2018

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Giuliana Vozza
Technological University Dublin

Minna Danish
Technological University Dublin

Hugh J. Byrne
Technological University Dublin, hugh.byrne@tudublin.ie

See next page for additional authors

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Recommended Citation

Ryan, S. et al. (2018) Application of Box-Behnken Experimental Design for the Formulation and Optimisation of Selenomethionine-Loaded Chitosan Nanoparticles Coated with Zein for Oral Delivery, *Int J Pharm.* 2018 Nov 15;551(1-2):257-269. doi:10.1016/j.ijpharm.2018.08.050

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Authors

Giuliana Vozza, Minna Danish, Hugh J. Byrne, Jesus Maria Frias, and Sinéad M. Ryan

Application of Box-Behnken experimental design for the formulation and optimisation of selenomethionine-loaded chitosan nanoparticles coated with zein for oral delivery

Giuliana Vozza^{a,b}, Minna Danish^{a,b}, Hugh J. Byrne^b, Jesús M. Frías^c, Sinéad M. Ryan^{d,*}

^a School of Food Science and Environmental Health, Dublin Institute of Technology, Marlborough Street, Dublin 1, Ireland

^b FOCAS Research Institute, Dublin Institute of Technology, Kevin Street, Dublin 8, Ireland

^c Environmental Science and Health Institute, Dublin Institute of Technology, Grangegorman,

^d School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland
Dublin 7, Ireland

* Corresponding author. E-mail: Sinead.Ryan@ucd.ie

Abstract:

Selenomethionine is an essential amino acid with a narrow therapeutic index and susceptibility to oxidation. Here it was encapsulated into a nanoparticle composed of chitosan cross-linked with tripolyphosphate for oral delivery. The formulation was optimised using a three-factor Box-Behnken experimental design. The chitosan:tripolyphosphate ratio, chitosan solvent pH, and drug load concentration were independently varied. The dependent variables studied were encapsulation efficiency, particle size, polydispersity index and zeta potential. For optimisation, encapsulation efficiency and zeta potential were maximised, particle diameter was set to 300 nm and polydispersity index was minimised. A 0.15mg/mL concentration of selenomethionine, chitosan solvent pH of 3, and chitosan:tripolyphosphate ratio of 6:1 yielded optimum nanoparticles of size $187\pm 58\text{nm}$, polydispersity index 0.24 ± 0.01 , zeta potential $36\pm 6\text{mV}$, and encapsulation efficiency of $39\pm 3\%$. Encapsulation efficiency was doubled to $80\pm 1.5\%$ by varying pH of the ionotropic solution components and by subsequent coating of the NPs with zein, increasing NP diameter to $377\pm 47\text{nm}$, whilst retaining polydispersity index and zeta potential values. Selenomethionine-entrapped nanoparticles were not cytotoxic to intestinal and liver cell lines. Accelerated thermal stability studies indicated good stability of the nanoparticles under normal storage conditions (23°C). In simulated gastrointestinal and intestinal fluid conditions, 60% cumulative release was obtained over 6 hours.

Keywords:

chitosan, zein, selenomethionine, nanoparticles, Box-Behnken design, oral delivery

Abbreviations

BBD, Box-Behnken design; **CL113**, PROTASAN™ UP; **Cs**, Chitosan; **DLS**, dynamic light scattering; **EE%**, Encapsulation efficiency; **GRAS**, Generally recognised as safe; **LDV**, laser doppler velocimetry; **MSC**, methylselenocysteine; **NP**, nanoparticle; **PDI**, Polydispersity; **pI**, Isoelectric point; **SeCys**, selenocysteine; **SeMet**, Selenomethionine; **TPP**, Tripolyphosphate; **ZP**, Zeta potential.

1 INTRODUCTION

Selenium is an essential micronutrient in human and animal nutrition (Rayman, 2000), that exists in a wide array of different formats, both organic and inorganic, better known as speciation. Selenomethionine (SeMet), the selenium analogue of methionine, is the predominant form of organic Se found in foods from the *Brassica* and *Allium* families (Reilly et al., 2014). SeMet is used for oral supplementation due to its capacity to be non-specifically incorporated into body proteins in place of methionine (Rayman et al., 2008). The potential health benefits of selenium are dependent on its chemical species, and several studies have suggested a possible role in cancer prevention (Nie et al., 2016), increased immunological status (Narayan et al., 2015) and increased fertility (Shanmugam et al., 2015). SeMet may also to have a number of benefits regarding oncology treatments due to its modulation of the therapeutic efficacy and selectivity of anticancer drugs (Evans et al., 2017), capacity to provide protection of normal tissues from the toxicities associated with chemotherapy and radiation treatments, in addition to enhancing their anti-tumour effects (Chintala et al., 2012; Mix et al., 2015; Panchuk et al., 2016). It may also have some potential in degenerative disease by decreasing oxidative stress of small molecule antioxidants used as a buffer for free

radicals in brain tissue (Reddy et al., 2017; Song et al., 2014). However, the oral delivery of SeMet can be challenging due to the distinctive electronegativity and atomic radius of the selenium atom (i.e. larger radius and lower electronegativity than sulphur,) that makes it easier for low valence state Se compounds to be more readily oxidised compared to their sulphur counter parts (Xu et al., 2013). SeMet is readily oxidised (Davies, 2016) and, even though it is less toxic than inorganic selenium (Se), it still has a low therapeutic index (Takahashi et al., 2017). Oral delivery formulations of SeMet therefore need to consider the balance between doses that exert beneficial effects and those which may potentially be toxic.

Inorganic Se species such as selenite (SeO_3^{2-}) and elemental selenium (Se_0), together with methylseleninic acid, have been formulated to nano-enabled delivery systems which exhibited improved bioactivity with reduced cytotoxicity *in vitro* (Forootanfar et al., 2014; Loeschner et al., 2014; Zhang et al., 2008). Nanoparticles (NPs) can be more biologically active due to their enhanced surface area per mass compared with larger-sized particles of the same chemistry (Oberdörster et al., 2005). By using NPs as a drug delivery vehicle, it might be possible to enhance a range of characteristics for a given bioactive, including; increased protection and stability (Nair et al., 2010) and suitability to increase bioavailability by non-parenteral routes of administration including oral, pulmonary and topical applications (Helson, 2013).

The natural polymer chitosan (Cs) is a mucopolysaccharide, closely related to cellulose and obtained by deacetylation of the compound chitin, predominantly found in the exoskeletons of crustaceans (Nagpal et al., 2010). Cs has been used for the development and formulation of nanoparticles by ionotropic gelation due to its physicochemical and biological beneficial properties (Mohammed et al., 2017; H. Zhang et al., 2015). Benefits include improved

adherence to mucosal surfaces, increased drug residence time (Ryan et al., 2012), and protection of the bioactive drug from intestinal proteases (Amaro et al., 2015; Ryan et al., 2013). In acidic medium, Cs can be dissolved, due to protonation of the amine residues present in the polymer backbone. Ionotropic gelation allows for the formation of NPs from Cs via crosslinking with oppositely-charged electrolytes under mild conditions in which amino acids and peptides will remain reasonably stable (Chen et al., 2013; Janes et al., 2001; Wang et al., 2011).

Zein a GRAS approved prolamine-rich protein derived from maize, has been used in the formulation and coating of peptide oral delivery systems (Y. Zhang et al., 2015), to increase encapsulation efficiency (Luo and Wang, 2014) and improve the control of gastric release of labile bioactives (Luo et al., 2010; Paliwal and Palakurthi, 2014). By exploiting the physical interactions between protein and polysaccharide (in this instance zein and Cs), it is possible to improve and broaden the physical and chemical stability properties of the NP delivery systems (Benshitrit et al., 2012). However, the formulation, characterisation and development of these multi-component systems can be more challenging than single component systems and as such, it is important to comprehensively optimise the formulation process. To the best of our knowledge, there are currently no reports which describe the formulation of biological Se species such as SeMet into a NP delivery system. The potential optimisation of this formulation could be significant, given that SeMet more effectively increases human and animal selenium levels and is less toxic than inorganic Se (Garousi, 2015).

In situations where several variables may influence system properties, a useful technique to identify the relationships between a given response and independent variables (or factors) and optimise the system, is Response Surface Methodology (RSM) (Anderson and Whitcomb,

2005). RSM is a more efficient approach to experimentation than one factor at a time (OFAT) experiments since it: 1) reduces the number of experimental runs typically required to gather the same information as OFAT, thus reducing resource requirements, 2) is useful in detecting interdependencies of variables that would not be typically identified during OFAT experiments and 3) improves the prediction of a response through use of gathered information from a larger parameter space. One of the most commonly applied RSM designs for process optimisation with a minimal experimental requirement is the Box-Benhken design (BBD), an independent quadratic design in which factor combinations are considered at 3 levels; the midpoints of edges of the process space and the centre (Traynor et al., 2013; Zolgharnein et al., 2013). After polynomial models for each of the different responses in a study have been completed, a desirability function may be constructed in order to estimate minima or maxima, provided such optima are within the design space (Bezerra et al., 2008).

In this study, SeMet was formulated into nanoparticles consisting of Cs and zein using ionotropic gelation. After evaluating the main variables which affect encapsulation efficiency, particle size and drug loading, a systematic approach (RSM) was used to optimise the formulation of nanoparticles suitable for oral delivery. A three-level, three-factor BBD was utilised to build polynomial models for the three responses and a desirability function was then constructed to optimise the system. Optimised SeMet NPs were prepared based on the predicted optimum levels of the independent variables of the factorial design. To ensure stability of the optimised formulation after lyophilisation, a cryoprotectant (trehalose) was also included (Danish et al., 2017a). The physicochemical properties, storage stability, cytotoxicity, and the release profile in a simulated intestinal buffer were assessed.

2 MATERIALS AND METHODS

2.1 Materials

The chitosan **ultrapure** PROTASAN™ UP (CL113, Mw=110-150kDa, DDA=85%, Endotoxins ≤ 100 EU/gram, Heavy metals ≤ 40 ppm) was purchased from NovaMatrix, FMC Corporations, Norway. DL-selenomethionine, D(+)-Trehalose dihydrate, and zein, of $\geq 99\%$ purity, were obtained from ACROS Organics™, Fisher Scientific, Ireland. Ultra-pure water $18\text{m}\Omega\text{cm}^{-1}$ was obtained from a Millipore simplicity 185 model instrument, UK, and was used for all aqueous solution preparations throughout. Sodium Tripolyphosphate (TPP) of **technical grade (85%)**, and all other reagents, chemicals and solvents were of analytical grade from Sigma Aldrich, Ireland.

2.2 Optimisation of nanoparticle formulation physicochemical properties

A BBD was used to optimise the formulation and EE% of SeMet into the nanoparticle. **Selected target physicochemical properties for oral delivery for the NPs were particle size of approximately 300 nm, PDI < 0.5 and ZP > 30 mV (des Rieux et al., 2006).** A three level, three factor BBD (Maleki Dizaj et al., 2015; Zhao et al., 2013) of 15 random order experiments was designed using Minitab™ 17 (Pennsylvania, USA). The 3 independent variables were, (X_1) the pH of the Cs solvent - the isoelectric point (pI) of SeMet, (pH-pI) (X_2), the load concentration of SeMet and (X_3) the ratio of Cs:TPP, while (Y_1) Particle size, (Y_2) PDI, (Y_3) ZP and (Y_4) EE% were the dependent variables. The variable ranges (Table 1) were based on an exploratory study.

Each dependant variable was independently assessed by linear regression using a 2nd degree polynomial model with 1st order interactions (Eq. 1).

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + \varepsilon$$

(Eq. 1)

where Y_i is the measure of the response associated with each factor level combination, b_0 is an intercept, b_1 - b_{33} are the regression coefficients, X_1 - X_3 are the coded independent variables and the X_iX_j and X_i^2 ($i, j = 1, 2, 3$) denote the interactive and quadratic terms, respectively. The linear regression and the significance ($p < 0.05$) test of independent variables and their interactions was assessed by statistical software (Minitab™, 17) to generate regression models. Through bidirectional elimination (testing at each step for variables to be included or excluded), non-significant terms were removed from the model in order to calculate regression equations with significant terms only (Wang et al., 2013). Desirability functions to optimise all responses were built the weighted geometric mean of individual desirabilities presented in Table 1.

Table 1: Variables and levels employed in the BBD with desirability function for optimisation of nanoparticle formulation.

Factor (Independent variables)	Levels used, (Actual coded)		
X_1 = pH of the Cs solvent – the pI of SeMet (pH- pI)	0.5	1.5	2.5
X_2 = SeMet concentration (mg/mL)	0.05	0.15	0.25
X_3 = ratio of Cs:TPP	4:1	6:1	8:1
Dependent Variables	Composite desirability		
Y_1 = Size (nm)	Target of 300nm		
Y_2 = PDI	Minimise		
Y_3 = ZP (mV)	Maximise		
Y_4 = Encapsulation efficiency (EE%)	Maximise		

Response surface plots, statistical testing of the linear models and identification of optimum formulations *via* feasibility and grid searches was performed to study the optimal area (Barrentine, 1999). Finally, repetitions ($N=4$) of the optimal point found were conducted experimentally to validate the study.

2.3 Preparation of SeMet loaded Cs:TPP nanoparticles

SeMet-entrapped NPs were produced using a modified ionic gelation method (Calvo et al., 1997). Briefly, Cs was dissolved in buffered pH medium (3, 4 or 5 pH) at a concentration of 3 mg/mL and filtered through a 0.22 µm syringe filter (Millex Millipore, UK) to remove undissolved Cs. A known amount of SeMet was then added to the Cs solution prior to crosslinking to obtain a final load concentration 0.05, 0.150 or 0.250 mg/mL. TPP was added dropwise to the solution under stirring at 700 rpm and room temperature to yield final mass ratios of Cs:TPP NPs of 4:1, 6:1 and 8:1. All of these experimental parameters (pH, concentrations and ratios) were prepared according to the BBD design. The NP suspension was stirred at 700 rpm for 30 min at room temperature for further crosslinking. After stabilisation, NPs were then transferred to a 30 kDa molecular weight cut off (Vivaspin 20, Sartorius) centrifugal filter and isolated by centrifugation at 3000 rpm for 30 min. Filtered H₂O (equivalent in volume to the recovered supernatant) was then added to the isolated NPs and sonicated at 35 % amplitude for 30 s with 5 s pulse intervals. Physicochemical properties of the NPs were then determined as per section 2.4, using a Malvern Zetasizer NanoZS (Worcestershire, UK) and the supernatant was retained for EE% determination as outlined in section 2.7. The optimised formulation has a mass ratio 6:1 (Cs:TPP), Cs media (pH 5), and a final SeMet load concentration of 0.15 mg/mL.

2.3.1 Increase of Ionisation/Protonation states of NP components during ionic gelation to increase EE% - Formulation I

After optimising the general physicochemical properties via BBD (section 2.2), the ionic gelation component preparation procedure was modified with the aim of increasing the EE %. Formulation I was produced as described in section 2.3 with one exception; SeMet and TPP were dissolved and diluted with NaOH (0.01 M) prior to crosslinking. The rationale for the pH adjustment was to induce higher electrostatic interactions (i.e. maximise the cationic

component of Cs and the anionic component of SeMet) between SeMet and Cs during the crosslinking process.

2.3.2 Coating NPs with zein to increase EE%

NPs were prepared as per formulation I (ratio 6:1 (Cs:TPP), Cs media (pH 3), TPP/SeMet NaOH solution (pH 11) and a final load concentration of 0.15 mg/mL), with the following modifications; after the NPs had stabilised, 8 mL of absolute EtOH were added dropwise to the formulation whilst the stirring speed of the solution was maintained at 700 rpm for 30 min at room temperature. Zein (10 mg/mL dissolved in 80 % EtOH and filtered) was added dropwise to yield zein:Cs mass ratios of 0.5:1, 1:1 and 2:1, stabilised at 700 rpm for 30 min and isolated as per section 2.3. The NP formulations were then concentrated under vacuum (175 mbar) at 40 °C until EtOH was completely removed. To ensure stability of the optimised formulation after lyophilisation, 10 mL of the cryoprotectant trehalose 5 % w/v in H₂O was added to each formulation and lyophilised for 36 hr (Danish et al., 2017a).

2.4 Nanoparticle Characterisation: Particle size, PDI and surface charge

Freshly prepared NP solutions were used for physicochemical analysis (Luo et al., 2010). The mean particle size and PDI of the NP formulations were determined by dynamic light scattering (DLS). The ZP values were measured with the use of laser doppler velocimetry (LDV). Both DLS and LDV analysis were performed in triplicate at 25 °C with a Zetasizer Nano series Nano-ZS ZEN3600 fitted with a 633 nm laser (Malvern Instruments Ltd., UK), using a folded capillary cuvette (Folded capillary cell-DTS1060, Malvern, UK). The values presented herein were acquired from three separate experiments, each of which included three replicates; N=3.

2.5 Scanning electron microscopy (SEM)

NP morphology was evaluated by scanning electron microscopy (SEM) (Hitachi, SU6600 FESEM, USA), at an accelerating voltage of 20 kV, unless otherwise stated, using the secondary electron detector. The fresh NP solutions were then spin coated onto Si wafers, dried at room temperature and then sputter coated with 4 nm Au/Pd prior to imaging (Mukhopadhyay et al., 2013).

2.6 Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of CL113, TPP, Cs:TPP NPs, SeMet and SeMet loaded Cs:TPP NPs were acquired via a Spotlight 400 series spectrometer (Perkin Elmer, USA), using the attenuated total reflectance spectroscopy method (ATR-FTIR), in the range of 650-4000 cm^{-1} . Prior to analysis, NP samples were lyophilised using a FreeZone 6 L bench top freeze dry system (Labconco, USA) at $-40\text{ }^{\circ}\text{C}$ for 20 hr. The dried solids were then placed on the ATR crystal prism (ZnSe), and 32 scans were acquired at 4 cm^{-1} resolution with background subtraction using the empty sample holder (Vongchan et al., 2011).

2.7 EE% of SeMet in Cs:TPP nanoparticles

The EE% of SeMet in the NPs was determined by the separation and quantification of SeMet left in the supernatant. This was performed by ultracentrifugation at 3000 rpm, $4\text{ }^{\circ}\text{C}$ for 30 min. SeMet in the supernatant was quantified by reverse phase high performance liquid chromatography (RP-HPLC), as previously described (Ward et al., 2012) with the following modifications. Samples were analysed with a Waters 2998 HPLC and Photodiode Array Detector, (Waters, USA), using a Poroshell 120, EC-C8 column, 3.0 x 100 mm, 2.7 μm , (Agilent Technologies, UK). Isocratic elution was carried out at a flow rate of 0.4 mL/min, column temperature $45.0 \pm 5.0\text{ }^{\circ}\text{C}$ with a mobile phase of water/methanol/trifluoroacetic acid

(97.9:2.0:0.1). Samples were monitored according to their UV absorbance at 218 nm. The encapsulation efficiency was calculated by Eq. 2 (Xu and Du, 2003);

$$EE\% = \frac{\text{Total amount of Se Met} - \text{Free amount of SeMet}}{\text{Total amount of SeMet}} \times 100$$

(Eq. 2)

2.8 MTS assay

The potential cytotoxicity of pure SeMet, SeMet loaded NPs and unloaded NPs (coated with zein) were examined on Caco-2 human epithelial cells, and HepG2 human liver hepatocellular cells. Both cell lines are routinely employed to assess the potential toxicity of orally delivered compounds (Brayden et al., 2015; Gleeson et al., 2015). Caco-2 and HepG2 cells, were seeded at a density of 2×10^4 cells/well and cultured on 96 well plates in Dulbecco's Modified Eagle Medium (DMEM) and Eagle's Minimum Essential Medium (EMEM) respectively, supplemented with 10 % foetal bovine serum, 1 % L-glutamine, 1 % penicillin-streptomycin and 1 % non-essential amino acids at 37 °C in a humidified incubator with 5 % CO₂ and 95 % O₂. Time points were selected with the intention to mimic *in vivo* conditions for each cell type. As the maximum time NPs will be exposed to the intestine, a 4 hr exposure time was used in Caco-2 cell lines (Neves et al., 2016), to mimic the liver, a 72-h exposure time was used for HepG2 cell lines (Brayden et al., 2014). Triton X-100™ (0.05%) was used as a positive control. The concentrations of the test compounds applied were 25, 50 and 100 µM. After exposure, treatments were removed and replaced with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Optical density (OD) was measured at 490 nm using a microplate reader (TECAN GENios, Grodig, Austria). Each value presented was normalised against untreated control and calculated from three separate experiments, each of which included six replicates.

2.9 Accelerated stability analysis

NPs were suspended at a concentration of 0.1 mg/mL, in aqueous KCl solution (10 mM) and stored at accelerated conditions; 60 °C for 720 min, 70 °C for 300 min and 80 °C for 120 min (Danish et al., 2017b). The particle size, PDI and ZP were measured using the Nanosizer ZS (Malvern Instruments Ltd, UK) over time intervals to determine the degree of degradation. The generated data was then analysed via R software (R Core Team, 2016). The temperature dependence of the kinetic parameters of SeMet-loaded NPs stability was measured by calculating the observed rate constants. This was plotted in an Arrhenius representation and apparent activation energy, E_a and reaction rate constant, k_{ref} were calculated according to Eq. 3;

$$P = P_o + e^{\ln(k) - \frac{E_a}{R}(\frac{1}{T} - \frac{1}{T_{ref}})} t \quad (\text{Eq.3})$$

where P is the property (particle size, PDI or ZP) at time t, P_o is the initial property conditions, k is the apparent zero order reaction constant, E_a is the energy of activation, R is the universal gas constant, T is the temperature of the experiment in Kelvin (K) and T_{ref} is the reference temperature (343 K).

2.10 *In vitro* controlled release studies

SeMet release from the NPs was carried out using a dialysis bag diffusion technique (Hosseinzadeh et al., 2012) over 6 hr (Calderon L. et al., 2013; Yoon et al., 2014). Freeze dried SeMet loaded NPs were suspended in 5 mL H₂O and sonicated at 35 % amplitude for 30 s with 5 s intervals and placed into a Float-A-Lyzer[®]G2 dialysis membrane with a pore size of 25 kDa (Spectrum Laboratories, USA). The sample was placed into 40 mL of simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) specified according to the British Pharmacopoeia (Pharmacopoeia, 2016). SGF was composed of 0.1 M HCL and SIF

was composed of 1 volume of 0.2 M trisodium phosphate dodecahydrate and 3 volumes of 0.1 M HCL (adjusted to pH 6.8), without enzymes (British Pharmacopoeia Commission, 2016). Samples were placed in a thermostatic shaker at 37 °C and agitated at 100 rpm. At predetermined time points, 1 mL of release fluid was analysed and replaced with simulated fluid to maintain sink conditions.

SeMet release was measured by RP-HPLC (section 2.7). Eq. (4) was used to determine the % drug release;

$$Drug_{rel} \% = \frac{C(t)}{C(l)} * 100$$

(Eq. 4)

where $Drug_{rel}$ is the percentage of SeMet released, $C(l)$ represents the concentration of drug loaded and $C(t)$ represents the amount of drug released at time t, respectively.

3 RESULTS AND DISCUSSION

3.1 Response Surface Modelling – Box Behnken design

The observed values for all 15 experiments described by the BBD yielded minimum and maximum values for Size (Y_1) (152, 318 nm), PDI (Y_2) (0.218, 0.554), ZP (Y_3) (26.0, 42.7 mV) and EE% (Y_4) of, (24.7, 41.4 %). The reduced models resulting from the analysis are presented in table 2. Response Y_1 showed no significant terms in the model, suggesting that there was no evidence in the sample population that could prove the association of the independent variables with particle size. This finding is unsurprising, as the NPs produced in this study fell within a narrow target region (152, 318 nm) and the regions of interest chosen

331 in this study (e.g. Cs:TPP 4:1-8:1, pH 3-5), to produce the Cs NPs, have been well
332 established for yielding particle sizes within 100-400 nm range (Hassani et al., 2015;
333 Mohammed et al., 2017; Sipoli et al., 2015).
334
335 Responses Y_2 - Y_4 (PDI, ZP and EE% respectively) all showed a curvature with regards to X_1 -
336 X_3 , as quadratic or interactive effects of some independent variables were statistically
337 significant in all the models.
338

Table 2: Coded variable estimated coefficients (Coef) with associated standard error (SE Coef.) and uncoded reduced regression equations for Y_2 - Y_4 .

Y ₂ (PDI)			Y ₃ (ZP)			Y ₄ (EE%)		
Term	Coef	SE Coef	Term	Coef	SE Coef	Term	Coef	SE Coef
Constant	0.30833	0.00630	Constant	35.534	0.293	Constant	31.731	0.814
X ₁ (pH-pI)	0.05150	0.00386	X ₁ (pH-pI)	6.405	0.300	X ₁ (pH-pI)	6.113	0.599
X ₂ (SeMet (mg/mL))	0.00838	0.00386	X ₂ (SeMet (mg/mL))	-	-	X ₂ (SeMet (mg/mL))	-	-
X ₃ (Ratio of Cs:TPP)	0.05213	0.00386	X ₃ (Ratio of Cs:TPP)	1.245	0.300	X ₃ (Ratio of Cs:TPP)	0.487	0.599
X ₁ *X ₁	-0.06129	0.00568	X ₁ *X ₁	-	-	X ₁ *X ₁	3.821	0.879
X ₂ *X ₂	0.10396	0.00568	X ₂ *X ₂	-	-	X ₂ *X ₂	-	-
X ₃ *X ₃	0.06446	0.00568	X ₃ *X ₃	-2.626	0.419	X ₃ *X ₃	-3.529	0.879
X ₁ *X ₂	-0.01750	0.00545	X ₁ *X ₂	-	-	X ₁ *X ₂	-	-
X ₁ *X ₃	0.01650	0.00545	X ₁ *X ₃	-	-	X ₁ *X ₃	-	-
X ₂ *X ₃	-0.04025	0.00545	X ₂ *X ₃	-	-	X ₂ *X ₃	-	-
Lack of fit	0.27		Lack of fit	0.09		Lack of fit	0.62	
R²adjusted	98.66%		R²adjusted	98.65%		R²adjusted	90.82%	
Eq. (5)	Y ₂ = 0.592 + 0.212 X ₁ - 1.565 X ₂ - 0.1495 X ₃ - 0.06 X ₁ *X ₁ + 10.40 X ₂ *X ₂ + 0.01611 X ₃ *X ₃ - 0.175 X ₁ *X ₂ + 0.00825 X ₁ *X ₃ - 0.2012 X ₂ *X ₃		Eq. (6)	Y ₃ = -1.21 + 6.405 X ₁ + 8.49 X ₃ - 0.6588 X ₃ *X ₃		Eq. (7)	Y ₄ = -2.06 + 5.35 X ₁ + 10.83 X ₃ + 3.821 X ₁ *X ₁ - 0.882 X ₃ *X ₃	

In table 2 (Eq. 5), PDI was mostly affected by the quadratic effects of $X_2^*X_2$, which showed a positive correlation such that, at -1 and +1 levels of X_2 , the PDI increased. It is also worth noting that the linear term of X_2 showed a negative coefficient, indicating that X_2 has a negative effect on PDI until a turning point is reached, whereafter $X_2^*X_2$ has a positive impact on PDI. Similar findings were reported by Masarudin *et al.*, (2015), who found that ratios of Cs:TPP less than 3:1 and greater than 12:1, resulted in a significant increase of the formed NPs PDI values (>0.75). Additionally, they found that the NPs produced at ratios between (median levels) the aforementioned upper and lower bounds resulted in NPs with PDI values ranging from 0.15-0.32. A contour plot based on the regression model is presented in Figure 1(A) and highlights this, showing that, at medium load concentrations of 0.15 mg/mL and medium/low ratios of Cs:TPP (4.75:1 to 5.5:1), a minimum value for PDI can be achieved. Conversely, as the concentration of TPP passes median levels PDI increases, which may be attributable to TPP inducing cross-linking between the nanoparticles and thus the

presence of smaller aggregates within the solution (Antoniou et al., 2015a; Hu et al., 2008). Furthermore, the reduction of PDI as the concentration of TPP approaches median levels, has been shown to relate with the increased availability of TPP molecules to interact with the free amino groups of chitosan, thus allowing for additional incorporation of the anion within the nanoparticle chitosan chains (Huang and Lapitsky, 2017). Although PDI can be minimised at these particular levels, it is worth noting that the PDI values for all formulations fell within optimum values for oral delivery (Wong et al., 2017).

The results presented in table 2 (Eq.6) show that ZP was not affected by X_2 , although it was affected by X_1 (pH-pI) and X_3 (Cs:TPP), indicating that the pH of the formulation medium and the electrostatic forces between the ionizable groups of Cs and TPP determine the net charge of the produced particles. It is likely that this is a direct consequence of the binding between the anionic phosphate groups of TPP with the positively charged amino acid moieties of Cs (Rampino et al., 2013), for example, a positive correlation was observed, such that ZP increased with increasing ratios. In addition, X_1 also showed a prominent effect on ZP, whereby an increase in ZP is observed as X_1 increases. The contour plot representing the reduced regression model (Figure 1(B)), demonstrates that low X_1 levels (pH 5 - 4.2) and low ratios of Cs:TPP (4.0/4.5:1) are the areas where NPs of ZP <30mV were obtained (with a minimum of 27 mV). As X_1 increases (approximately pH 3.5), an increment in ZP was observed and maximum values of >39 mV are achieved between X_3 (ratio) of 4.5:1 - 7.5:1. This is in agreement with previous studies by Antoniou *et al.*, (2015), that showed at Cs: TPP mass ratios of 7:1, the ZP of the produced NPs decreased almost linearly with increasing pH of the formulation medium. As it has been shown that high ZP (either negative or positive), requires higher energy for bringing two particles in contact with each other (Dora et al., 2010), this pH-responsive behaviour can be attributed to the protonation of the primary amino

groups present on the Cs chain, resulting in an increase of electron density and repulsion forces between the crosslinked Cs chains (Lai and Guo, 2011). For example, at pH 3.5, the charge interaction between these two molecules becomes strong enough, and stable Cs NPs are obtained. On the contrary, at pH approaching the pI of Cs, reduced availability of protonated amine residues ($-\text{NH}_3^+$) present on the Cs polymer backbone chain results in a lower surface charge of the formed NPs (Huang et al., 2015).

EE% was not affected by load concentration (X_2) but was mostly affected by the pH of the Cs media (pH-pI (X_1)) (table 2 (Eq.7)), demonstrating the notable effect of SeMet loading with the charge of both polyelectrolytes (Cs and TPP) and their subsequent interaction during the crosslinking process. As can be seen, a negative correlation was observed, indicating that, as X_1 increases, the EE% decreases, most likely attributable to the reduced protonation of the primary amines present on Cs (Umerska et al., 2014). It is also worth noting that the quadratic term $X_1 * X_1$ showed a positive coefficient, indicating X_1 had a negative effect on EE% until a turning point was reached, where after X_1 had a positive impact on EE%. He *et al.*, (2017), reported that by exploiting the ionic nature of insulin through modulation of the formulation media, the EE% of insulin into Cs:TPP NPs could be significantly enhanced, going from 37 to 94 %. This is evident in the contour plot produced by the regression model (Figure 1(C)) showing that, at maximum X_1 levels (approximately 2.5 unit distance from pI of SeMet) and minimum to median/high ratios of Cs:TPP (4:1 - 7.5:1), maximum EE % (>40 %) can be achieved, indicating that pH plays a significant role in the EE%. These findings agree with those of other research groups (Antoniou et al., 2015b; Janes et al., 2001), that reported a strategy to increase ionisable proteins (such as bovine serum albumin (BSA) and insulin) EE% (>80%) within a Cs NP matrix, by dissolving the load protein at a pH above its isoelectric point. By doing this, deprotonation of the hydroxy groups present on the load

cargo occurs, inducing a predominantly negative state and thus has a higher affinity to Cs and increased EE%. Several other studies have mirrored these findings, showing that the electrostatic interactions between the acidic groups present on insulin and the amino groups of Cs play a role in the association of insulin to the Cs-NPs by mediation of the ionic interaction between both macromolecules (Mattu et al., 2013; Pan et al., 2002).

Figure 1: Contour Plot of (A) PDI against ratio of Cs:TPP (X_3) and SeMet (mg/mL) (X_2), with pH-pI (X_1) held at median level (1.5), (B) ZP against pH-pI (X_1) and the ratio of Cs:TPP (X_3) and (C) EE% against pH-pI (X_1) vs ratio of Cs:TPP (X_3) for the RSM models presented in Table 2.

3.2 Optimisation

Employing the models constructed with the BBD evaluation in Table 2, response optimisation was employed in order to establish a formulation strategy to yield NPs with minimum PDI values, ZP of ≥ 30 mV and maximum EE% (des Rieux et al., 2006). As the range of NP sizes within all experimental runs fell within recommended target values for oral delivery, this response was excluded from the optimisation analysis.

Figure 2 shows the optimisation plot of the desired responses and indicates that, when the variable settings of X_1 , X_2 and X_3 are fixed at 2.5 (pH-pI), 0.15 (mg/mL) and 6 (Cs:TPP), respectively, NPs with the desired properties can be produced. Additionally, the 95 % confidence interval ranges of the predicted NP properties that these conditions would produce are presented in Table 3.

Figure 2: Desirability profiles for optimisation of the formulation parameters; X_1 (pH-pI), X_2 (load concentration) and X_3 (Ratio of Cs:TPP) - maximising ZP and EE%, whilst minimising PDI.

Table 3: 95% confidence interval for particle characteristics that optimal conditions would produce under the present experimental conditions of uncertainty

Response	Fit	95 % CI	Actual values
Y ₂ (PDI)	0.299 ± 0.007	(0.282, 0.317)	0.284 ± 0.044
Y ₃ (mV)	42.2 ± 2.0	(41.6, 42.7)	39.7 ± 2.6
Y ₄ (EE%)	41.7 ± 1.0	(39.5, 43.8)	39 ± 3

To verify the validity of the proposed models, n = 4 replicates of the optimised formulation were prepared, and each experimental response was compared with the predicted one. Table 3 shows the validation results of the model, whereby NPs presented sizes of 187 ± 58 nm, PDI of 0.284 ± 0.044 , ZP of 39.7 ± 2.6 mV and a max EE % of 39 ± 3 %. No statistical significant values ($p > 0.05$) for predicted and measured responses (Y₂-Y₄) were observed, indicating that the models fit the data satisfactorily and has adequate precision for the prediction of NP ZP, PDI and EE% in the chosen space of independent variables (in the domain of levels chosen for the independent variables). Additionally, several consuming and laborious laboratory studies were eliminated in this study by using the BBD, rather than a OFAT approach.

3.3 EE% optimisation I: protonation and ionisation

The results from the BBD were promising in terms of the NPs produced and their physicochemical properties. However, even when optimised, EE% remained low at approximately 40 %. A second study was undertaken with the aim of maximising the EE%, by controlling the ionisation process, given that this property primarily influenced the encapsulation efficiency in the desirability profile (Figure 2). In the previous BBD, it was noted that the highest EE% of SeMet within Cs:TPP NPs was obtained when the pH of the Cs medium was maintained at pH 3 and the SeMet load was dissolved within it prior to crosslinking. The fundamentals of ionotropic gelation exploit the opposing charges between

the protonated amine residues found on the glucosamine residue unit of Cs against the deprotonated hydroxyl groups on TPP (Janes et al., 2001). In order to increase the EE%, a modulation of the ionisation state of SeMet was achieved by dissolving it (and TPP) in a basic solution prior to crosslinking with Cs. The rationale for this approach is the observation that the pKa for the acid moiety of SeMet is pH 2.6, whereas the pKa for the basic moiety is at pH 8.9 (Foulkes, 2003) and as such, further electrostatic interactions can be induced at time of complexation (Qi et al., 2010). The effect of dissolving the TPP and SeMet in 0.01 M NaOH (pH 12) rather than H₂O prior to crosslinking in the optimised formulation (ratio Cs:TPP 6:1, SeMet load concentration of 0.15 mg/mL and Cs medium pH of 3) was investigated, as a means to elicit greater interaction between Cs and SeMet and subsequently increase EE%. N= 3.

By altering the pH of the formulation, the EE% increased from 39 ± 3 % to 66 ± 1 %, whilst the physicochemical properties of the NPs remained within the target range for oral delivery (Table 4) (des Rieux *et al.*, 2006). This was most likely attributed to the electrostatic interactions between the now further ionised TPP and SeMet groups and protonated amine residues found on the glucosamine unit of Cs. This is consistent with the work by Pan et al., (2014) which showed curcumin (CUR) successful encapsulation (>90%) into casein NPs, through deprotonation of CUR at pH 12, enabling for its re-association and thus subsequent encapsulation into the NP matrix.

3.4 Improved Encapsulation Efficiency using zein

The results from the first EE% optimisation study (Table 4) were promising, in terms of increasing EE% in addition to maintaining the desired NP physicochemical properties.

478 Nevertheless, the maximum EE% obtained by modifying the pH remained at 66 ± 4 %.

479 Therefore, a subsequent step, involving the coating of the optimised NPs with zein (at

480 varying zein:Cs mass ratios), was pursued as a means to increase the encapsulation efficiency

481 of SeMet into the Cs: TPP NPs.

482

483 As shown in Table 4, as the ratio of zein in the formulation increased, the EE% improved. At

484 0.5:1, zein:Cs, the average EE% achieved was 75 % and approximately 5 % increments in

485 EE% were observed with subsequent increments of zein:Cs. The physicochemical properties

486 of the NPs (PDI and ZP) were still within the target range for oral delivery for formulations

487 with zein:Cs ratios $\leq 1:1$, although an increase in NP size was observed upon increasing zein

488 concentrations. At a ratio of 2:1, zein:Cs, the physicochemical properties of the NPs were less

489 than ideal, as large particle sizes (721 nm average) and high PDI values (0.783) were yielded,

490 most likely as a consequence of a denser and thicker coating (Luo et al., 2010) provided by

491 the partial deposition of the negatively charged protein on the particle surface, thus reducing

492 the total net charge and inducing particle swelling (Rampino et al., 2013). For example, as the

493 zein concentration increases, so does the viscosity of the dispersion, which can affect the

494 nucleation process leading to the production of larger sized NPs (Zhong and Jin, 2009).

495 Similar results have been observed by others, whereby, an increase in zein concentration led

496 to an increase in particle size of 6,7-dihydroxycoumarin loaded zein NPs (Podaralla and

497 Perumal, 2012) or alpha-tocopherol loaded zein NPs, stabilised with Cs (Luo et al., 2012).

498 Lastly, the ZP of this formulation was the lowest observed (average of + 6 mV), which may

499 be attributed to the increasing zein concentration, causing increased masking of the free

500 positively charged amino groups of Cs (Krauland and Alonso, 2007). It is also possible that

501 agglomeration occurred as a result of the reduced electrostatic repulsion between the NPs in

502 suspension (Liu and Gao, 2009).

Table 4: Physiochemical results for SeMet loaded NPs (Ratio 6:1, SeMet in NaOH (0.15 mg/mL load), Cs in pH 3) coated with zein. Size, PDI, ZP and EE% are presented for each NP using different mass ratio combinations of zein and Cs. N=3.

Zein:Cs	Size (nm)	PDI	ZP (mV)	EE (%)
0:1	227 ± 17	0.448 ± 0.049	32 ± 1	66 ± 4
0.5:1	319 ± 19	0.221 ± 0.040	27 ± 6	74 ± 1
1:1	377 ± 47	0.325 ± 0.136	35 ± 6	81 ± 1
2:1	721 ± 108	0.783 ± 0.281	6 ± 4	85 ± 1

3.5 Characterisation of SeMet loaded and unloaded Cs:TPP NPs with and without zein coating - Fourier transform infrared spectroscopy (FTIR)

Figure 3 shows the FTIR spectra of (A) zein, (B) TPP, (C) Cs, (D) SeMet:Cs:TPP NPs and (E) SeMet:Cs:TPP:zein NPs. The zein spectrum (Figure 3, A) shows characteristic peaks at 3289, 2929, 1644, 1515 and 1233 cm^{-1} corresponding to; NH stretching vibrations, CH stretching, amide I (C=O stretch), amide II (C-N and C-N-H/ in plane bending) and amide III respectively (Podaralla and Perumal, 2012). Characteristic peaks for the phosphate ion (P=O) in TPP (Figure 3, B) were observed at 1121 cm^{-1} and 886 cm^{-1} , respectively (Rampino et al., 2013). Cs spectra (Figure 3, C) showed characteristic peaks at 3240, 2879, 1625, 1514, 1376 and 1063 cm^{-1} . corresponding to NH Stretch / OH in pyranose ring, CH₂ in CH₂OH group, C=O in NHCOCH₃ group (amide I), NH₂ in NHCOCH₃ group (amide II), CH₃ in NHCOCH₃ group and C-O-C (glycosidic linkage) respectively (Luo et al., 2010; Mohammed et al., 2013).

Figure 3: FTIR spectra of (A) zein, (B) TPP, (C) Cs, (D) SeMet:Cs:TPP NPs and (E) SeMet:Cs:TPP:zein NPs. Spectra are offset for clarity.

525 The FTIR spectrum of SeMet:Cs:TPP NPs (Figure 3, D) is different to that of the Cs matrix,
526 as a result of intermolecular interactions of the constituent components. If interaction
527 between the Cs and TPP had occurred, it will lead to frequency shifts or splitting in
528 absorption peaks (Gan et al., 2005). For example, the peaks at 1514 cm^{-1} in the Cs spectrum
529 have been shifted to 1560 cm^{-1} , indicating electrostatic interaction between the phosphate
530 groups of TPP and the amino groups present in the Cs NP matrix (Papadimitriou et al., 2008).
531 Additionally, the broadening and the increased absorbance of the peak at 3272 cm^{-1} indicate
532 hydrogen bonding has been enhanced (Luo et al., 2010). The band observed at 1644 cm^{-1} in
533 the zein FTIR spectrum (assigned to N-H bond), has been shifted to 1648 cm^{-1} in the zein/Cs
534 NPs spectrum (Figure 3, A and E respectively), indicating an interaction among the zein and
535 Cs chains, possibly through hydrogen bonding among the amino groups present on both Cs
536 and zein chains (Müller et al., 2011). Another indication of Cs:zein interaction arises from the
537 amide III bands at 1406 cm^{-1} , not visible in the zein spectrum, but now visible in the zein/Cs
538 NPs (Figure 3, A and E respectively) spectrum, most likely as a result of the C-N stretching
539 and out of plane N-H deformation being highly sensitive to structural changes (Sessa et al.,
540 2008).

541

542 Lastly, the formation of zein/Cs NPs (E) is also characterised by the appearance of a band at
543 1041 cm^{-1} (assigned to C-O-C (glycosidic linkage)), which was observed in Cs/SeMet NPs
544 (D) at 1028 cm^{-1} but not observed in the zein spectrum (A), supporting the presence of Cs
545 within the NP matrix (Figure 3) (Müller et al., 2011). The crosslinked Cs also showed a peak
546 for P = O at 1151 cm^{-1} (Bhumkar and Pokharkar, 2006), further indicating electrostatic
547 interaction between Cs and TPP (Figure 3, E). No significant SeMet peaks in either
548 Cs/TPP/SeMet or Cs/TPP/SeMet/zein NPs spectra were observed, most likely due to the fact,

that only 0.07 % of the dried formulation is contributed by SeMet and it is consequently masked by the other formulation components.

3.6 Scanning electron microscopy

Figure 4 shows the SEM images of uncoated and zein coated Se loaded NPs. The particle size of the NPs after spin coating was in good agreement with the DLS measurements taken on the freshly prepared NPs. Spherical, well distributed particles for both coated and uncoated SeMet loaded NPs were observed. However, it is interesting to note that the zein coated NPs displayed a smoother surface than that of the uncoated. This may be attributed to the use of the acetic acid buffer used during the formulation process, as similar results have been observed in spin-cast zein films prepared from an aqueous ethanol solvent compared to an acetic acid solution, indicating that a rough and hydrophilic surface was acquired for the former, whilst the zein film produced from an acetic acid solution appeared to be smooth, featureless and more hydrophobic (Shi et al., 2009; Y. Zhang et al., 2015).

Figure 4: SEM image of (A) SeMet:Cs:TPP NPs and (B) SeMet:Cs:TPP NPs coated with zein

3.7 Accelerated stability analysis of SeMet-loaded NPs coated with zein

The principle aim of accelerated stability testing is to provide reasonable assurance that a pharmaceutical or food consumable will remain at an acceptable level of quality throughout its timespan in the market place (Bajaj et al., 2012; Waterman and Adami, 2005). Real-time, retained sample, cyclic temperature and acceleration, are the four categories into which stability testing procedures fall (Bhagyashree et al., 2015). In the latter, the product is subjected to elevated temperatures and/or humidity well above ambient values, to determine the temperature at which product failure (i.e. degradation) will occur.

573

574 The Arrhenius equation, upon which the interpretation of accelerated stability testing is
575 based, allows for the determination of the activation energy and consequently, the
576 degradation rate of a product at lower temperatures (i.e. ambient, refrigerated etc.). In this
577 instance, the data acquired can then be used to project the shelf life of the product in a much
578 shorter time than that of real time assessments (Ali et al., 2013; Bhagyashree et al., 2015).
579 This is a beneficial approach to stability testing, as it results in a greatly reduced product
580 development schedule.

581 Figure 5 shows the kinetic behaviour of the NP properties; size (A), PDI (B) and ZP (C) at
582 temperatures ranging from 60-80 °C. The stability of the NPs decreased with increasing
583 temperature. Little change was detected for all properties at 60 °C, over the course of 720
584 min, whereas a more pronounced increment in size and PDI and a decrease in ZP was
585 observed at 70 °C after 300 min. At 80 °C, destabilisation of the NP complexes was evident
586 across all properties, whereby size increased from approximately 300 nm to > 800 nm, PDI
587 from approximately 0.2 to >0.9 and ZP reduced from approximately 32 mV to < 18 mV,
588 indicating that aggregation of the NPs had occurred (Wu et al., 2011).

589

590 **Figure 5: (1) Particle size, (2) PDI and (3) ZP analysis of SeMet loaded NPs exposed to**
591 **(a) 80 °C, (b) 70 °C and (c) 60 °C, over time periods of 120, 300 and 720 min,**
592 **respectively. N=3**

593 The one-step nonlinear regression analysis of the kinetic experiments shows that particle size
594 and PDI fit to a zero-order kinetic behaviour, with an Arrhenius dependence of $\ln(k_{\text{ref}}@70$
595 $^{\circ}\text{C}) = 1.66 \pm 9.44 \text{ min}^{-1}$ and $E_a = 452.57 \pm 570.59 \text{ kJ/mol}$ for size, and a $\ln(k_{\text{ref}}@70^{\circ}\text{C}) =$
596 $0.029 \pm 0.014 \text{ min}^{-1}$, and an $E_a = 182.31 \pm 42.64 \text{ kJ/mol}$ for PDI respectively. In terms of ZP,

an apparent first order mechanism fits the data better than that of an apparent zero order model, with an Arrhenius dependence of $\ln(k_{\text{ref}}@70\text{ }^{\circ}\text{C}) = 0.038 \pm 0.010\text{ min}^{-1}$ and $E_a = 205.71 \pm 25.65\text{ kJ/mol}$. Additionally, as can be seen in figure 6, a linear correlation is evident between $1/T$ and $\ln k$, indicating that the formulations will be stable under normal storage conditions. This was expected, as previous reports have shown that zein coatings can increase the colloidal stability of iron phosphate NPs (Van Leeuwen et al., 2014) and, when stabilised with Cs, results in high thermal resistance of the NPs over prolonged periods of time (Luo et al., 2013).

Figure 6: Arrhenius plots for the (A) ZP, (B) PDI and (C) size accelerated studies of SeMet loaded NPs. N=3.

3.8 Cytotoxicity assessment of SeMet-loaded NPs

The potential cytotoxicity of SeMet in its native form and unloaded or SeMet loaded NPs coated with zein, at different test concentrations (25, 50 and 100 μM), were examined on Caco-2 human epithelial cells, and HepG2 human liver hepatocellular cells, using the MTS assay. As the NPs will become exposed to the intestinal epithelia following oral delivery (leading to its facilitated transport and uptake), time points were selected with the intention to mimic *in vivo* conditions for each cell type, to assess the potential cytotoxicity of the formulated NPs (Gleeson et al., 2015; Brayden et al., 2015). Caco-2 cells were exposed for 4h (figure 7(A)) and HepG2 cells for 72h (figure 7(B)). In Caco-2 exposures, no cytotoxicity was observed for unloaded or SeMet loaded NPs, in comparison to the negative control, across all tested concentrations. For HepG2 exposures, no cytotoxicity was observed for unloaded or SeMet loaded NPs coated with zein. Additionally, the lower concentrations (25 and 50 μM) of native SeMet showed no cytotoxicity to either cell line, whereas a reduction in

HepG2 cell viability (figure 7(B)) for native SeMet, at the 100 μ M test concentration, was observed (approx. 66% cell viability).

Similar results were observed by Takahashi, Suzuki and Ogra, 2017, whereby SeMet showed no significant change on the viability of Caco-2 cell lines, although it did show marginal toxicity to HepG2 cells at concentrations > 80 μ g/mL after prolonged exposure (48 hr). This finding is also in agreement with other works that showed SeMet toxicity occurred at concentrations $\geq$ 40 μ M in various hepatoma cell lines (Kajander et al., 1991). It has previously been shown that Cs nanoparticles can enhance the delivery of inorganic Se compounds whilst reducing its toxicity (C. Zhang et al., 2015). In this work, SeMet loaded NPs elicited no significant reduction in viability of either cell line at equivalent concentration (100 μ M), indicating that, by encapsulating SeMet within the Cs NP matrix, the cytotoxic effects of pure SeMet, can be reduced.

Figure 7: Cytotoxicity assessment of SeMet, unloaded NPs with zein coating and SeMet loaded NPs with zein coating, exposed for (a) 4h in Caco-2 cell lines and (b) 72h in HepG2 cell line at SeMet equivalent concentrations (25 μ M, 50 μ M and 100 μ M). TritonTM X-100 (0.05%) was used as positive control and percentage (%) of MTS converted was compared to untreated control. 1-Way ANOVA with Dunnetts's post-test *** $P < 0.001$, ** $P < 0.01$, Each value presented was normalised against untreated control and calculated from three separate experiments, each of which included six replicates. N=3

3.9 *In vitro* release studies

In vitro techniques are advantageous for modelling potential interactions between NPs and the *in vivo* environment of the GI tract. Simulated gastric fluid and membrane analysis models enable assessment of *in vivo* environments without the use of human cell lines (Gamboa and Leong, 2013). Figure 8, shows the cumulative release profile of SeMet loaded NPs coated with zein, after subjection to 2 hr in an SGF environment (pH 1.2) representative of the stomach, followed by a compartmental change to SIF (pH 6.8), representative of the intestine, for 4 hr. As can be seen, 25 ± 1 % of SeMet was released after 2 hr in SGF, followed by 33 ± 3 % in SIF for 4 hr. No significant difference in the release profile of SeMet loaded NPs without zein coating was observed (data not shown). However, it was necessary to keep zein in the formulation due to the increase in EE (≥ 80 %).

The target site of absorption of SeMet is the jejunum, in the small intestine. Therefore, it is important to withstand the acidic environment of the stomach. Three basic mechanisms that are typically applied to describe the release of drugs from polymeric particles, are swelling/erosion, diffusion, and degradation (Liechty et al., 2010). In this work, the total cumulative release of SeMet from the zein coated NPs, after 6 hr in simulated gastrointestinal tract environments, was 58 %, indicating that degradation of the NP was slow and thus the mechanism may be diffusion/relaxation oriented. As such, the release kinetics of SeMet NPs, under the SGF and SIF sequential controlled release experiments, were fitted using the following diffusive models derived from swellable systems (Siepmann and Peppas, 2011; Danish et al., 2017a).

For the SGF:

$$\frac{M_t}{M_\infty} = ks_1 * (\sqrt{time}) + ks_2 * time \quad (Eq.8)$$

where M_t is the diffused mass at a given time, M_∞ is the asymptotic diffused mass at infinite time, and ks_1 and ks_2 are the diffusive and relaxation rate constants respectively.

For the SIF:

$$\frac{M_t}{M_\infty} - \frac{M_{120}}{M_\infty} = ki_1 * (\sqrt{time - 120}) + ki_2 * (time - 120) \quad (Eq.9)$$

where M_{120} is the predicted diffused mass at the time of changing from SGF to SIF (120 min), ki_1 and ki_2 are diffusive and relaxation rate constants.

Table 5: Swellable model parameters for kinetic release studies SeMet NPs. ks represents the stomach compartment and ki the intestinal compartment, divided into diffusion and relaxation mechanisms (i_1 and i_2). All parameters listed were statistically significant, $*P < 0.001$; $*P < 0.05$.**

Parameters	Estimate	Std. Error	t-value	Significance code
Ks_2	0.17082	0.04092	4.175	***
Ki_1	0.69145	0.33863	2.042	*
Ki_2	0.10817	0.02105	5.140	***
R^2_{adj}			0.984	

Table 5 presents the fitted values for the rate constants in SGF (ks) and SIF (ki) for SeMet NPs, with ks_1 , representing a diffusion mechanism and ks_2 a relaxation mechanism (Eq. 8, 9). In terms of the stomach compartment (SGF, pH 1.2), no statistically significant ks_1 parameter was found, indicating that the primary mechanism for release in the stomach was via

relaxation, i.e. slower release, approaching zero-order kinetics. After 2 hr subjected to the SGF environment, a compartmental change was employed to mimic the movement of the NPs to the intestinal environment (SIF, pH 6.8), whereupon a combination of diffusion (k_{i1}) and relaxation (k_{i2}) mechanisms were observed ($p < 0.05$). Overall, the model employed (Eq. 8, 9) predicted the experimental data well, with an $R^2_{adj} > 0.98$. These results were expected, as polysaccharides generally undergo solvent penetration, swelling and chain disentanglement and relaxation, resulting in their ultimate dissolution (Fu and Kao, 2010). Additionally, this result is in agreement with previous studies, reporting a diffusion and zero order kinetic profile for IPP and LKP loaded CsNPs, coated with zein (Danish *et al.*, 2017a) and that of other researchers, who observed that zein proved to be a good coating for NPs, whereby, the stronger the interaction of the load material (in this instance phenolic monoterpenes) with that of the wall material (zein) was evidenced by its controlled release over time (da Rosa *et al.*, 2015).

Figure 8: Release kinetics of SeMet NPs coated with zein after 2 hr in SGF (pH 1.2) and 4 hr in SIF (pH 6.8).

4 CONCLUSION

In this study, SeMet-loaded Cs NPs were produced via ionotropic gelation. BBD was used to identify optimum formulation variables that would result in NPs with physicochemical properties thought to be suitable for oral delivery. BBD highlighted the optimum conditions for NP production, although EE% remained relatively low. By varying the formulation media pH, increased electrostatic interaction between the crosslinking polyelectrolytes and drug were achieved, resulting in an increase in EE %. Coating the NPs at a 1:1 mass ratio of Cs:zein, resulted in NPs with a doubled EE accompanied by an increase in diameter. These

NPs were then characterised *via* FTIR analysis, which identified the presence of key functional groups of the native components and identifying shifts in the crosslinked matrixes. SEM analysis showed that spherical, well distributed particles were observed. MTS cytotoxicity studies showed no decrease in cellular viability in either Caco-2 or HepG2 cell after 4 and 72 hr exposures, respectively. Accelerated thermal stability of the loaded NPs indicated good stability under normal storage conditions. Lastly, after 6 hr exposure to simulated intestinal buffers, the release profile of the formulation showed that $\leq 60\%$ of the drug had been released. These findings infer that encapsulation of SeMet into a NP delivery system comprising food-derived components reveals an oral administration approach for this molecule.

Funding:

This work was supported by Department of Agriculture, Food and Marine under FIRM (Food Institutional Research Measure). Project Ref: 13F510.

Conflict of interest:

All authors have approved the final manuscript, and the authors declare that they have no conflicts of interest to disclose.

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1055 **Graphical abstract:**

The above graphic depicts the formulation methodology used to produce selenomethionine loaded chitosan nanoparticles, coated with zein, for *in vitro* assessment. Briefly, to produce the nanoparticles, chitosan was protonated by dissolving in acidic buffer (pH 3), then crosslinked with ionised tripolyphosphate and selenomethionine in NaOH (0.1 M). Zein coating was then employed to coat the nanoparticles and purification was achieved by removing unencapsulated formulation components through ultracentrifugation.

Figure 1: Contour Plot of (A) PDI against ratio of Cs:TPP (X_3) and SeMet (mg/mL) (X_2), with pH-pI (X_1) held at median level (1.5), (B) ZP against pH-pI (X_1) and the ratio

of Cs:TPP (X_3) and (C) EE% against pH-pI (X_1) vs ratio of Cs:TPP (X_3) for the RSM models presented in Table 2.

Figure 2: Desirability profiles for optimisation of the formulation parameters; X_1 (pH-pI), X_2 (load concentration) and X_3 (Ratio of Cs:TPP) - maximising ZP and EE%, whilst minimising PDI.

Figure 3: FTIR spectra of (A) zein, (B) TPP, (C) Cs, (D) SeMet:Cs:TPP NPs and (E) SeMet:Cs:TPP:zein NPs. Spectra are offset for clarity.

Figure 4: SEM image of (A) SeMet:Cs:TPP NPs and (B) SeMet:Cs:TPP NPs coated with zein

Figure 5: (1) Particle size, (2) PDI and (3) ZP analysis of SeMet loaded NPs exposed to (a) 80 °C, (b) 70 °C and (c) 60 °C, over time periods of 120, 300 and 720 min, respectively. N=3

Figure 6: Arrhenius plots for the (A) ZP, (B) PDI and (C) size accelerated studies of SeMet loaded NPs. N=3.

Figure 7: Cytotoxicity assessment of SeMet, unloaded NPs with zein coating and SeMet loaded NPs with zein coating, exposed for (a) 4h in Caco-2 cell lines and (b) 72h in HepG2 cell line at SeMet equivalent concentrations (25 uM, 50 uM and 100 uM). Triton™ X-100 (0.05%) was used as positive control and percentage (%) of MTS converted was compared to untreated control. 1-Way ANOVA with Dunnetts's post-test * P< 0.001, ** P< 0.01, Each value presented was normalised against untreated control and calculated from three separate experiments, each of which included six replicates. N=3**

Figure 8: Release kinetics of SeMet NPs coated with zein after 2 hr in SGF (pH 1.2) and 4 hr in SIF (pH 6.8).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

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1119 **Figure 8**