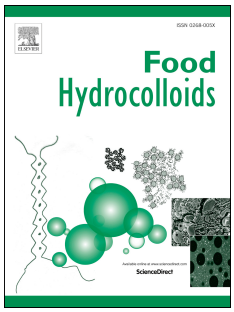

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Layer by Layer Self-Assembly for Coating a Nanosuspension to Modify Drug Release and Stability for Oral delivery

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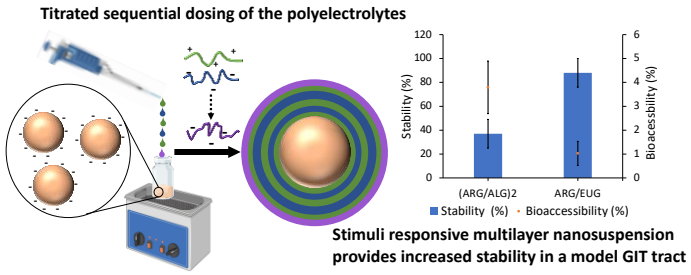
Graphical Abstract



Abstract

Layer-by-layer (LbL) modification is an effective way to tune the properties of particles. However, the traditional LbL process involves repeated washing steps which are not compatible with nanoparticles with a partial solubility. In this work, we demonstrate the use

of a titration method for producing LbL coatings onto a nanosuspension of curcumin (a compound with a limited aqueous solubility). The aim of the work was to show how LbL can be used to enhance the release behaviour and stability of the curcumin in a nanosuspension form. Coated nanosuspension samples were produced using biocompatible and biodegradable polyelectrolytes, poly-L-arginine, and sodium alginate and was sizes approximately 500 nm. A stimuli-responsive nanosuspension was prepared by coating the nanosuspension with 5 layers of poly-L-arginine and alginate and Eudragit L100 as an outmost layer. The *in vitro* release of these nanosuspensions revealed that the use of a pH-responsive layer (Eudragit L100) as an outermost shell resulted in delay the release of curcumin (5 %) in acidic pH and facilities its release in neutral pH (11%) over 72 h. Additionally, the bioaccessibility study showed that increasing the number of layers resulted in increasing the stability curcumin in nanosuspension when exposed to conditions that mimic the gastrointestinal tract from 20 % for uncoated nanosuspension to 40 % and 90 % for 4-layered coated nanosuspension and stimuli-responsive (6-layered) coated nanosuspension, respectively. The cytotoxicity of LbL-coating of nanosuspension revealed a reduction in the toxicity of curcumin. This work shows how a titrated LbL modification approach could be used to tailor the stability and the release behaviour of nanosuspensions for oral drug delivery applications.

Keyword: Layer by Layer self-assembly, Titration method, Curcumin, Nanosuspension, LbL-coated nanosuspension, Stimuli-responsive nanosuspension.

Introduction

Oral drug delivery has gained much interest to treat many chronic diseases, because these diseases required a long duration of treatment and a good medical adherence (Belali et al., 2019). However, there are some obstacles facing a drug during its passage through the gastrointestinal tract (GIT) before reaching its target side via oral administration. One of the main obstacles for any orally administrated drug is the harsh stomach environment such as acidic pH (1-2) (Lu et al., 2016). An appealing approach to overcome these obstacles for oral drug delivery is the development of nanomedicine or nanoscale drug delivery systems. The design of nanomedicines provides the opportunity to tailor the properties of nanoparticles to

be potentially provide a systemic enhancement, protection, targeting, controlled and sustained drug release. During the last few years, 51 nanomedicine products has been approved by FDA with many other nanomedicine products in clinical trials (Bobo et al., 2016).

Many drugs and active pharmaceutical ingredients (APIs) are poorly water soluble, and this property can limit the oral availability of the API (Khan et al., 2022). One attractive approach to address bioavailability issues in poorly water soluble APIs is to formulate the API as a nanosuspension (also known as solid drug nanoparticles), a nanoscale dispersion of the API in the solid form (Rabinow, 2004). The nanoparticles of the nanosuspension provide an increase in the specific surface area of the API which facilitates accelerated dissolution and ultimately enhances bioavailability (Giardiello et al., 2016; Guo et al., 2019; McDonald et al., 2014). Additionally, nanosuspensions are being increasingly explored for use as long-acting injectables, in which they provide a concentrated depot of the drug that slowly dissolves. Such systems offer the opportunity to avoid patient adherence issues by removing the requirement for repeated oral dosing (Ferretti & Boffito, 2018; Flexner et al., 2021; Hobson et al., 2021; Town et al., 2017, 2019; Williams et al., 2015).

Curcumin is a poorly water-soluble compound that is the principle active ingredient in turmeric, and it is responsible for its yellow colour, physiochemical properties, and biological activities. Several studies showed that curcumin possesses an anticancer effect on different cancers such as colon cancer, breast cancer, lung cancer and prostate cancer (Lee et al., 2013). Curcumin is also a useful model compound for investigating the release of poorly soluble APIs. We have previously shown that curcumin can be formulated into nanosuspension to enhance its physicochemical properties. The resultant nanosuspension exhibited an improved chemical stability and sustained drug release (Elbaz et al., 2021a).

The ability to tune and tailor the properties of nanosuspensions would be a valuable tool in the nanomedicine toolbox. Modifying the surface of the nanoparticles within a nanosuspension would allow the dissolution of the API to be controlled, the API to be protected and potentially alter the biological interactions with cells. Layer-by-layer self-assembly is an appealing technique for assembling of polyelectrolytes multi-layered shells into a charged core, driven by electrostatic interactions (Yan et al., 2014). Based on the nature

of polyelectrolytes, the architecture of the charged polyelectrolytes shells and the number of layers, the coated nanoparticle can exhibit different properties such as pH or temperature-responsive behaviour (Agarwal et al., 2008; Souto et al., 2013). However, the traditional LbL self-assembly involves intermediate washing steps to remove the excess polyelectrolytes after each layer deposition. These intermediate washings are time consuming, strongly limiting the yield as it caused particles and drug losses and are less adaptable to common manufacturing process (Santos et al., 2019a). An approach to circumvent these drawbacks is by using a titration method, an alternative approach, where LbL coating is obtained by the alternated deposition of polycations and polyanions to charged nanoparticles without using intermediate washings (Bantchev et al., 2009). Due to the versatility of LbL coating on the nanoparticles surfaces and its simplicity, a titration method could be considered as an enforceable technology for large-scale production (Santos et al., 2015, 2019a).

Herein, we demonstrated that a curcumin nanosuspension can be modified with a LbL titration method to produce a coated nanosuspension (Figure 1A). The LbL-coated curcumin nanosuspension was designed using underlying layers of poly-L-arginine (ARG) and sodium alginate (ALG) with different number of layers to study the impact of varying the number of layers on the stability and release behaviour. A stimuli-responsive drug delivery system was also produced with ARG, ALG and Eudragit L100 (EUD) (Figure 1B). All developed formulations were fully characterised including z-average diameter, polydispersity index (PDI), zeta potential (surface charge), morphology, colloidal stability, *in-vitro* release studies, *in-vitro* bioaccessibility, and *in vitro* cytotoxicity studies.

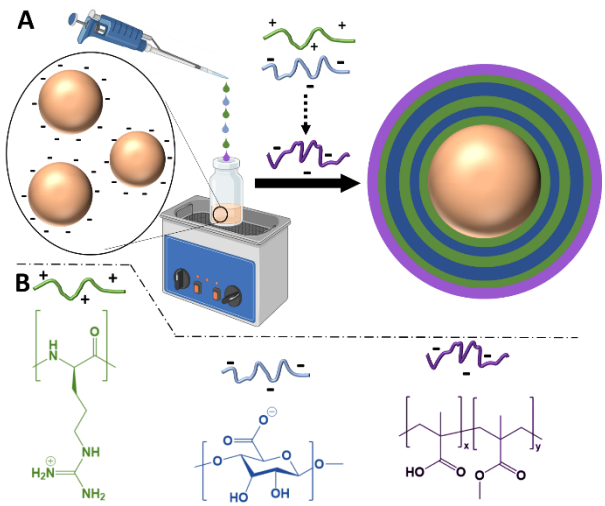


Figure 1. Preparation of stimuli-responsive nanosuspension by the titration method using oppositely charged polyelectrolytes. A) stimuli-responsive nanosuspension were also prepared using poly-L-arginine (ARG) and sodium alginate (ALG), and Eudragit L100 (EUD L100) as an outmost shell using titration method, a washless approach with no intermediate washing following the layer additions. B) The chemical structures of the polyelectrolytes used in the work.

Materials and Methods

Materials

Curcumin, Polyethylene glycol (Mw 10,000), Poly-L-arginine hydrochloride (M_w >70,000), sodium alginate (from brown algae, medium viscosity), ethanol, phosphate buffered saline (PBS), pepsin, mucin, pancreatin, bile extract, calcium chloride, potassium chloride, potassium acetate, aluminium nitrate, glacial acetic acid, and dialysis tube (cellulose membrane, cut off 20,000 MW) were purchased from Sigma-Aldrich. Hydrochloric acid, sodium chloride, acetonitrile, ethanol, and dichloromethane were purchased from thermo-fisher scientific. Eudragit L100 (EUD L100) was obtained as a gift from Evonik Industries, Germany. All chemicals were used without further purification. Deionised water was used in all experiments.

Methods

Emulsion-templated freeze drying for preparing curcumin nanosuspension

Curcumin nanosuspension was prepared at concentration of 10 mg/mL in a mixture solution of dichloromethane and ethanol with a v/v ratio 9:1 as mentioned in the earlier study (Elbaz et al., 2021b). Briefly, aqueous stock solutions of various excipients were prepared with concentration of 22.5 mg/mL. 400 µl of the excipient was added to 100 µl of curcumin solution (water: oil ratio 4:1 v/v). The mixture was emulsified with a Covaris S2x for 15 seconds and then the samples were cryogenically frozen with liquid nitrogen followed by lyophilisation using Virtis benchtop K freeze dryer for 48 hours. The samples were sealed immediately until analysis.

Titration method for coating curcumin nanosuspension

Curcumin nanosuspension were coated with multilayers using titration method described in a previous study,(Santos et al., 2019b) instead of traditional LbL self-assembly technique. Polyelectrolytes of concentration 0.1 % w/v were used. Both polyelectrolytes were dissolved in sodium chloride concentration of 0.01 M and the pH was adjusted to 6. In this process, nanosuspension was re-dispersed in 1 mL deionized water followed by the addition of a certain volume of poly-L-arginine and then sonication for 30 seconds. Then, sodium alginate was added dropwise and then sonicated for 30 seconds. The addition of polyelectrolytes was repeated until 4-layered capsules were formed.

Plotting titration curve

By using concentration of polyelectrolytes (0.1 %) and salts (0.01 M), different volumes of polyelectrolytes were added to nanosuspension to a plot a titration curve. A polyelectrolyte concentration of 0.1 %, salt concentration of 0.01 M and pH 6 were used. 1 mg of nanosuspension was dispersed in 1 mL deionized water, different volumes (10, 20, 30, 40, and 50 µl) of poly-L-arginine was then added and continuously sonicated for 30 seconds. Zeta potential was monitored after the addition of each volume followed by the addition of sodium alginate with different volumes along with continuous monitoring of the zeta potential. After

plotting the titration curve for first and second layers, same steps were repeated to plot a titration curve for third and fourth layers.

Study the effect of varying polyelectrolytes concentration on the formation of LbL-coated nanosuspension

A series of experiments were carried out using different concentrations of poly-L-arginine and sodium alginate. The preparation took place as follows: various concentrations 0.1, 0.2, 0.4 and 0.6 % w/v of poly-L-arginine and sodium alginate were dissolved in 0.01 M NaCl, and pH was adjusted to 6. After dispersing 1 mg of nanosuspension in 1 mL deionized water, 20 µl of poly-L-arginine was then added and continuously sonicated for 30 seconds. Then, an individual volume of sodium alginate solution was added and continuously sonicated for 30 seconds.

Study the effect of varying NaCl concentration on the formation of LbL-coated nanosuspension

The concentration of NaCl was then varied to identify its effect on the nanosuspension z-average diameter and polydispersity index. A range of experiments were carried out using different concentrations of NaCl (0.05, 0.01, and 0 M) along with using polyelectrolytes concentration of 0.1 %, and pH 6. 0.1 % of both polyelectrolytes were prepared in each concentration (0.05, 0.01, and 0 M) of NaCl.

Preparation of poly-L-arginine/Alginate (LbL) nanosuspension

Based on the pervious experiments, the optimum conditions showed the smallest z-average diameter and narrowest PDI were chosen were: 0.1% for polyelectrolyte concentration, 0.01 M for NaCl concentration, volume of polyelectrolytes were 20 µl for poly-L-arginine and 30 µl for sodium alginate. Therefore, the preparation of poly-L-arginine/alginate modified nanosuspension was carried using these optimum experimental parameters. Poly-L-arginine and sodium alginate were dissolved in 0.01 M NaCl, and pH was adjusted to 6. 1 mg of nanosuspension was re-dispersed in 1 mL of deionized water followed by adding 20 µl of poly-L-arginine, and sonicating for 30 seconds. Afterwards, 30 µl of sodium alginate, and sonicated for 30 seconds. The layer deposition steps were repeated until forming 4-layered capsules. The resultant sample were named as $(ARG/ALG)_2$.

Preparation of stimuli responsive (LbL) nanosuspension

By using the same experimental parameters used for preparing pol-L-arginine/alginate modified nanosuspension, stimuli-responsive LbL nanosuspension was prepared using the following polyelectrolytes: Eudragit L100, sodium alginate, and poly-L-arginine. The nanosuspension sample with sizes 200 nm were used and coated with 5-layers were made of poly-L-arginine/sodium alginate as mentioned above. Finally, 30 µl Eudragit L100 (0.1%w/v) dissolved in 0.01M NaCl (pH 7) was added and sonicated for 30 seconds. The resultant sample were named as (ARG/EUG).

Stability study

The stability of 4-layered modified nanosuspension $(ARG/ALG)_2$ and stimuli-responsive nanosuspension (ARG/EUG) samples was carried out in water under dark conditions. 1 mL of each LbL-modified nanosuspension sample (1 mg/mL) was dispersed in 1 mL deionized water to give a final concentration of 1 mg/mL The z-average diameter and PDI of the samples measured by DLS at day 0, 1 2, 3, 4, 7, 14, and 21. All the samples were vortexed before taking the measurements.

In vitro release study

In vitro release studies were carried out using dialysis tubes in three pH values that mimic stomach (0.1 M HCl, pH 1.2) and small intestine (phosphate buffer pH 7.4) under dark conditions. 1 mL of each sample, curcumin nanosuspension (1mg/mL), 4-layered modified nanosuspension $(ARG/ALG)_2$ (0.9 mg/mL), and stimuli-responsive nanosuspension (ARG/EUG) (0.87 mg/mL), were placed in dialysis tube. The release study was performed individually for each pH value as follows: the dialysis tube containing the sample were placed in the release media and then shaken in shaking incubator at 37 °C under mild mixing at 100 rpm. Following the addition of the dialysis tube in the release media, 1 ml of the release media was withdrawn at fixed time interval and replaced by 1 mL of a freshly prepared release media1260 HPLC (Agilent Technologies, Santa Clara, CA, USA) was used to determine the concentration of drug in the release media. The concentration of the released drug was measured using 1260 HPLC according to the following equation (1):

Cumulative drug release (%)= $\frac{Amount\ of\ drug\ \ in\ release\ medium}{Initial\ amount\ of\ drug\ loaded\ into\ nanocapsules} \times 100$ (1)

In vitro model for bioaccessibility

The *in-vitro* bioaccessibility of curcumin was evaluated by passing curcumin nanosuspension, 4-layered modified $(ARG/ALG)_2$ nanosuspension and stimuli-responsive (ARG/EUD) nanosuspension through a stimulated gastrointestinal tract (GIT) consist of mouth, stomach and small intestine (Mao & McClements, 2012). Briefly, 1 mg of each sample dispersed in 1 mL deionised water was mixed with 1 mL of the stimulated saliva fluid and shaken for 10 minutes at 37°C. 2 mL of stimulated gastric fluid and shaken at 100 rpm for 2 hours. Then, the pH of the samples was adjusted to pH 7 using 2 M sodium hydroxide solution followed by adding 4mL of small intestinal fluids. After passing through the stimulated fluids, the release media were collected and centrifuged at 1200 g for 40 minutes at 4°C. The resulting supernatant was collected and assumed to be the amount of curcumin solubilised in bioaccessible form. This solubilised curcumin was diluted with acetonitrile and determined by 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV-vis detector. The stability and bioaccessibility were determined using the following equation (2) and (3):

$$Stability\ \ (\%)\ = \frac{CR}{CI} x100 \text{ (2)}$$

$$Bioaccessibility\ (\%) = \frac{CN}{CI} x100\ (3)$$

CR is the concentration of curcumin in the release media after exposure to the stimulated GIF (assuming that there is no degradation occur in the stimulated GIT), CN is the concertation of curcumin in the nanoparticles, and CI is the initial concentration of curcumin in the system. The initial concentration of curcumin was assumed to be the total amount of curcumin that would be present in the small intestine if there were no losses due to the chemical degradation. It is important to note that this simple *in-vitro* GIT model cannot accurately stimulate the complex process takes place in the gastrointestinal tract, but it is a useful way for rapid screening different samples and identify their physicochemical properties.

In vitro cytotoxicity MTT assay

The cytotoxic effects of 4-layered $(ARG/ALG)_2$ nanosuspension and stimuli-responsive multi-layered nanosuspension (ARG/EUG) at the concentrations of 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µM were investigated on HT-29 and Caco-2 cells using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates at a density of 10^4 cells/well and incubated for 48 hours with various concentrations of all formulations. Following 48 hours treatment, the media were discarded, and the cells were washed and stained with 0.4% MTT for 30 minutes. Following incubation, the MTT solution was discarded, and the plates were washed with 1% acetic acid. The optical density (OD) of each well was measured at 540 and 630 nm with ELISA microplate reader. The experiment was performed in triplicate and the percentages of cell viability were determined using the following Equation.

$$Cell\ viability\ (\%) = \frac{OD\ sample - OD\ blank}{OD\ control}\ x\ 100 \quad (4)$$

Statistical Analysis

GraphPad Prism software version 9 was used for data handling and statistical analyses. The data were presented as mean ± SD, unpaired t- test was used to compare the data.

Characterisation techniques

Scanning Electron Microscope (SEM)

The morphology of all formulations was characterized with Tescan FIB SEM S8000G. For Tescan FIB SEM S8000G, the samples were imaged in ultra-high vacuum at 5 kV. All SEM samples were prepared as follows: carbon tape was deposited on the aluminium stub followed by adding a glass slide. Then, each sample of nanosuspension were dispersed in 1 mL deionized water and a drop of each sample was added on the cover slide and subsequently dried in air. Afterwards, the samples were coated with gold layer. The coating was performed by Quorum Q150T ES using gold as a target with thickness 5-10 nm and current 10 mA for 30 seconds.

Dynamic Light Spectroscopy (DLS)

The z-average diameter and zeta potential of curcumin nanosuspension and LbL-coated nanosuspension was measured using dynamic light scattering (DLS, Malvern Zetasizer ZS instrument). Each sample of were dispersed in 1 mL water where the sizes of each sample was tested in triplets at 25 °C and the results averaged. These samples were dispersed in water and each measurement was done using disposable polystyrene cuvettes. The zeta potential measurement was performed with Malvern Zetasizer Nano ZS by adding 1 mL of each sample in a disposable folded capillary cell using automatic measurement optimisation. The layer deposition was monitored with DLS and Zeta potential measurement. Both DLS and Zeta potential measurements used Malvern Zetasizer software version 7.11 for analysis.

High performance Liquid chromatography (HPLC)

HPLC was used to quantify the amount of curcumin released from the coated nanosuspension, accordingly the cumulative release percentage was determined. All the samples were dissolved in acetonitrile with ratio 1:1. A 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with pump and an ultraviolet-visible spectroscopy detector at 425 nm was used to plot a calibration curve for curcumin and determine the drug release percentage. The mobile Phase was a mixture of 0.1% formic acid and acetonitrile at ratio 1:1 and the flow rate were 1 mL min^{-1}. A ZORBAX C18 column (Stablebond Analytical, C18 4.6 x 250 mm) was used, and the injection volume was 20 µl. (further details about the HPLC protocol see the supporting information).

Results and Discussion

Using the titration method to produce layer-by-layer coatings on nanosuspensions

Unlike traditional LbL self-assembly, the titration method is a washless approach where there are no purification steps or intermediate washings between the layer depositions. In the titration method, the deposition of polyelectrolytes layers was carried out in equilibrium by the assessment of the required amount of polyelectrolyte for each layer of the multi-layered shell. This required amount of polyelectrolyte was determined by a polyelectrolyte titration curve in which the zeta potential was measured with increasing volumes of polyelectrolyte

added (figure 2). The complete adsorption of polyelectrolytes was indicated by the curve plateau and is known as the adsorption saturation point, where the adsorbed layer of polyelectrolytes remained constant due to the surface charge saturation. In figure 2A, the titration of nanosuspension (1 mg/mL) with poly-L-arginine (ARG) reached the plateau at 20 µg (equivalent to 20 µl), where the initial negative charge of curcumin nanosuspension become positive and reached saturation. This confirmed the complete deposition of poly-L-arginine layer and thus conferred colloidal stability. Sodium alginate (ALG) was then added to nanosuspension-ARG (Figure 2B) and resulted in a charge reversal from positive to negative values. The highest zeta potential was achieved at 30 µg of sodium alginate (equivalent to 30 µl). Afterwards, poly-L-arginine was added to 2-layered coated nanosuspension and the plateau has started again at 20 µg (Figure 2C) followed by the addition of sodium alginate to 3-layered coated nanosuspension resulting in the charge reversal from positive to negative and the plateau started at 30 µg (Figure 2D). Following the formation of the first four layers, the titration procedures of the subsequent layers were straightforward since it was possible to predict the volume and quantity of polyelectrolytes required for each layer without any additional adjustments. Therefore, 20 µg for poly-L-arginine and 30 µg for sodium alginate were selected and used as the optimal concentration of polyelectrolytes to obtain a complete surface coverage and forming multi-layered shell.

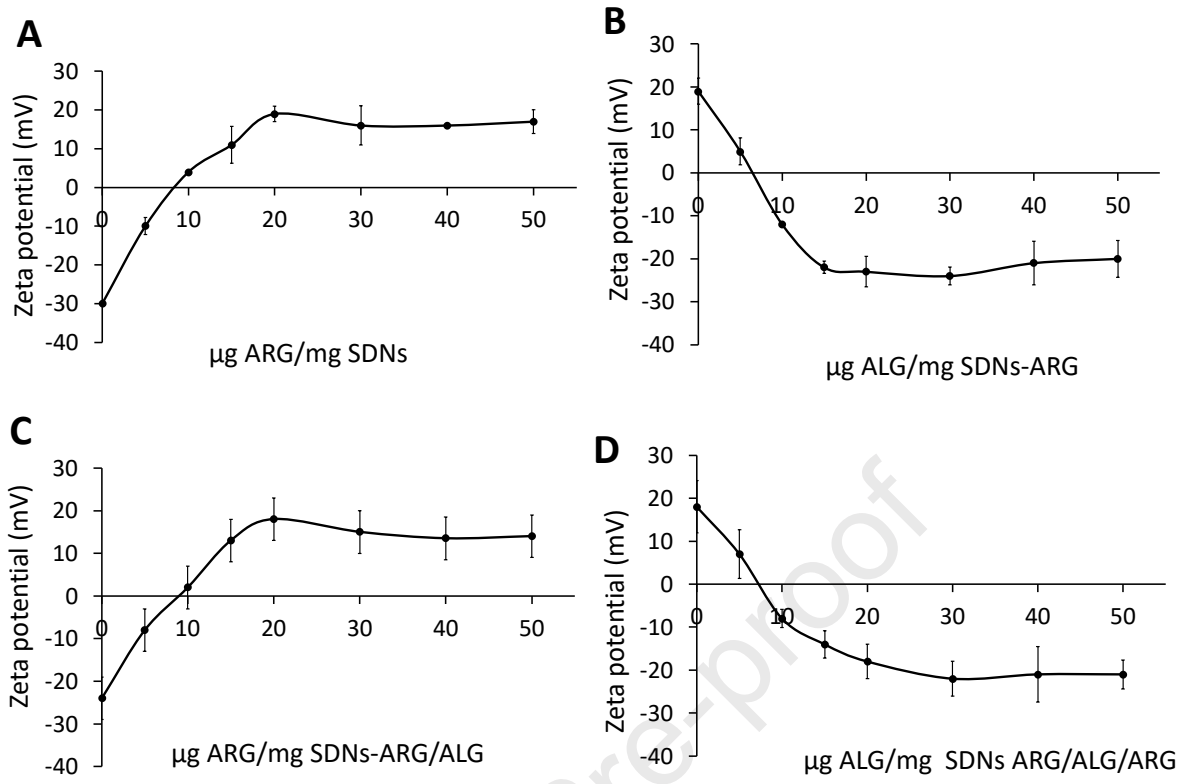


Figure 2. Titration curve for zeta potential against polyelectrolyte solution volume for nanosuspension dispersed in 1 mL deionised water (1 mg/mL) during titration process. Where the salt solution concentration (0.01 M) and polyelectrolyte solutions concentrations (0.1% w/v) were constant. A-C showed the poly-L-arginine concentration (20 µg) which required to achieve the adsorption saturation point, a complete surface coverage. B-D showed the sodium alginate concentration (30 µg) which required to achieve the adsorption saturation point.

The concentration of NaCl and polyelectrolytes used during LbL can have a significant effect on the formation of the layers and the colloidal stability of the nanosuspension. We used concentrations of 0.01 M and 0.1% w/v for the salt and polyelectrolyte respectively as identified from the work of Santos *et al* (Santos et al., 2019a). However, in order to check that these concentrations were appropriate for our samples, we investigated three different salt concentrations and three different polyelectrolyte concentrations and identified that concentrations of 0.01 M and 0.1 % w/v for the salt and polyelectrolyte respectively did indeed result in coated nanoparticles with the lowest polydispersity index (PDI) (see

supporting information figure S2). Using the optimised conditions including the salt concentration, polyelectrolyte concentration, and polyelectrolyte volume, 4-layers of alternating poly-L-arginine and sodium alginate and this sample will be referred to as $(ARG/ALG)_2$. The zeta potential, z-average diameter and PDI (figure S3) were monitored during layer coating to guarantee the successful layer deposition and the narrow size distribution of the coated nanosuspension.

To further demonstrate the viability of the titration method, the nanoparticles in the curcumin nanosuspension were coated with 4-layers of poly-L-arginine and sodium alginate and 6-layers of poly-L-arginine and sodium alginate and these sample will be referred to as $(ARG/ALG)_2$ and $(ARG/ALG)_3$, respectively. These samples were designed to validate the possibility of tuning the number of deposited layers using titration method. In addition, we would like to study the influence of layer thickness/ number on the stability and release profile of the nanosuspension. The zeta potential was measured after the addition of each layer (figure 3A). This analysis showed a charge alteration upon the subsequent addition of oppositely charged layers, which confirmed the layer deposition. Analysis of the sample by DLS showed that after the addition of each of the six layers of poly-L-arginine and alginate onto nanosuspension, there was an increase in the z-average diameter of nanosuspension reaching a maximum of reached 500 nm (figure 3B). Along with the increase in diameter associated with the formation of the layers around the nanoparticles, the PDI decreased to below 0.1 for the six layers, which confirmed the stability of nanosuspension (Figure 3B). Characterisation of the morphology of 6-layered nanosuspension by SEM (Figure 3C) showed that the coated nanosuspension were spherical with a diameter of around 300 nm. This diameter was smaller than the sizes determined by DLS, where the size distribution for the same samples with 6 layers of polyelectrolytes showed monomodal distribution with a mode of 500 nm (Figure 3D). This discrepancy in the diameters can be attributed to the solvation sphere that is included in a DLS diameter measurement but not in the samples dried for SEM analysis (M et al., 2019; Santos et al., 2019c). Our work agrees with the limited number of other studies that have investigated the titration method for modifying nanoparticles, Santos et al. used coated ibuprofen nanoparticles (cores) with poly (allylamine hydrochloride) (PAH) and polystyrene sulfonate (PSS) using titration method. The titration curve was plotted to determine the required amount of each polyelectrolyte that cover the core surface and

conferred colloidal stability (Santos et al., 2015). In another study, resveratrol nanocrystals were formed and coated with PAH and dextran sulphate by plotting titration curve to determine the amount for each polyelectrolyte. The required amount of each polyelectrolyte was also determined via plotting a titration curve (Santos et al., 2019c). Our data revealed that the titration method was a simple protocol, where future preparations were not time consuming and were easy to scale up.

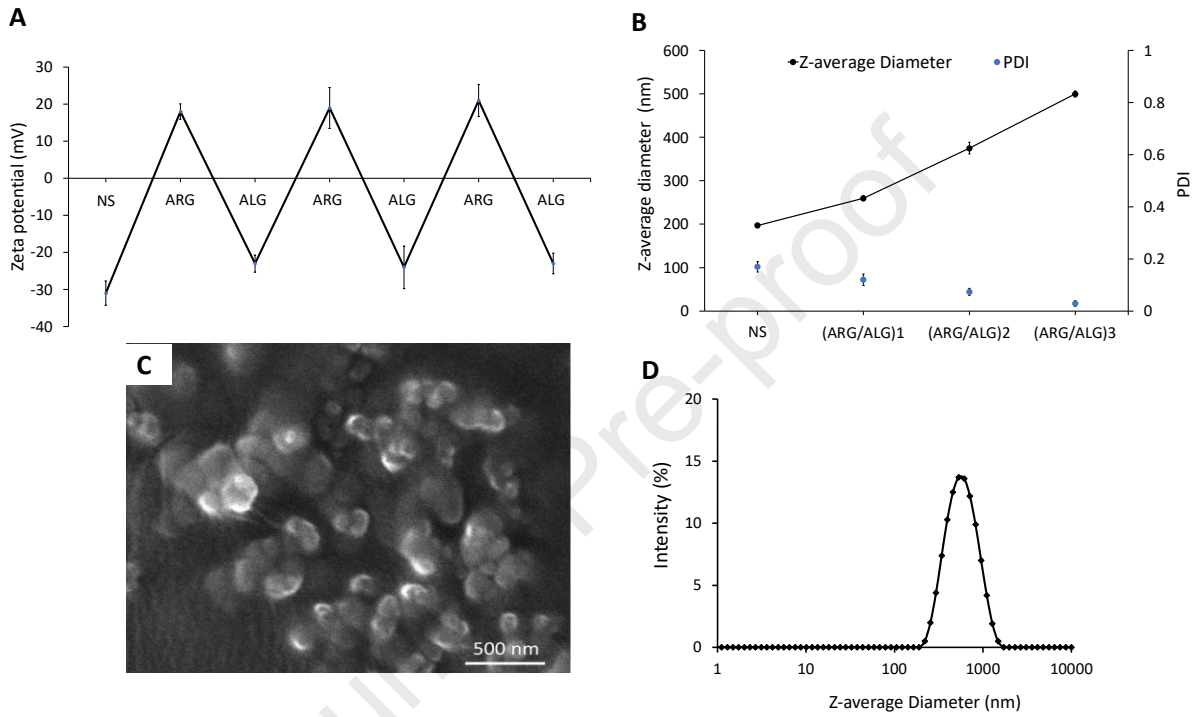


Figure 3. Curcumin nanosuspension coated with six layers of alternating ARG and ALG $(ARG/ALG)_3$ produced using a LbL titration method. A) The zeta potential of nanosuspension was monitored after the addition of each layer by DLS at 25 °C in water. B) The z-average diameter and PDI of nanosuspension (1 mg/ml). were monitored after the addition of each bilayer by DLS at 25 C in water. C) SEM images of 6-layered nanosuspension made of poly-L-arginine and sodium alginate. D) Size distribution diagram of 6-layered nanosuspension obtained by DLS after the deposition of six layers.

<u>Preparing stimuli-responsive multi-layered nanosuspensions</u>

The titration LbL approach is also versatile in terms of the polyelectrolytes used, we demonstrated this by preparing another sample in which the sixth layer was Eudragit L100 (referred at as EUD from hereon) (rather than ALG). EUD is a pH responsive polymer, which is well-known for its potential to protect the drug in the stomach. It is soluble in the pH of the small intestine, which is above 6, and hence could aid in formulating an oral delivery system for curcumin (Cetin et al., 2010). This sample with a pH responsive outer layer will be referred to as the stimuli-responsive nanosuspension (ARG/EUG) from hereon. The ARG/EUG sample was formed successfully with very similar characterisation data to the sample with the six alternative layers of ARG and ALG (ARG/ALG) showing alternation in the zeta potential, a gradual increase in diameter and monomodal particles (figure 4).

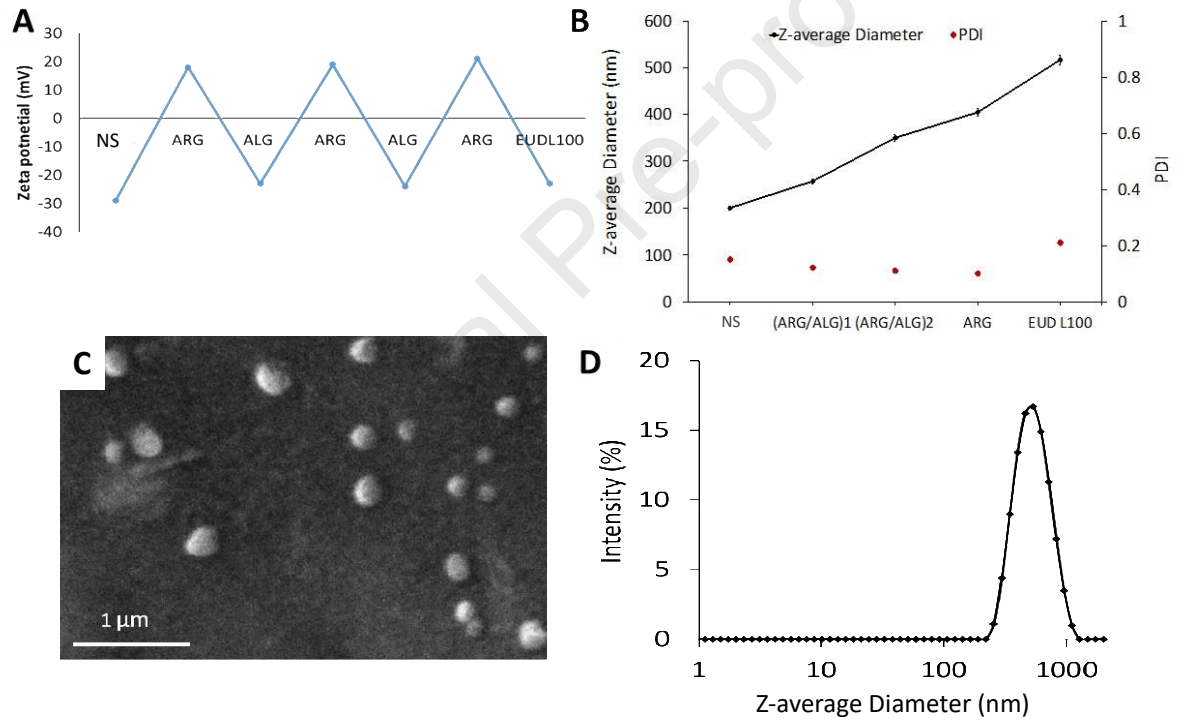


Figure 4. Curcumin nanoparticles coated with five layers of alternative ARG and ALG with a final layer of EUD produced using a LbL titration method. A) The zeta potential of nanosuspension was monitored after the addition of each layer by DLS at 25 °C in water. B) The z-average diameter and PDI of nanosuspension were monitored after the addition of each bilayer by DLS at 25 C in water (1 mg/mL). C) SEM images of prepared stimuli-responsive multi-layered nanosuspension with fifth layer of poly-L-arginine and alginate and Eudragit L100 as an outermost shell. D) Size distributions obtained by dynamic light scattering of (ARG/EUD) sample.

Colloidal stability of the coated nanosuspensions

To investigate whether the coating of the nanosuspension by the LbL titration process influenced the colloidal stability of the nanosuspensions, three samples were analysed by DLS over time. These samples were the two six layered samples prepared previously, $(ARG/ALG)_3$ and ARG/EUD and an additional sample with only four layers of poly-L-arginine and alginate and referred to as $(ARG/ALG)_2$. These four layered samples were included to see if coating thickness had an impact on stability. The three samples were studied in water under dark conditions to assess the stability in storage condition. The 4-layered $(ARG/ALG)_2$ nanosuspension showed a fluctuation in the z-average diameter and PDI for 4 days followed by a rapid increase in PDI from 0.3 to 0.8 on day 7 and a decrease in z-average diameter was observed indicating particle aggregation or sedimentation (Figure 5A). The 6-layered nanosuspension (Figure 5B) showed z-average diameter and PDI fluctuation over 21 days which indicating particles instability. The stimuli-responsive nanosuspension formulation (ARG/EUG) containing poly-L-arginine and Eudragit L100 showed a fluctuation in the z-average diameter and PDI over the 21 days (Figure 5C), with a relatively narrow PDI over the first 4 days, afterwards the z-average diameter and PDI started to increase indicating instability of these nanosuspensions. The destabilisation of these formulations could be due to the establishment of bridging interactions by the polyelectrolytes between nanoparticles leading to particle aggregation.

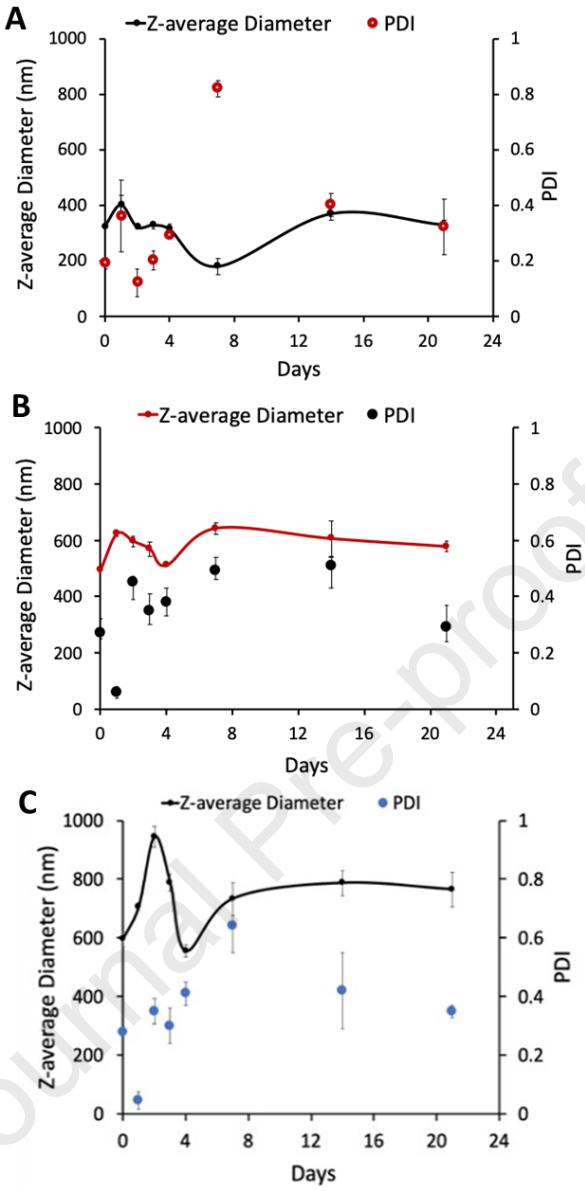


Figure 5. Investigating the colloidal stability of the coated samples, the z-average diameter of LbL-modified nanosuspension was monitored over 21 days by dynamic light scattering at 25 °C in water. (A) 4-layered coated nanosuspension $(ARG/ALG)_2$, (B) 6-layered nanosuspension $(ARG\text{-}ALG)_3$, and (C) Stimuli-responsive nanosuspension (ARG/EUG).

In vitro studies were carried out to investigate the effect of pH on the curcumin release behaviour of nanosuspension coated with 4-layers $(ARG/ALG)_2$ and the 6-layered samples with a pH responsive outer layer (ARG/EUD). These samples were selected to give insight into the effect of coating thickness and the responsive behaviour of the EUD, they were compared with curcumin nanosuspension (uncoated). The *in vitro* study was performed at 37 °C and pH

values selected to mimic different regions of the GIT tract such as stomach (pH 1.2) and small intestine (pH 7.4). Under acidic conditions, after 24 hours, the percentage of curcumin released from uncoated curcumin nanosuspension, 4-layered coated nanosuspension $(ARG/ALG)_2$ and stimuli-responsive (ARG/EUG) was 2.1 %, 2.0 %, and 0.8 %, respectively (Figure 6A). The uncoated curcumin nanosuspension, 4-layered nanosuspension and stimuli-responsive (ARG/EUG) showed a release percentage of 37.5 %, 8.9 %, and 4.9 % over 72 hours. This data showed that the coating of the nanoparticles slowed the release of the curcumin over time periods >48 hours. The delay in curcumin release of both coated nanosuspension could be associated with the impact of shell thickness on nanosuspension and the presence of Eudragit L100, an acid resistant polymer, in one of the formulations.

In stimulated intestinal fluid (pH 7.4) in the first 24 hours, 0.8%, 4.2% and 1.2 % of curcumin were released from uncoated curcumin nanosuspension, 4-layered nanosuspension $(ARG/ALG)_2$ and stimuli-responsive (ARG/EUG) samples respectively (figure 6B). This difference could be due to presence of EUD L100 that was not fully detached from the nanosuspension. After 72 hours, the percentage of curcumin released from uncoated curcumin nanosuspension, 4-layered nanosuspension $(ARG/ALG)_2$ and stimuli-responsive (ARG/EUG) was 2.2 %, 8.9 % and 11.7 %, respectively. The release data showed that 4-layered coated nanosuspension $(ARG/ALG)_2$ and stimuli-responsive (ARG/EUG) showed similar release rates after the complete detachment of EUD L100 that resulting in the ionisation and swelling of underneath layers resulting in drug release. Therefore, the use of the stimuli responsive polyelectrolyte as the outer layer allowed the nanosuspension to show the slowest release under acidic conditions while also delivering accelerated release at pH 7.4.

Comparison of the curcumin release at pH 7.4 and pH 1.2 is shown in figure 6C; the use of both the 4-layered coating $(ARG/ALG)_2$ and the 6-layered coating with the pH responsive polymer (ARG/EUD) showed a significant improvement for facilitating selective drug release under pH 7.4 (the conditions in the intestine). The (ARG/EUD) sample delivered more than a 2-fold enhancement in the selective release at both the 48- and 72-hour time points. These data show that the use of LbL modification can alter the dissolution of a nanosuspension, additionally, the selection of a pH responsive polyelectrolyte as the outer layer can be used to further slow dissolution of the nanosuspension under acidic conditions.

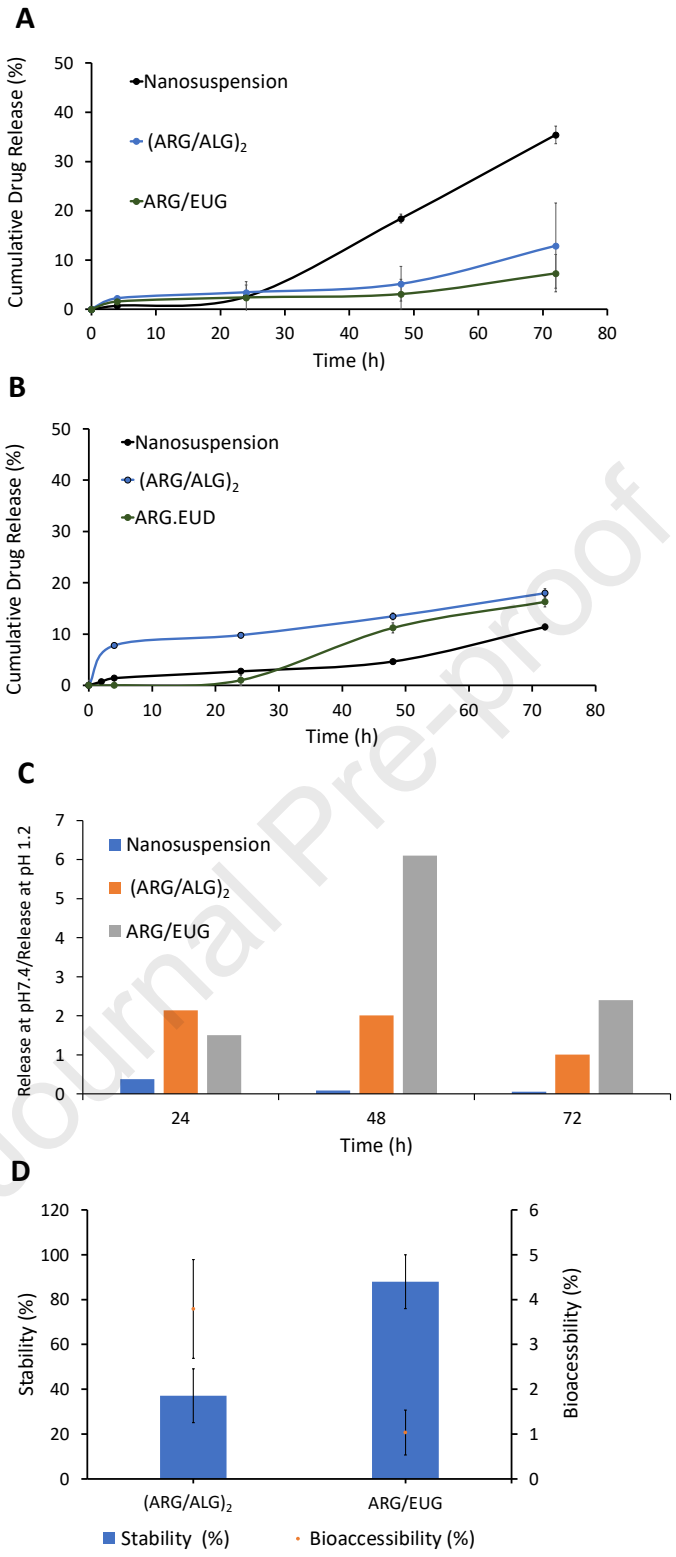


Figure 6: *In vitro* curcumin release behaviour. A-C) Cumulative release of curcumin nanosuspension, curcumin 4-layered coated nanosuspension $(ARG/ALG)_2$ and stimuli-responsive nanosuspension (ARG/EUG) at different pH values which mimics (A) stomach, pH 1.2, and (B) small intestine, pH 7.4. HPLC was used for qualifying the percentage release of curcumin. Each of the samples was analysed in triplicate. C) Effect of pH on ratio of curcumin

493 release at pH 7.4/pH 1.2 for the three formulations. D) Percentage of stability and
494 bioaccessibility of 4-layered nanosuspension $(ARG/ALG)_2$ and stimuli-responsive
495 nanosuspension (ARG/EUG). HPLC was used for qualifying the percentage of stability and
496 bioaccessibility and all samples were carried out in triplicate.
497
498 To access the potential for using the LbL coating approach to alter oral drug delivery from
499 nanosuspensions, as *in-vitro* model for bioaccessibility was used. This model has previously
500 been reported for investigating the bioaccessibility of curcumin, (Cheng et al., 2017; Peng et
501 al., 2018; Zou et al., 2015) the method involves passing the samples through a stimulated
502 gastrointestinal tract (GIT) using different pH, salts, and enzymes to mimic the conditions of
503 month saliva, stomach, and small intestine. The stability (S*) and bioaccessibility (B*) were
504 determined by HPLC. The stability (S*) defined as the percentage of curcumin remaining in
505 the overall media after passing through the stimulated GIT. Bioaccessibility (B*) defines as the
506 fraction of curcumin in the small intestine phase only that is available for absorption (Peng et
507 al., 2018). Two LbL coated nanosuspension formulations samples were selected for this study,
508 which were the 4-layered nanosuspension $(ARG/ALG)_2$ and ARG-EUD to see if the difference
509 in the release kinetics might influence the stability and bioaccessibility of the formulation. The
510 stability of different coated nanosuspension were 37% and 88% for 4-layered nanosuspension
511 and stimuli-responsive nanosuspension (ARG/EUG), respectively (Figure 6D). The 4-layered
512 nanosuspension $(ARG/ALG)_2$ showed the lowest stability and therefore the highest
513 degradation through exposure to different GIT conditions such as acidic environment and
514 different digestive enzymes. Our prior study showed that curcumin nanosuspension have
515 small dimensions and large surface area to volume ratio therefore they have highdissolution
516 rate and are highly susceptible to enzymatic degradation (Elbaz et al., 2021b). The
517 nanosuspension coated with 4-layers of poly-arginine and sodium alginate showed a higher
518 stability compared to the (uncoated) curcumin nanosuspension in our previous study which
519 had a value of 20%, likely due to the layers of the ARG/ALG-4 coating causing a reduction in
520 the dissolution rate (Elbaz et al., 2021b). The stimuli-responsive nanosuspension (ARG/EUG)
521 showed much higher stability (89%), indicating the effect of the shell wall thickness on
522 curcumin protection as this formulation has additional layers of poly-L-arginine and Eudragit
523 L100, which in turn offers higher curcumin protection. The bioaccessibility was 4% for 4-
524 layered nanosuspension, and below 1 % for stimuli-responsive nanosuspension (ARG/EUG)

525 suggesting the tendency of extra layers in reducing the degradation and delay the drug
526 dissolution and release. These findings revealed 4-layers nanosuspension $(ARG/ALG)_2$
527 potentially exhibited insufficient wall thickness to protect curcumin as compared to stimuli-
528 responsive nanosuspension (ARG/EUG).
529
530 Finally, to investigate whether the coating of the curcumin nanosuspension influenced the
531 cytotoxicity of the nanosuspension, an *in vitro* cytotoxicity was performed using MTT assay to
532 measure cell viability after exposure to the $(ARG/ALG)_2$ and ARG/EUD nanosuspensions. Two
533 cell lines were used; Caco-2 cells were used in the light of the intended oral administration
534 and HT-29 cells were used to assess any anticancer effect of developed nanosuspensions. The
535 study was performed by exposure the cells to different concentrations of coated
536 nanosuspension and incubated for 48 hours. With the Caco-2 cells, the IC_{50} value of 4-layered
537 nanosuspension was 90 µM effectively the same as the uncoated curcumin nanosuspension
538 (88 µM as previously reported under the same conditions (Elbaz et al., 2021b)) confirming no
539 additional cytotoxicity attributed to the presence of the LbL construct (Figure 7A). These data
540 were in line with the previous studies proving the non-toxicity and biocompatibility of poly-L-
541 arginine and sodium alginate.(Lan et al., 2008; Yang et al., 2018) The IC_{50} value of stimuli-
542 responsive nanosuspension formulations (ARG/EUG) was above 100 µM (Figure 7B). The
543 samples with six layers were slightly less toxic compared to the 4-layered nanosuspension
544 $(ARG/ALG)_2$ showing the additional safety provided by the extra layers (Santos et al., 2015,
545 2019a; Yu & Pishko, 2011). This behaviour may be due to the additional layers slowing the
546 curcumin release, which may have reduced the exposure for the cells to the curcumin.
547 However, further studies would be required to understand if the toxicity was associated with
548 presence of soluble curcumin, or the accumulation of nanosuspension or a combination of
549 mixed factors. When dosed to the HT-29 cells the behaviour was very similar to that with the
550 Caco-2 cells. The 4-layered nanosuspension $(ARG/ALG)_2$ showed the highest cytotoxicity with
551 an IC_{50} value of 71 µM (Figure 7C) and the stimuli-responsive nanosuspension formulations
552 (ARG/EUG) showed no IC_{50} value, because there was no significant reduction in cell viability
553 at the concentrations of nanosuspension tested (Figure 7D). These data show the LbL
554 approach does not lead to any increase in toxicity for the nanosuspension, indeed it may be
555 that the slowing of the dissolution of the layers coated the nanosuspension reduces the
556 toxicity of the dosed curcumin.

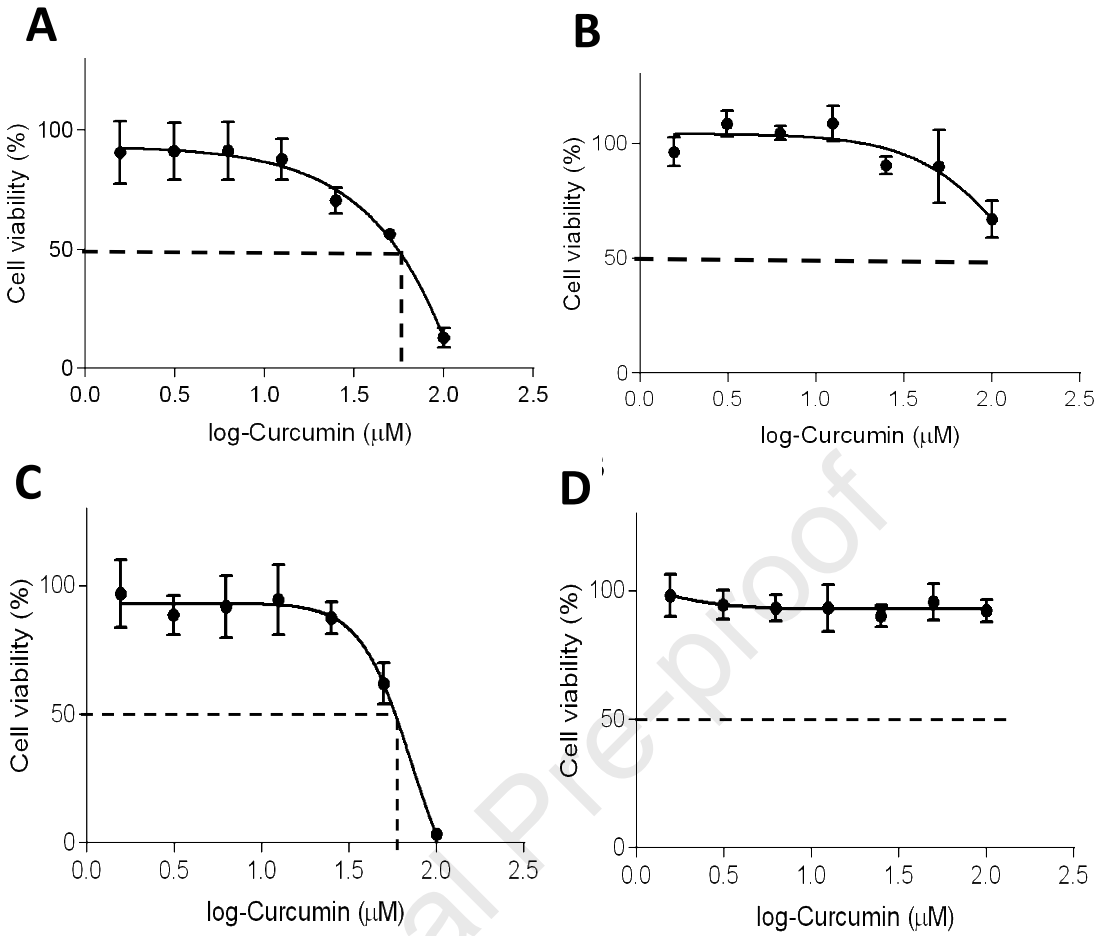


Figure 7. Cell viability with Caco-2 cells or HT-29 cells after 48 h incubation either 4-layered nanosuspension $(ARG/ALG)_2$ and stimuli-responsive formulations (ARG/EUG) with concentrations varying from 0-100 µM. (A) 4-layered nanosuspension with Caco-2 cells and (B) stimuli-responsive formulations (ARG/EUG) with Caco-2 cells. (C) 4-layered nanosuspension $(ARG/ALG)_2$ with HT-29 cells and (D) stimuli-responsive formulations (ARG/EUG) with HT-29 cells. All measurements were carried out in triplicate.

Conclusions

LbL-coated nanoparticles pose a desired category of materials due to their tuneable and multi-functional traits. The titration method, a simple washless approach, was used for coating curcumin nanosuspensions. To precisely design the LbL-coating nanosuspension, titration method was optimized by using polyelectrolytes titration curve by monitoring the zeta potential. Moreover, the z-average diameter and PDI were monitored during layer deposition to ensure obtaining a maximum colloidal stability for each coating layer and avoid nanoparticle aggregation. Following the formation of 4- layered and 6-layered made of poly-

L-arginine and sodium alginate, stimuli-responsive nanosuspension made of poly-L-arginine and sodium alginate as the underlying layers and a final layer of pH responsive EUD was formed. The *in vitro* release and bioaccessibility model studies revealed that the use of Eudragit L100 as an outermost shell in the one formulation (ARG/EUG) resulted in a significant protection of curcumin nanosuspension in stimulated stomach fluid compared to 4-layered nanosuspension and thus proposed to formulate a promising oral delivery system for curcumin nanosuspension. The cytotoxicity profile of chosen LbL-construct showed reduced the toxicity of curcumin on both cell lines tested. Further biological studies are still required to investigate the effect of the addition of layers on nanosuspension such as studying the cellular uptake and permeability. The stimuli-responsive nanosuspension (ARG/EUG) seems to be very promising for oral administration because of its delay drug release in the gastric- and intestinal pH. To the best of our knowledge, this is the first time of curcumin nanosuspension formulation to be coated with LbL using various polyelectrolytes to manipulate the nanosuspension release and protect it in different GIT conditions for oral administration**.**

Declarations of competing interest

There are no conflicts to declare.

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Highlights:

- Titration method is a simple and wash-less approach for tuning the nanostructures properties.
- Optimization of the titration method enabled the production of 4-layered and 6-layered coated curcumin nanosuspensions.
- A stimuli-responsive nanosuspension was successfully produced using 5 layers of poly-L-arginine and alginate, with Eudragit L100 as the outermost layer
- The stimuli-responsive nanosuspension revealed a delay in the release under neutral pH compared to acidic pH due to the use of a pH-responsive layer (Eudragit L100) as an outermost shell.
- The titrated LbL modification approach could be used to tailor the stability and the release behavior of nanosuspensions for oral drug delivery applications.

Declarations of competing interest

There are no conflicts to declare.