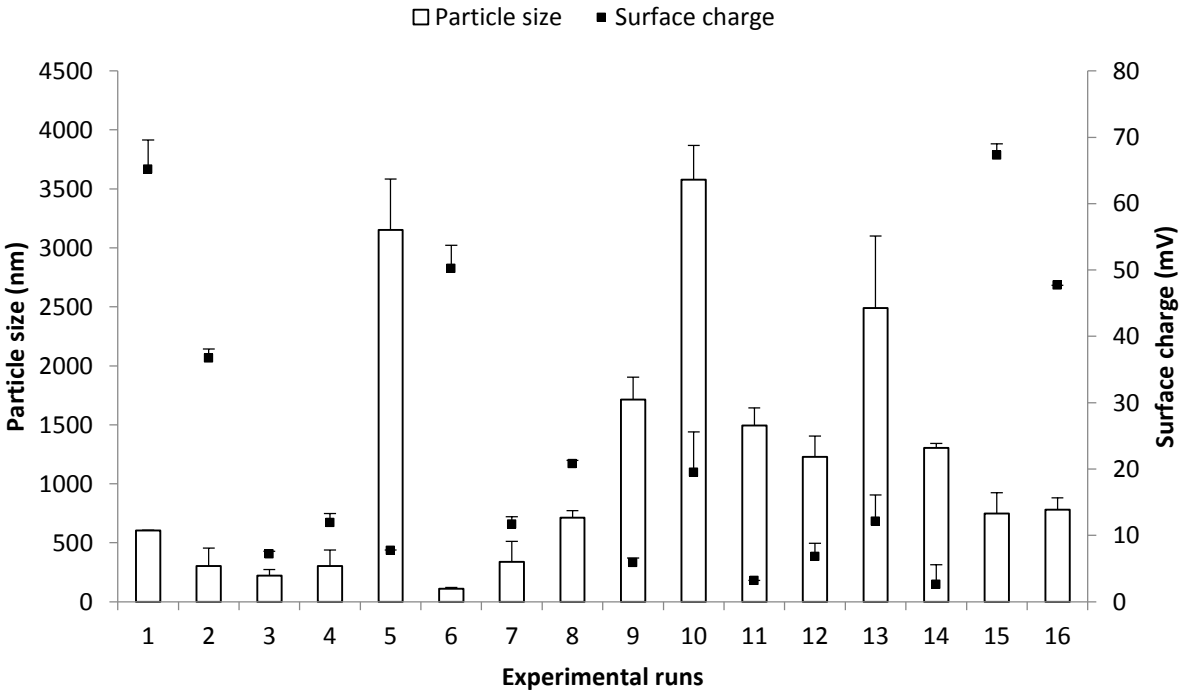


Fig 2:- Summary of the effect of various variables on the particle size and surface charge

3.1.1. The effect of different parameters on particle size

Particle size is an important determinant of drug bioavailability as it is believed that nanoparticles with size less than 100 nm has 3-fold arterial uptake compared to larger particles (Song, Labhasetwar, Cui, Underwood, & Levy, 1998). From formulation aspect, particles with smaller size will have a larger surface area and increasing the surface area will enhance the ability of the particles to withstand the compression

force during the tableting process by decreasing the overall compression pressure per particle, hence optimising the particle size is a mandate in the current study. Formation of CS nanoparticles depends on the ability of the polymer to form intermolecular cross-linkages with polyanions such as TPP. The extent of Intermolecular cross-linkages between the phosphate groups of TPP and the amino groups of CS will control and modulate the properties of CS nanoparticles prepared. The current study looked at the effect of six independent variables on the size of CS particulate system. The DoE study demonstrated that particle size is dramatically influenced by most of the variables; CS concentration, acetic acid concentrations, drug concentrations and TPP concentrations. On the other hand, stirring time and CS:TPP ration did not show any impact on the size of CS nanoparticles (Figs 3 &4).

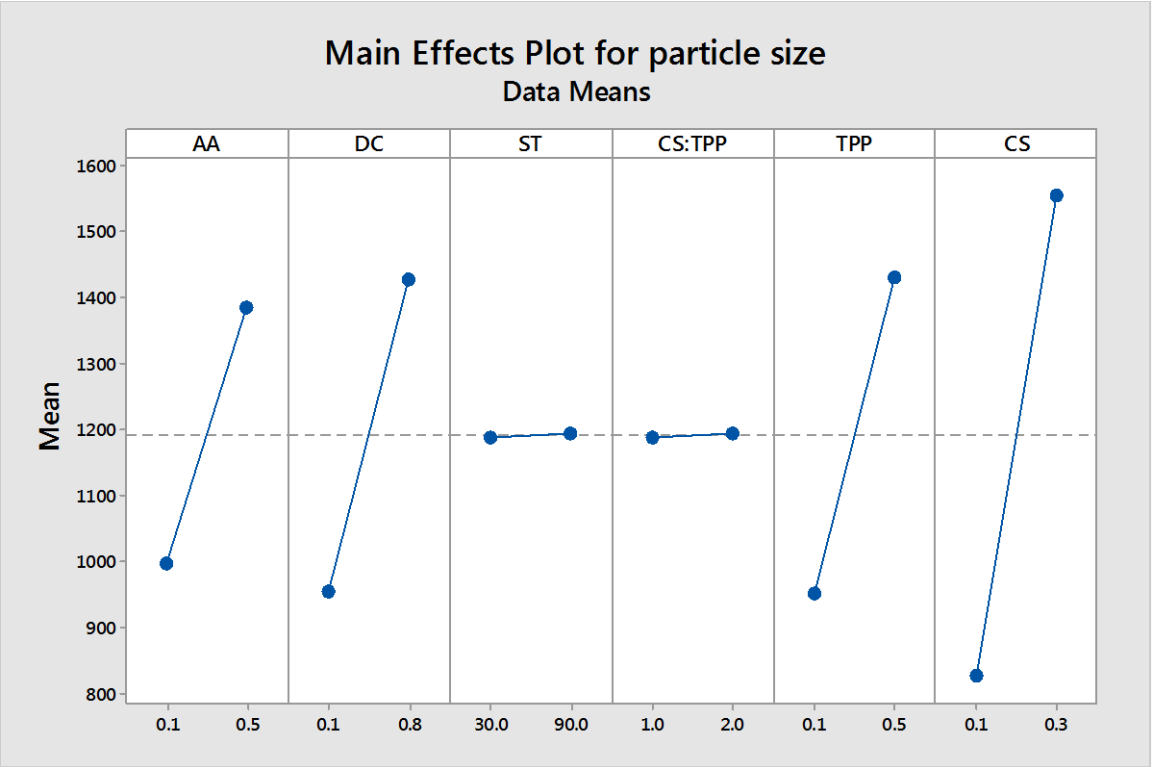


Fig 3:- Main effects plot showing the influence of the independent variables on CS particle size

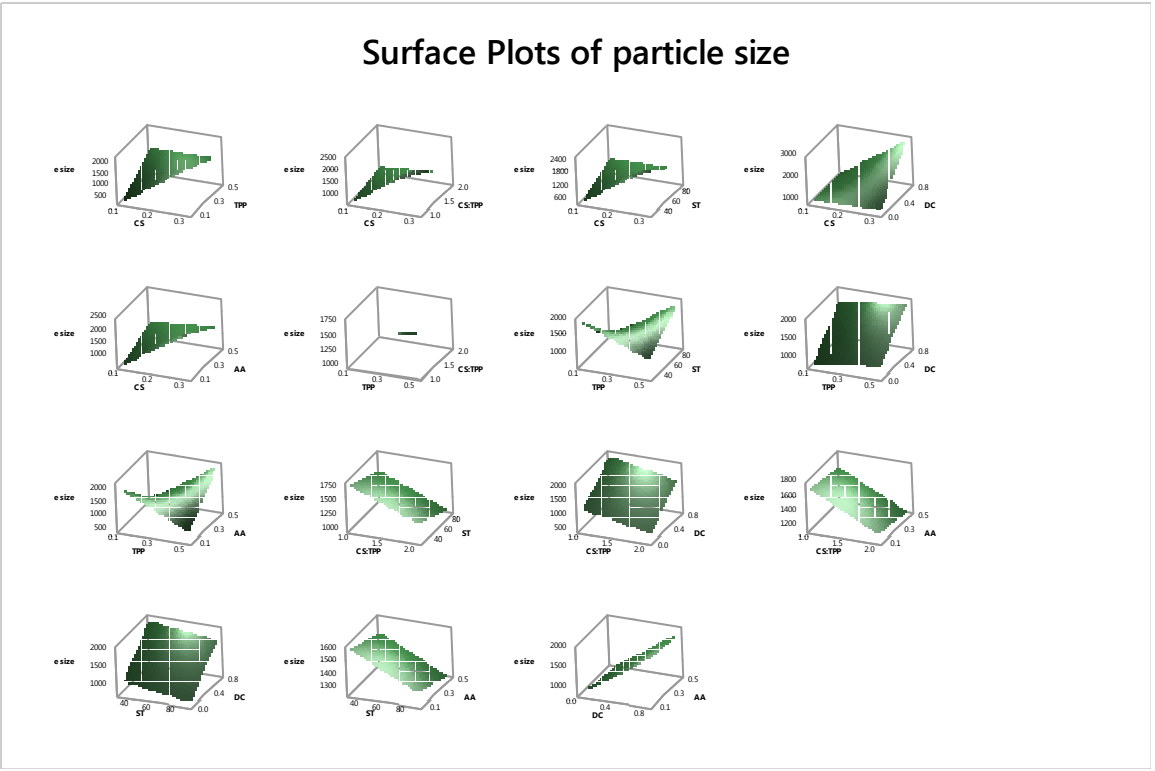


Fig 4:-Response surface plots of interaction effects between different variables and their effect on CS particle size. Hold values are 0.20 for CS, 0.30 for TPP, 1.50 for CS:TPP, 60.00 for ST, 0.45 for DC and 0.30 for AA.

According to the main effect plot (Fig 3), CS concentrations had the foremost influence on the particles size of the prepared nanoparticles. Increasing CS concentration was associated with an increase in the average particle size of the nanoparticles. Possibly increasing the concentration of CS results in a viscosity increase, which in turn will affect the shear capacity of homogenization leading to the formation of aggregates with larger particle size (Hong et al., 2014; Bugnicourt, Alcouffe, & Ladavière. 2014). Similar findings were also reported by Bugnicourt et al., 2014 (Bugnicourt, Alcouffe, & Ladavière. 2014). Looking at the effect of TPP concentration on particle size, it was demonstrated that the higher the concentration of TPP, the larger the particle size, this is because of the stiffening of the cross-linking bonds between TPP and CS associated with the rise of the tripolyphosphoric ions (Patel, Parikh, & Aboti, 2013). The increase in the drug concentration led to a decrease in the particle size, this could be attributed to the competition between PMZ and CS cations to bind with TPP phosphoric ions which in turn will decrease the intermolecular cross linkage between CS and TPP and hence the formation of larger particles. Similar pattern was observed when higher concentration of acetic acid was used to solubilise CS; increasing the drug concentration will increase the negative charge in the sample, which will interact with CS and promote the production of nanoparticles in the media.(Hong et al., 2014; Patel, Parikh, & Aboti, 2013; Luo, Zhang, Cheng, & Wang, 2010; Bugnicourt, Alcouffe, & Ladavière. 2014)**.** On the other hand, stirring time does not show any effect on the particle size. Although it was reported in literature that stirring speed affected the particle size as the increase in the speed resulted in smaller particle size, this could be based on the increase in homogenization speed results in smaller particles (Hong,et al., 2014; Bugnicourt, Alcouffe, & Ladavière. 2014 ; Patel, Parikh, & Aboti, 2013).

3.1.2. The effect of different parameters on surface charge (zeta potential)

The presence of glucosamine group on CS backbone contributes to the creation of positive charge on the surface of the polymer in acidic solutions. CS positively charged surface plays an important role in improving drug targeting and mucoadhesion properties. CS nanoparticles' surface charge was affected by most of the variables, but it was clearly shown in the plot that the CS, TPP and drug concentration were the main factors influenced the change of surface charge (Fig 5& 6).

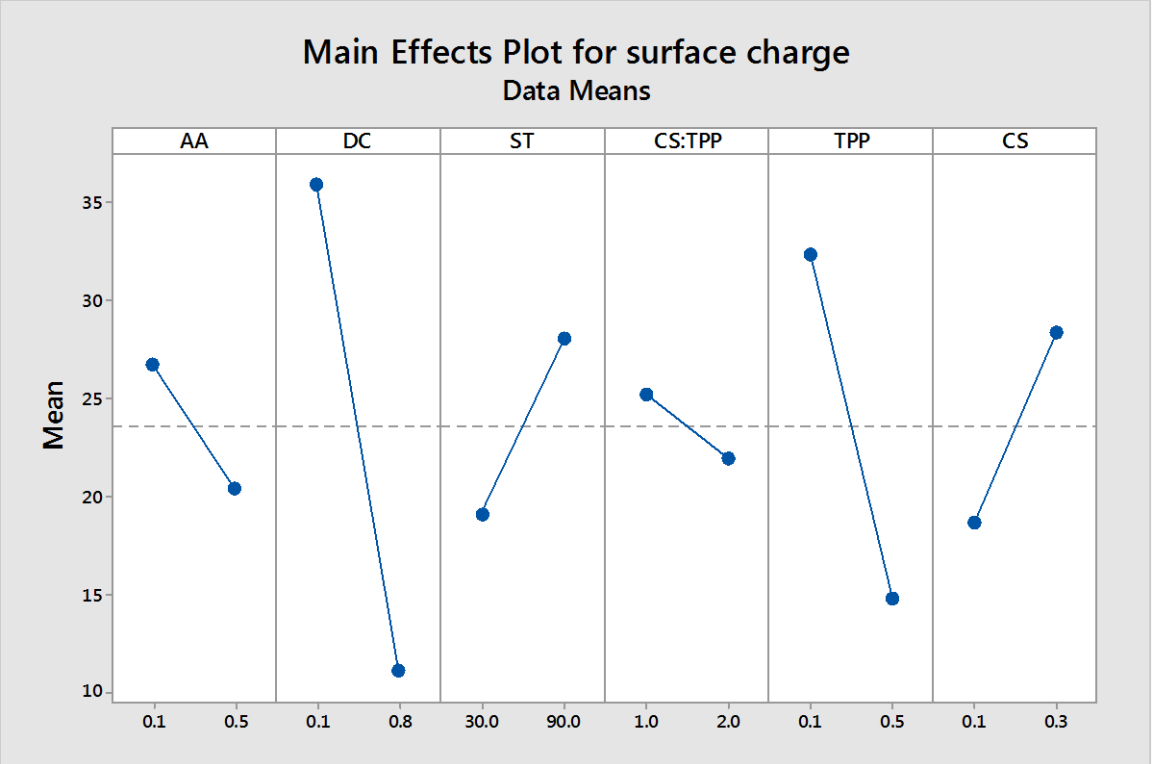


Fig 5:- Main effects plot showing the influence of the independent variables on CS surface charge.

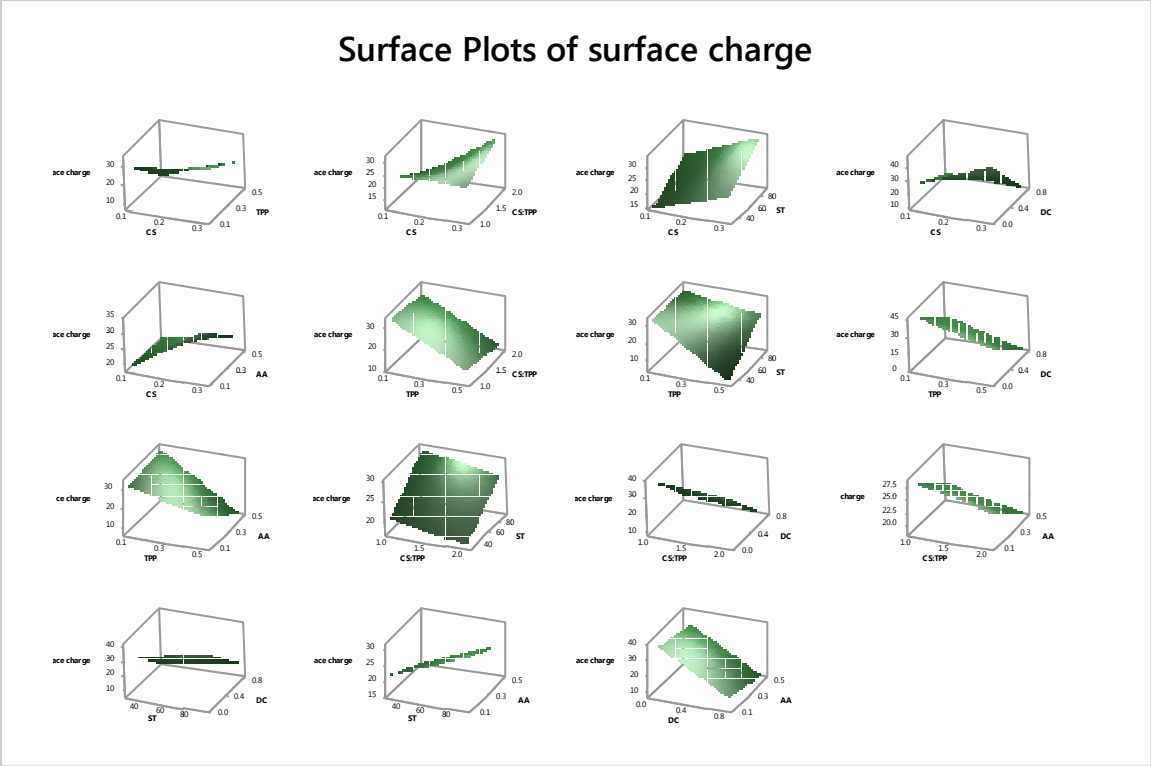


Fig 6:- Response surface plots of interaction effects between different variables and their effect on CS surface charge. Hold values are 0.20 for CS, 0.30 for TPP, 1.50 for CS:TPP, 60.00 for ST, 0.45 for DC and 0.30 for AA.

The increase in CS concentration will be accompanied with an increase in protonized $–NH3^{+}$ which increases the positive charge on the surface of the nanoparticles (Hong et al., 2014; Patel, Parikh, & Aboti, 2013; Luo, Zhang, Cheng, & Wang, 2010; Bugnicourt, Alcouffe, & Ladavière. 2014). Contrariwise, increasing TPP concentration will increase the interaction between CS and TPP and reduce the overall surface charge on the particles due to the presence of the negative charge on the surface. In addition, the increase in the drug concentration resulted in a drop in zeta potential, which can be explained by the competition between CS and the drug to bind to TPP. Acetic acid did not have a significant effect (Fig 5), as it did not have a dramatic effect on the pH, all samples had a pH range of (pH 3.3-3.6).

3.1.3. The effect of different parameters had on encapsulated efficiency

The encapsulated efficiency was detected by measuring the amount of drug (PMZ) in the supernatant, after centrifugation of the nanoparticles. The current study looked at 2 concentrations of PMZ; 0.4mg/ml, 0.8mg/ml and the results obtained indicated an EE range of (95-99%).

The obtained results outlined that EE was significantly affected by the CS:TPP ratio and drug concentration (FigS 7&8). Increasing DC was associated with increasing the entrapment efficiency. Nonetheless, all the prepared formulations had entrapment efficiency greater than 95%. Previous studies had demonstrated that the nature of the drug -whether hydrophilic or hydrophobic- will not have an effect on the encapsulation efficiency (Cafaggi, et al., 2007; Bugnicourt, Alcouffe, & Ladavière. 2014; Klancke 2003). Moreover, the study conducted by Yan Wu et al., claimed that the drug concentration has no effect on the EE despite using similar concentration range (0.2-0.8mg/ml) to our study (Wu, Yang, Wang, Hu, & Fu, 2005)

There is a debate on the effect of CS concentration on EE of CS nanoparticles, previous studies conducted by Vandenberg et al., 2001 and Hassani 2014 reported that the increase in CS lead to the increase in drug encapsulation, mainly due to an increase in the CS concentration leading to an increase in the ion gelation hence better entrapment efficiency. In contrast a study by Wu et al. 2005 indicated that the increase of CS decreases the EE (Wu, Yang, Wang, Hu, & Fu, 2005). Nevertheless, our study demonstrated that CS concentration has no significant effect on PMZ entrapment ($p<0.05$).

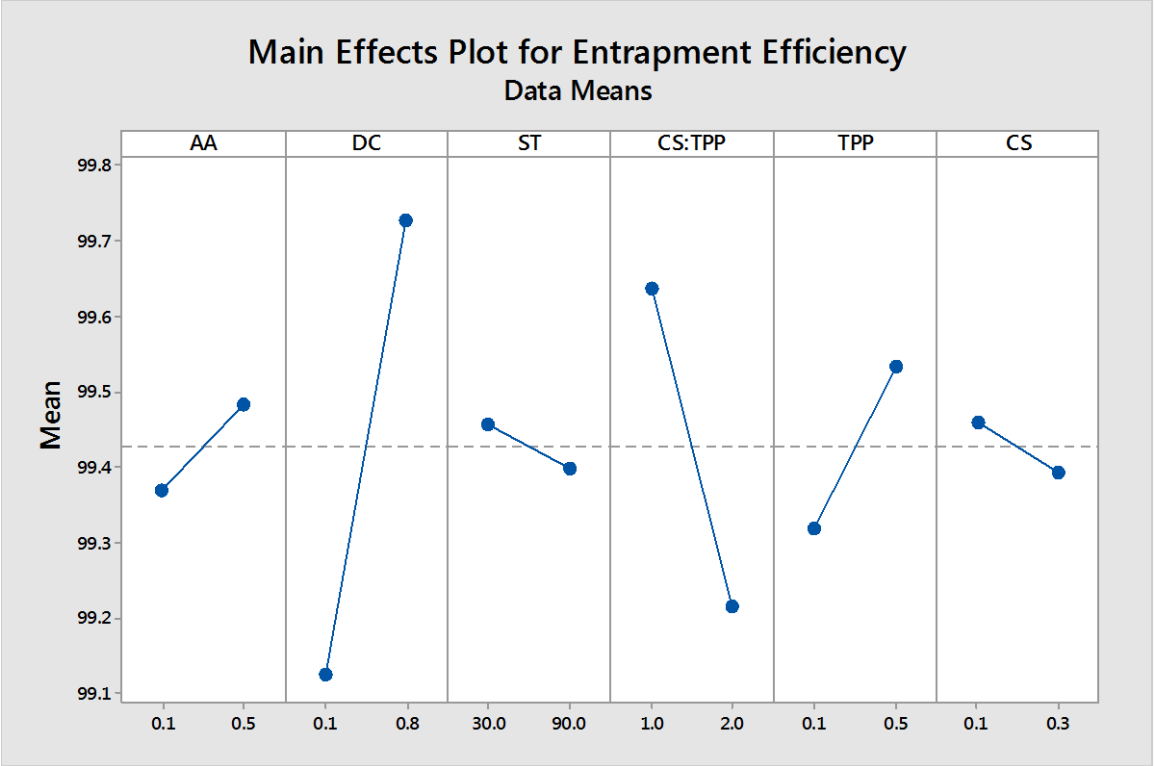


Fig 7:- Main effects plot showing the influence of the independent variables on the entrapment efficiency of PMZ

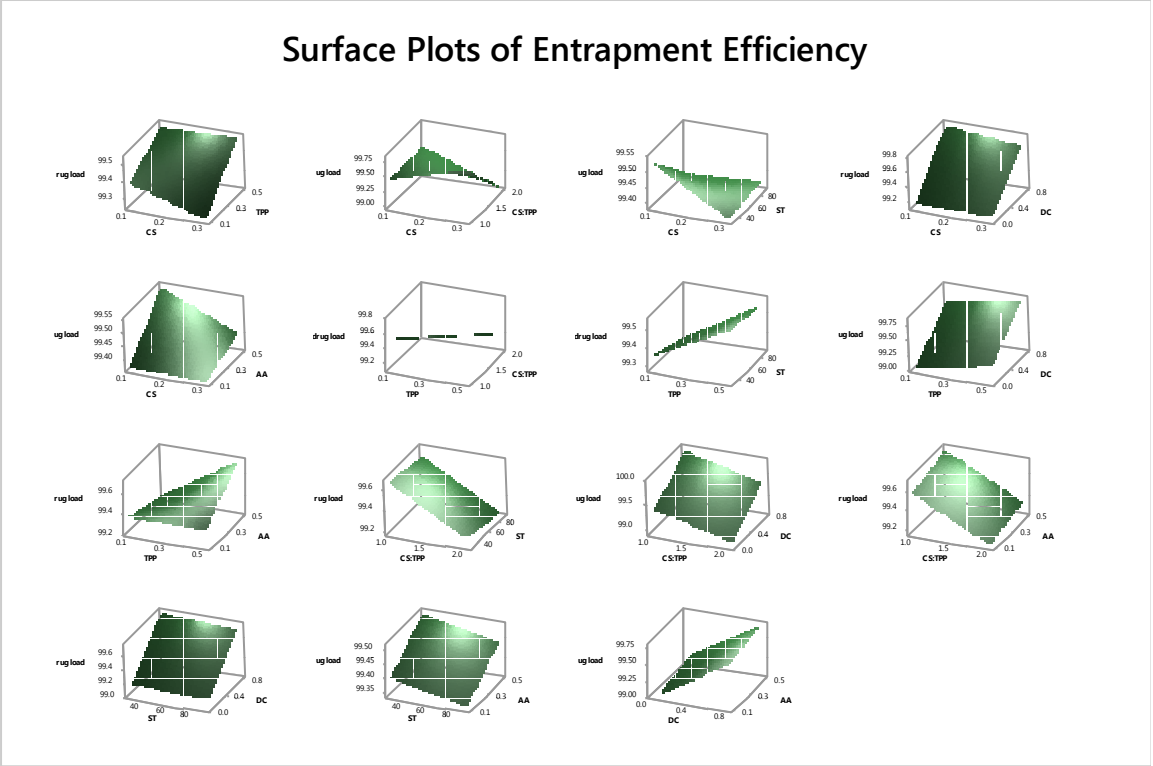


Fig 8:- Response surface plots of interaction effects between different variables and their effect on the entrapment efficiency of PMZ. Hold values are 0.20 for CS, 0.30 for TPP, 1.50 for CS:TPP, 60.00 for ST, 0.45 for DC and 0.30 for AA.

After identifying the effect variables on the responses, an optimization study was performed by Minitab (Table 2), to determine the optimum conditions to produce the nanoparticles with maximum drug loading and targeted particle size of 250 nm. Nanoparticles were prepared using the optimal conditions and evaluated to determine the accuracy of the conditions produced; the results obtained from the sample showed a particle size of 280nm, with EE of 99% and zeta potential of 20.8 mV. Where Minitab predicted a particle size of 250nm and EE of 94%, this can clearly conclude the precision of the optimization study by Minitab.

Table 2:- Summary of the optimised conditions for preparing CS-nanoparticles

Concentration of CS	**Concentration of TPP**	**CS:TPP**	**Concentration of PMZ**	**Concentration of acetic acid**	**Stirring time**
0.1%	1%	5:2	0.8mg/ml	1%	90min

3.2. Characterisation of coated CS nanoparticles

The fast advances of polymeric sciences led to the introduction of a number of new polymers into the pharmaceutical industry and resulted into the production of a number of novel sustained release drug delivery systems. After optimisation of chitosan nanoparticles using the DOE approach, the optimised nanoparticulate system was coated with three polymers namely; PVP, PEG and PEAA which have cationic,

non-ionic and anionic nature, respectively. The nature of the coating polymer might affect the surface charge, particles size and loading capacity of chitosan nanoparticulate system. Both PVP (Park 2003) and PEG (Park 2001) were co-grafted with chitosan to improve the low solubility of the hydrophobic polymer in aqueous solutions. The new grafted polymers were used for delivery of DNA molecules and showed responsiveness (Park 2001; Park 2003). The particles size for non-coated CS nanoparticles was not affected (*p>0.05*) by the incorporation of PMZ (Fig 9 and Table 3). Non-coated particles showed particle size of 151.4±6.9 nm to 153.8±14.0 nm for non-coated PMZ nanoparticles and PMZ-free nanoparticles respectively. Nonetheless, incorporation of the coating polymers during the manufacturing of CS nanoparticles has affected both the particles size and surface charge (Fig 9). Addition of PVP was associated with an increase (*p<0.05*) in the particle size which reached 186±19 nm. The cationic nature of PVP might be the reason of increasing the particle size of CS nanoparticles as the polymer might compete with chitosan to interact with TPP during the manufacturing process which will reduce the ionic gelation capacity of chitosan ,therefore larger particles were formed. On the other hand, addition of PEG decreased the size of the particles prepared (*p<0.05*). This can be explained by the ability of the electronegative oxygen atom of PEG to form intramolecular hydrogen bonding with the electropositive amino hydrogen on CS as reported by (Kim and Lee, 1995) which in turn tighten the nanoparticle structure, therefore a smaller size (124±5.2 nm; PDI 0.32±0.04) was obtained. In a similar pattern, the surface charge on PEG-CS coated nanoparticles has decreased significantly (*p<0.05*) to 21.3±6.8 mV when compared to non-coated PMZ nanoparticles (31.4±0.9 mV). Similar trend was reported by Wu et al (2005) and Quellec et al., (1998). PEAA is the third polymer used to coat CS nanoparticles. PEAA did not have any effect on the particle size (*p>0.05*) or the surface charge of the prepared nanoparticulate system (Fig 9). This could be attributed to the weak acidic nature of the polymer (pKa of 4.25) which has a minimal effect on the pH of CS acetic acid solution and hence minimal effect on the characteristics of the nanoparticles as suggested by (Wu, Yang, Wang, Hu, & Fu, 2005; Bugnicourt, Alcouffe, & Ladavière. 2014; Quellec et al., 1998).)

Table 3:- Summary of particle size, surface charge, PDI and entrapment efficiency of coated and non-coated CS-nanoparticles (mean±SD)

Sample	particle size (nm)	surface charge (mV)	PDI	EE%
PVP-coated nanoparticle	186±19.00	39.7±1.50	0.27±0.19	99.69±0.04
PEAA-coated nanoparticle	153.8±5.40	28.6±6.60	0.47±0.10	99.74±0.00
PEG-coated nanoparticle	124±5.20	21.3±6.80	0.32±0.04	99.77±0.06
Non-coated PMZ nanoparticles	151.4±6.90	31.4±0.90	0.67±0.08	99.77±0.06
PMZ-free nanoparticles	153.8±14.00	38.6±2.60	0.42±0.29	-

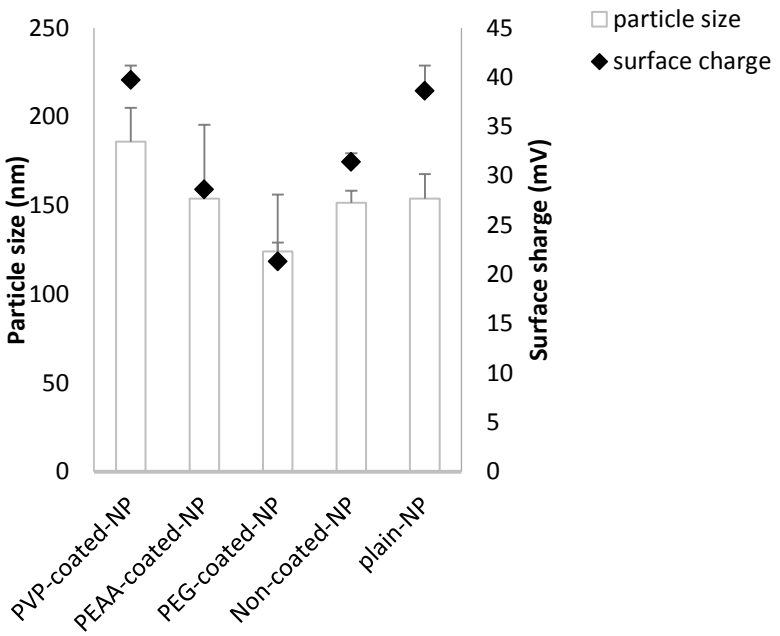
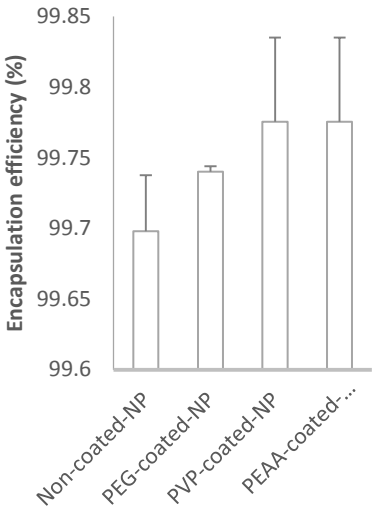


Fig 9:- Effect of coating polymers on the particle size and surface charge of CS nanoparticles.

Encapsulation efficiencies (EE) of coated nanoparticles were evaluated as well (Fig 10). All coated nanoparticulate systems showed high percentage of EE ranged around (99.5%-99.9%), which suggest that different coating polymers did not affect the encapsulation efficiency of CS nanoparticles (*p>0.05*).



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450 *Fig 10:-Effect of coating polymers on the encapsulation efficiency of CS nanoparticles.*

451 **3.3. Scanning electron microscopy (SEM)**

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453 In order to investigate the morphology and surface properties of the prepared nanoparticles, SEM was used.
454 (Fig11) shows SEM images of PMZ HCl, chitosan polymer, plain CS-nanoparticles, PEG-coated nanoparticles,
455 PVP-coated nanoparticles, PEAA-coated nanoparticles and non-coated CS nanoparticles. PMZ HCl showed
456 cubic crystals with a wide range of particle size ranging from few µms to 200 µm. Small and large crystals
457 aggregate together forming raspberry like aggregates (Fig11B). Chitosan particles were irregular in shape
458 with some folds on their surface. CS particles showed large particle size greater than 400 µm (Fig 11C). All
459 the prepared nanoparticles; coated and non-coated were spherical in shape and showed particles size in
460 nano-range as suggested by DLS studies. Plain CS-nanoparticles showed a smooth surface without any
461 evidence of aggregate formation; probably the high surface charge (ξ=38.6±2.6 mV) prevented any
462 aggregation through electrostatic repulsion between the positively charge particles. Similarly, only few
463 aggregates were observed when nanoparticles were coated with PVP (Fig11F). In contrary, loads of
464 aggregates appeared under the microscope when PEAA was used as a coating polymer (Fig11G), this could
465 be attributed to the anionic nature of PEAA which decreased the overall charge on the CS nanoparticles
466 (ξ=28.6±6.6 mV). Similar trend was observed with PEG-coated nanoparticles (Fig 11E).

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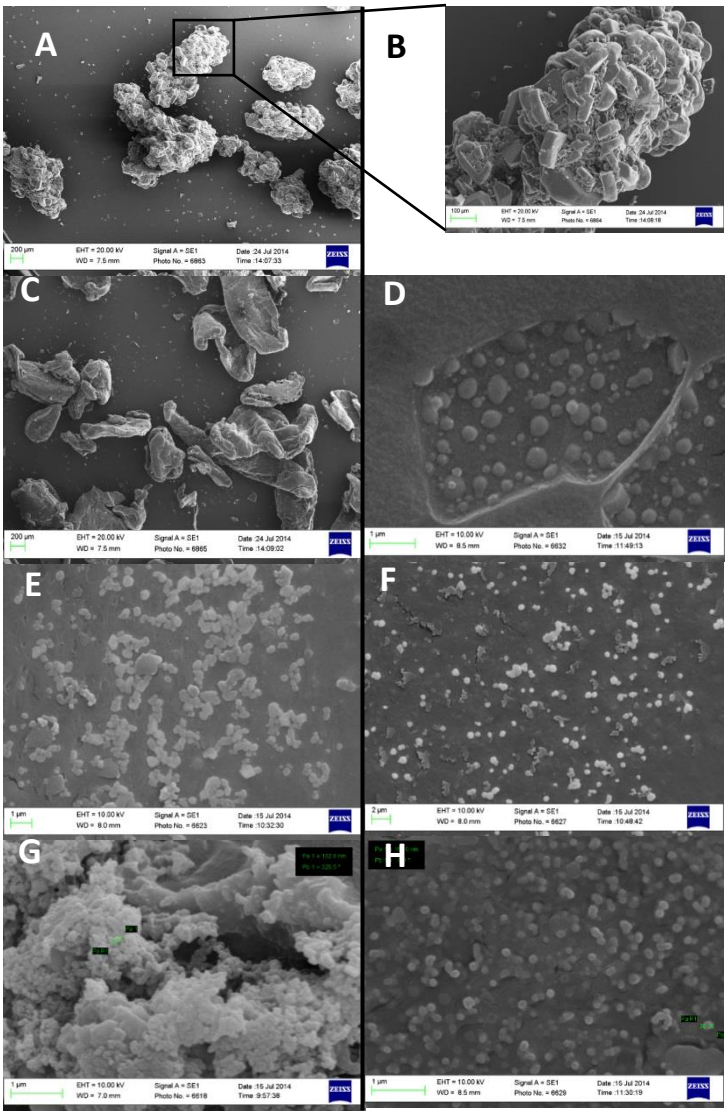

Fig 11:- SEM images of PMZ HCl at low magnification (A) and high magnification (B), chitosan polymer (C), plain CS-nanoparticles (D), PEG-coated nanoparticles (E), PVP-coated nanoparticles (F), PEAA-coated nanoparticles (G) and non-coated CS nanoparticles (H).

3.4. Thermal analysis

Differential scanning calorimeter is used to determine any change in the physic-chemical properties of the material by measuring the energy transfer from and to PMZ. Moreover, differential scanning calorimetry will enable investigation of any interaction between PMZ and CS or the coating polymers used in this study.

(Fig12) shows the rate of heat absorption for PMZ, CS, PEG-coated nanoparticles, PVP-coated nanoparticles, PEAA-coated nanoparticles and non-coated CS nanoparticles. DSC has shown a sharp endothermic peak at 234 ^{o}C corresponding to the melting of PMZ HCl salt (Fig12A) (Lutka, A 2002; Ambrogi, Nocchetti, & Latterini, 2014). Chitosan thermal scans has shown a broad endothermic peak between 60 and 140 ^{o}C and this is attributed to evaporation of water that is associated with the hydrophilic groups of CS (Figure 12 B). Coupling DSC scans with TGA can confirm this finding as a weight loss of (19 %) was observed between 60-140 ^{o}C (Fig13B). Similar findings were reported earlier by (Dong, Ruan, Wang, Zhao, & Bi, 2004; Mladenovska et al., 2007). PMZ-CS nanoparticles (Fig12F) did not show any endothermic or exothermic peaks and PMZ HCl endothermic peak disappeared which suggests possible interaction between the drug and CS by Van der Waals force within the nanoparticles. Moreover, it was reported that spaces between CS chain provide favourable conditions for dispersing drug within CS nanoparticles (Sarmento, Ferreira, Veiga, & Ribeiro, 2006; Dos et al., 2011)

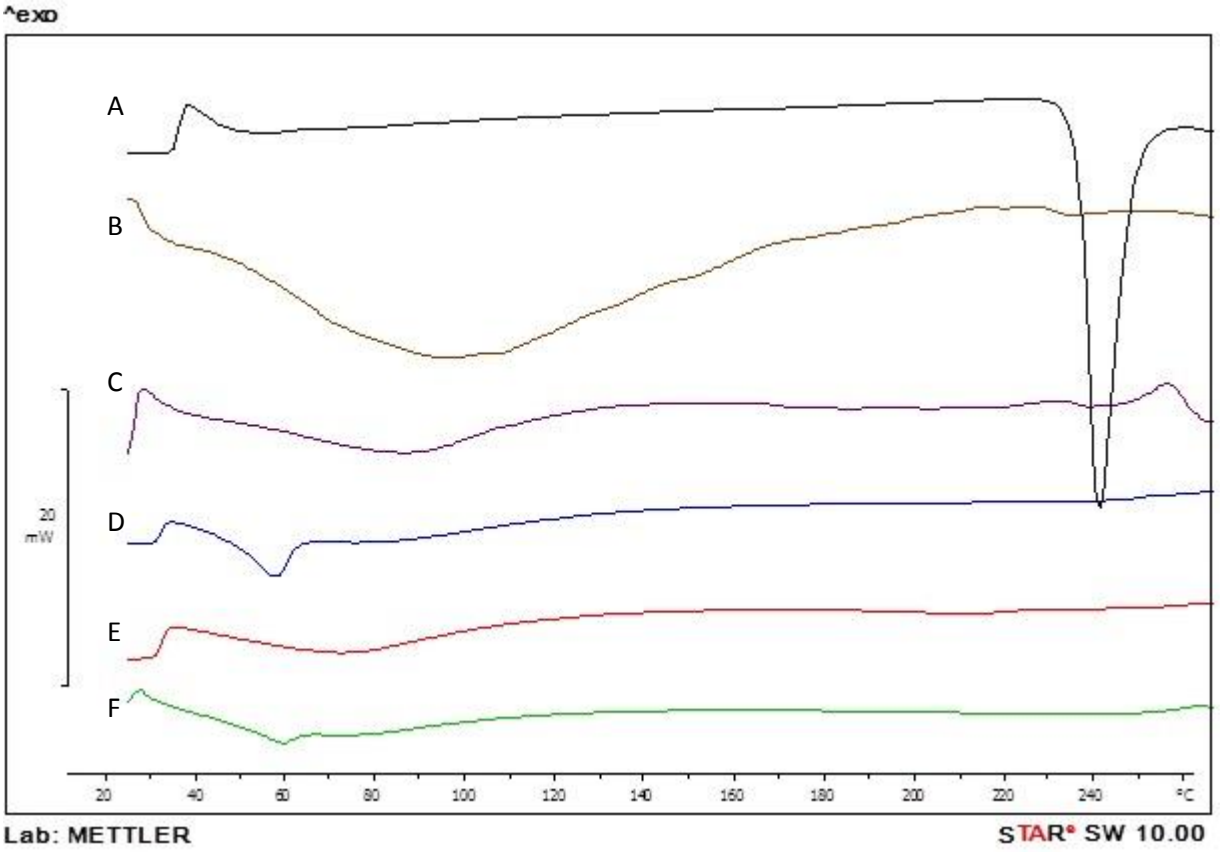

Fig 12:- DSC scans of PMZ HCl salt (A), CS (B), PEG-coated nanoparticles (C), PVP-coated nanoparticles (D), PEAA-coated nanoparticles (E) and non-coated CS nanoparticles (F).

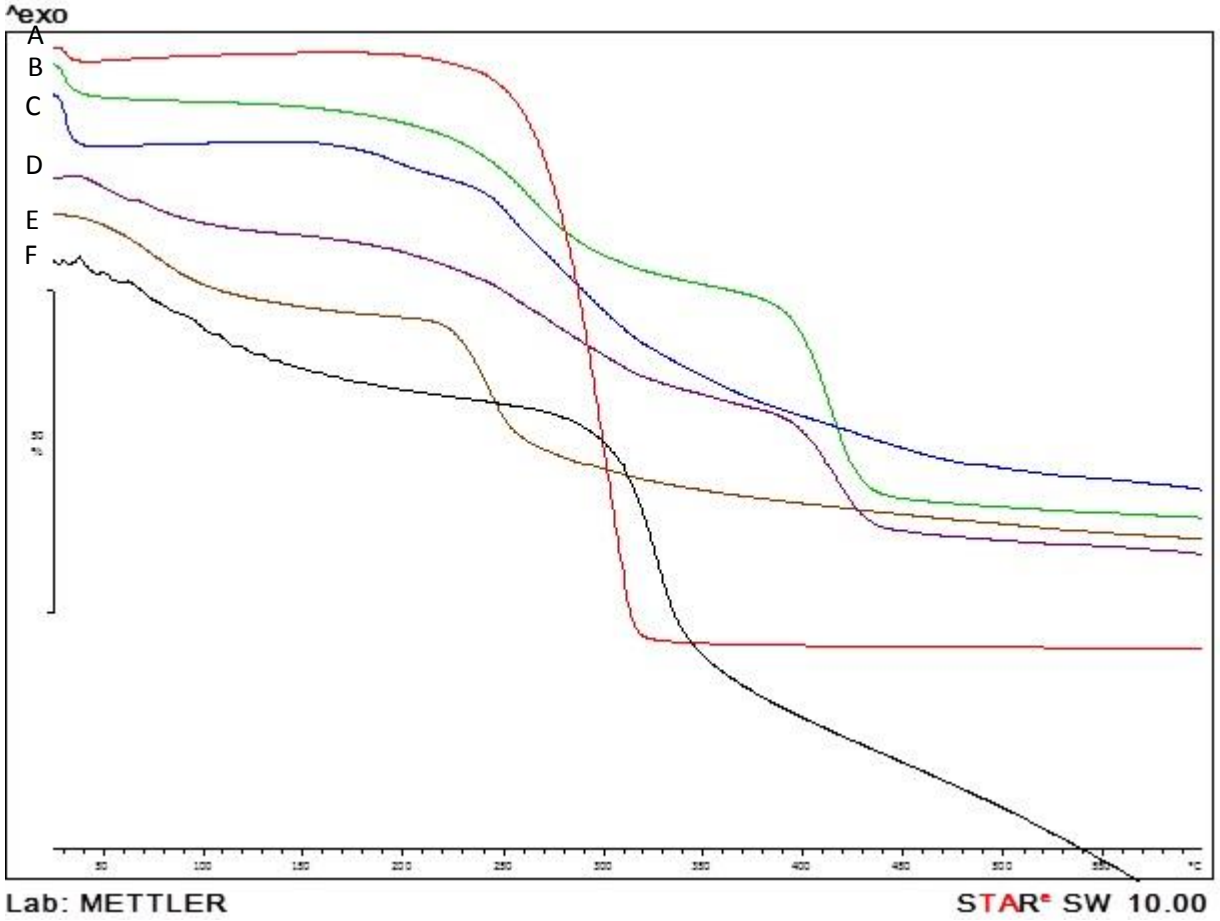

Fig 13:- TGA scans of PMZ HCl salt (A), PEAA-coated nanoparticles (B), non-coated CS nanoparticle (C), PVP-coated nanoparticles (D) PEG-coated CS nanoparticles (E) and non-coated CS nanoparticles (F).

CS-coated nanoparticles showed similar scans to non-coated CS-nanoparticles as PMZ HCl endothermic peak disappeared because of the dispersion of the drug between CS and the coating polymer used.

3.5. ODTs preparation and evaluation

After preparation and characterisation of various CS-nanoparticles, the particles were incorporated into orally disintegrating tablet matrix adapted from (ElShaer, A, Butt, U, Rauf, I , Sohaib Saboley, & Gawad, M 2014) and based on the following formulation 25% LH-B1, 1% Magnesium stearate, 5% PMZ and 69% D (+)-Lactose Monohydrate. After the preparation of ODTs, the tablets were then evaluated for their hardness, friability, disintegration time, dissolution profiles.

3.5.1. Hardness, disintegration time and friability

Hardness and friability tests were performed to determine if the tablets produced have a significant mechanical strength to stand fraction and erosion. The mechanical strength of ODTs is a critical parameter, as ODTs are prepared under low compression in order to form highly porous compress for fast disintegration. Nonetheless, the preparation process together with the excipients used might results in producing a friable/ brittle tablet. Control ODTs did not contain any nanoparticles within their matrix and showed fast disintegration time of 34±1.4 sec and high tensile strength of 2.7±0.25 N/mm^2. Addition of

coated and non-coated CS nanoparticles into ODTs tablet matrix did not distort the characteristics of the tablets (Fig 14) and Table (4). All ODTs showed disintegration time between 25- 35 sec and tensile strength ranging from 2.5-3 N/mm^2. All tablets showed no significant effect ($p<0.05$) in disintegration time comparing to each other ($p>0.05$) , but had a statistical significant affect comparing to the disintegration time of the control tablet($p<0.05$). All the prepared tablets passed the friability test (Table 4) with highest friability of 0.9% exhibited by ODTs containing PVP coated nanoparticles.

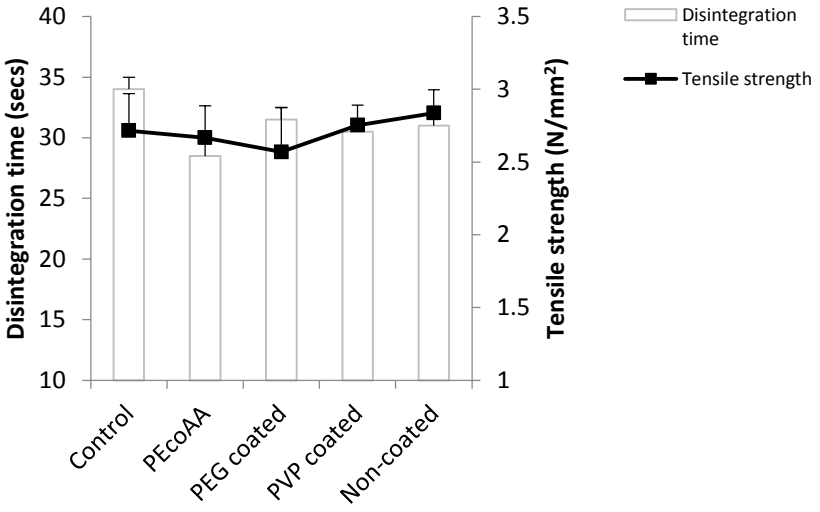

Fig 14:- Tensile strength and disintegration time of ODTs containing non-coated CS-nanoparticles, PEAA coated CS-nanoparticles, PEG coated CS nanoparticles, PVP coated CS nanoparticles and control ODTs.

Table 4:- Thickness, diameter (mean±SD) and friability of ODTs tablets containing coated and non-coated CS nanoparticles.

sample	Thickness (mm)	Diameter (mm)	Friability (%)
Control tablet	13.1±0.04	2.71±0.25	0.75%
PEAA coated nanoparticle tablet	13.02±0.04	2.66±0.22	0.5%
PEG coated nanoparticle tablet	13±0	2.56±0.30	0.7%
PVP coated nanoparticle tablet	13.06±0.054	2.66±0.05	0.9%
Non-coated nanoparticle tablet	13.04±0.054	2.68±0.04	0.6%

3.5.2. Dissolution test

In order to evaluate the release profile across CS-nanoparticles containing tablets, *in vitro* dissolution studies were performed. Control tablets showed a fast release of PMZ as 46.1±0.3 % of the drug was released within 20 mins of the dissolution study and 97.3±0.13% was released at 60 mins. On the other hand, tablets containing CS-nanoparticles showed a slower release profile that became even slower upon coating the nanoparticles (Fig15). Non-coated chitosan nanoparticles managed to sustain the drug release for 24 hours with only 35.5±0.14% and 68.8±3.3% after 2 and 6 hours respectively. Similar release profiles were reported

by (Lu et al., 2009) when using CS nanoparticles to deliver aminoglycosides such as gentamicin and tobramycin. As more than 60% of the drugs were retained inside CS nanoparticles for 6 hours at pH of 1.2. Unmodified CS has been used intensively to sustain the drug release for several therapeutic agents such as ammonium glycyrrhizinate.(Wu, Yang, Wang, Hu, & Fu, 2005) dorzolamide hydrochloride, and pramipexole hydrochloride (Papadimitriou, Bikiaris, Avgoustakis, Karavas, & Georgarakis, 2008) ciprofloxacin (Jain, & Banerjee,. 2008) and even for peptides and proteins (Jiang, Pan, Cao, Jiang, Hua, & Zhu, 2012). Nevertheless, CS-nanoparticles fail to sustain the drug release for longer time as the acidic conditions in the stomach solubilise chitosan (George, & Abraham, 2006). Therefore a second coating polymer was used in this study. The *in vitro* dissolution test indicated that coated nanoparticles had a slower release profile compared to non-coated, even after 24hr the drug release from the nanoparticle was not complete Fig (15).

PEAA coated nanoparticles showed a burst effect as 45±0.9 % of PMZ was release within 2 hours of the dissolution study and the drug release remained below 58.6±0.23% during the time course of the experiment. Despite the weak acidic nature of PEAA which was believed to reduce its dissolution under the acidic conditions of this study (0.1N HCl), PEAA-CS particles exhibited a burst effect, possibly because some of PMZ was attached to the surface of the nanoparticles and released ring the first few hours of the dissolution study as suggested earlier by (Patel, Parikh, & Aboti, 2013). On the other hand, PVP and PEG coated nanoparticles showed the slowest amount of drug release over 24hr. PEG and PVP-coated CS nanoparticles released 13.86±0.13% and 7.6±0.54 % after 6 hours of the dissolution study respectively. And less than 45% of PMZ after 24 hours of the dissolution study.

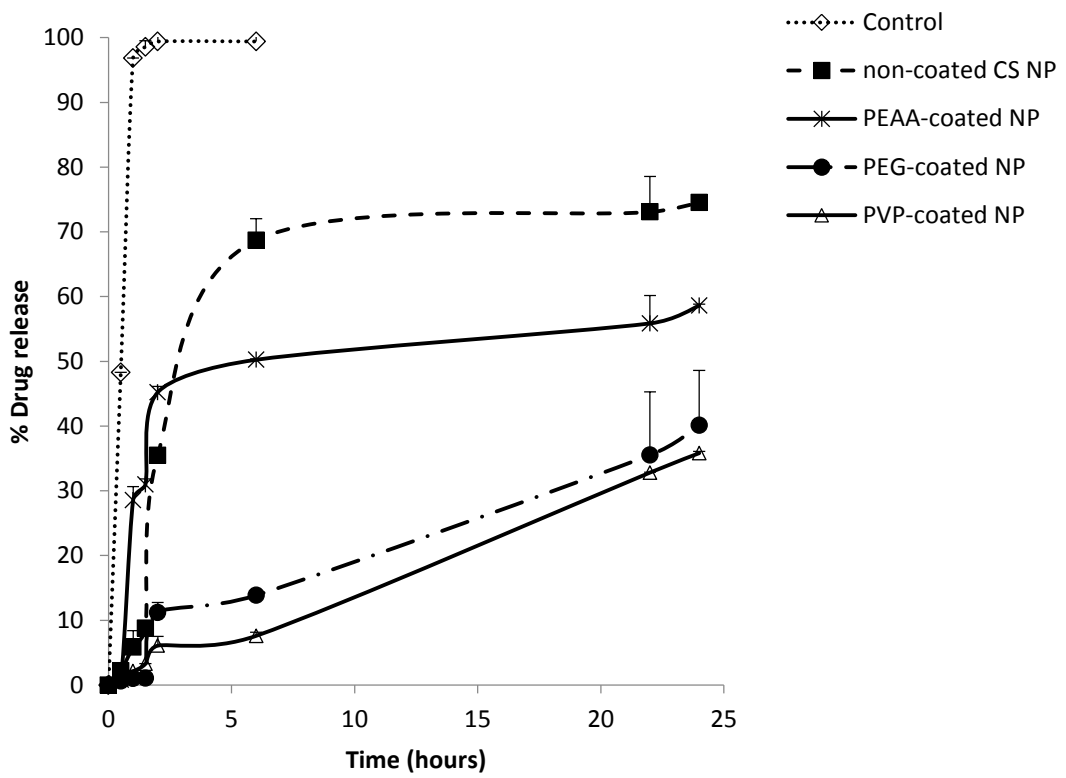

Fig 15:- In vitro *dissolution study of orally disintegrating tablets containing PMZ (control), non-coated CS NPs, PEAA-coated CS-NPs, PEG-coated CS-NPs and PVP-coated CS-NPs.*

3.5.2.3. Sulforhodamine B (SRB) cytotoxic assay.

The SRB assay was used to study the cytotoxic effect of the prepared nanoparticles. Figure (16) illustrate the cell viability of Caco-2 cell lines after 24 hours incubation with different concentration of the nanoparticles compared to the negative control. The average cell viability for the highest concentration (40 mg/mL) of the PVP coated nanoparticles was 85% ($p<0.05$) compared to the untreated cell (negative control), similar findings were suggested earlier by Lara et al (2010). Likewise, the cell viability of PEG coated nanoparticles were significantly ($p<0.05$) reduced to 80%, this could be ascribed to the ability of PEG to form hydrogen bonding with surrounding water which in turn increases the osmotic pressure of the surrounding media. This osmotic shock will be associated with disorganisation of the nuclear chromatin cells of the Caco-2 cells by hyper-condensation of the nuclear chromatin and accumulation of cytoplasmic vesicles as suggested by Gilles et al., 1995 and Parnaud et al., 2001. On the other hand, lower concentrations of both PVP and PEG (20 mg/mL and 10 mg/mL) showed no signs of toxicity on the mammalian cells (Fig 16). In contract to PVP and PEG behaviors, 40 mg/mL of the chitosan and PEAA nanoparticles had no significant ($p>0.05$) effect on the cells' viability. The average cell viability for the chitosan and PEAA coated nanoparticles were 92% and 96% respectively. These results are in agreement with previous studies conducted by (Huang et al., 2004) suggesting that higher concentrations of CS is associated with cell toxicity because of the higher surface charge density which is a high contributor to cell death. Other concentration of the prepared nanoparticles had no significant effect on the Caco-2 cell lines after 24 hours incubation period (*$p>0.05$*)

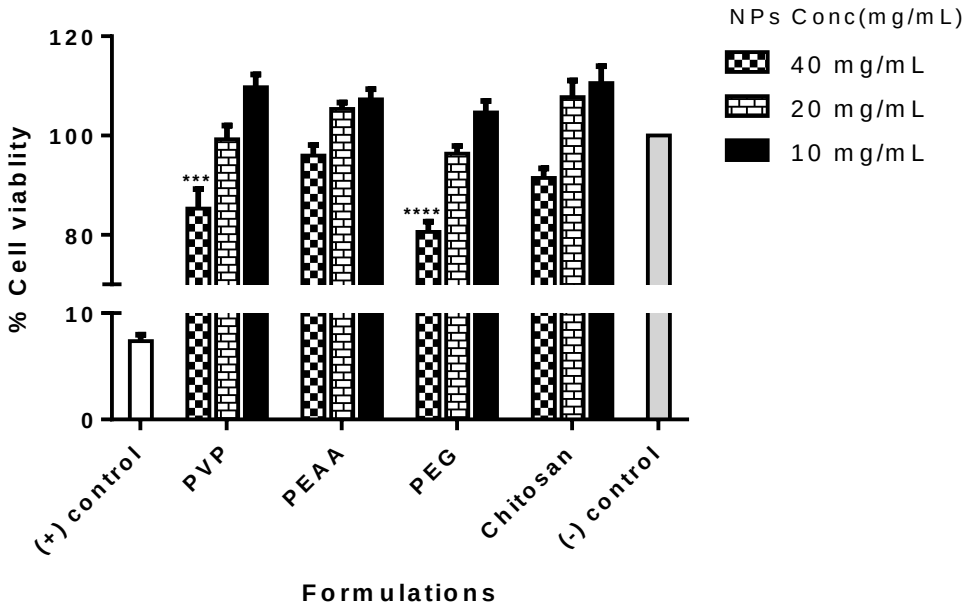

*Figure 16:- SRB cytotoxic assay for the effect of chitosan and coated nanoparticles on Caco-2 cell line after 24 hours incubation. Results express as mean value ±SD (n=6), ***p<0.001, ****p<0.0001*

Conclusion

The properties of CS nanoparticles was engineered using Minitab in order to manufacture a new formulation of SR-ODTs. Minitab studies revealed that the nanoparticles' particle size is affected by most of the independent variables. The concentration of TPP and CS was associated with an increase in the particles size and this is possibly due to the stiffening of the cross linking bonds between TPP and CS, and the increase in the viscosity which will affect the shear capacity of homogenization leading to the formation of aggregates with large particle size, respectively. Drug concentration and CS:TPP ratios were the two main variables affecting the encapsulations efficiency. The engineered nanoparticles were further characterised using SEM which revealed that all the samples were spherical in shape with smooth surface and had particle size ranging between 100- 200 nm that goes in line with DLS results. Optimised CS-nanoparticles were further coated with polyvinylpyrolidine (PVP), polyethylene glycol (PEG) and polyethylene co-acrylic acid (PEAA). The coated nanoparticles were incorporated into ODTs. All tablets had passed the friability test and showed good tensile strength despite disintegrating in less than 40sec. The drug release profile was studied in 0.01M HCL solution showing that tablets containing PVP and PEG coated nanoparticles managed to sustain the drug release over 24hr, yet showed a slight toxic effect on Caca-2 cell lines at high concentrations of 40 mg/mL. On the other hand, non-coated and PEAA nanoparticles showed a faster rate of release without any pronounced effect on the viability of Caco-2 cells.

Conflict of Interest:

Authors declare that there is no conflict of interest.

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