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Venturi-based rapid expansion of supercritical solution (Vent-RESS): synthesis of liposomes for pH-triggered delivery of hydrophilic and lipophilic bioactives

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Abstract

Multivitamin-loaded and surface-modified liposomes tailored for simultaneous intestinal delivery of both lipophilic and hydrophilic bioactives were synthesized from sunflower phosphatidylcholine (SFPC). Liposomes (SL) were generated with the aid of a novel, organic solvent free, and environmentally benign process which utilizes venturi-based rapid expansion of supercritical solution (Vent-RESS). Vitamins E and C were used as model lipophilic and hydrophilic bioactives and demonstrated an average encapsulation efficiency of 92 and 70 %, respectively. Synthesized liposomes were coated with a pH-responsive double-wall of chitosan and β-lactoglobulin (βlg-Cs-SL) to develop a biocompatible vehicle for pH-triggered delivery of bioactive cargo(s). To compare the efficacy of this newly developed dual-coating, SL was also coated with a commercially available pH responsive polymer, Eudragit® S100 (Eu-SL). No organic solvent was used during the surface coating of SLs with these two different types of enteric coatings. The performance of these two coatings was studied by conducting morphological characterization through diameter and ζ-potential measurements along with confocal laser scanning and freeze-fracture cryogenic scanning electron microscopies. The stability of coated and uncoated SFPC liposomes was determined in simulated gastrointestinal fluids. For βlg-Cs-SL and Eu-SL, after 2 h of incubation in simulated gastric condition, less than 5 % of the encapsulated vitamins C and E were released, whereas for SL, 41 and 28 % of vitamins C and E were released within 2 h of incubation period. In simulated intestinal fluid, coated liposomes released most of their remaining payload when incubated for 4 h. The newly developed dual coating was found to be as effective as its commercially available counterpart, Eudragit® S100 coating; nevertheless, the biocompatible, non-toxic, and non-synthetic nature of this coating makes it an attractive alternative. Modeling the release kinetics of vitamins from coated liposome showed that the release of payload from surface coated liposomes proceeded through a multistep structural disintegration involving both Fickian and non-Fickian types of diffusion. The ability of these surface-coated liposomes to maintain

structural integrity under the gastric condition followed by site-specific, pH-triggered release of encapsulated cargo in the intestine will make them highly suitable for oral administration of bioactive compounds in pharmaceutical and food applications.

1. Introduction

Liposomes are microscopic artificial vesicles of spherical shape with one or more phospholipid bilayer(s) encompassing an aqueous core. Liposomes' unique amphiphilic nature enables encapsulation of both hydrophilic and lipophilic compounds respectively in the aqueous core and inside the phospholipid bilayer. [1,2] Liposomes are also remarkable for their biocompatibility, minimal toxicity, non-immunogenicity, and biodegradability. Liposomal delivery systems have been extensively studied for customization of their attributes to fit the specificity of a desired application.[3-5] Liposomal surface modification involves coating them with additional carbohydrates,[6-8] polymers,[9,10] surfactants,[11,12] or other amphiphilic molecules to protect the encapsulated bioactive-payloads from enzymatic degradation along with proliferation of intestinal retention and mucus-penetrating abilities; liposomes may also be modified via enhancement of their bilayer stability by incorporation of other molecules (i.e., cholesterol, bile salts, polymers).[13] Owing to these advantageous attributes, liposomes have been extensively used to increase solubility, bioavailability, therapeutic effects, and for targeted delivery and controlled release of different bioactive compounds throughout pharmaceutical and cosmetic industries, and more recently in the food industries. [14-17]

Some of the conventional liposome synthesis methods comprise thin-film hydration (TFH),[18] solvent injection,[19] hydration of pro-liposomes,[20] reverse phase evaporation,[21] and the detergent removal method.[22] One of the major drawbacks in these methods is the use of toxic-organic solvents to dissolve the lipophilic part, which requires a post-processing step to remove the organic solvent. This acts as a limiting factor, because any residual organic solvent left in the liposomes can make the formulation cytotoxic and make them unsuitable for approved biological applications. Thus, an ideal synthesis method should involve utilization of generally recognized as safe (GRAS) solvents, which would eliminate the need for any post-processing solvent removal step. Consequently, in recent years, novel liposome production methods have been developed to eliminate or reduce the use of toxic

organic solvents, reduce processing time, maintain reproducibility and homogeneity, and increase liposomal encapsulation efficiency. These approaches include microfluidics and supercritical fluid-based systems.[23,24] Carbon dioxide (CO_2) is the most common fluid used in the supercritical fluid-based liposome formation systems since it is non-toxic, abundant, and inexpensive, along with having a mild critical temperature (31 °C) and pressure (7.4 MPa).[25] As for liposome synthesis, supercritical-CO_2 (SC-CO_2) has been adapted for several purposes: as a solvent, antisolvent, or dispersing agent.[26,27] Our group has previously reported a novel and environmentally benign procedure for liposome synthesis, exploiting the rapid expansion of supercritical solution using a venturi-based system (Vent-RESS) for concomitant vacuum driven cargo loading, based on Bernoulli's principle.[28,29] In this approach, phospholipids and cholesterol, along with other lipophilic bioactives, are dissolved in SC-CO_2, followed by its rapid expansion and thorough mixing with an incoming stream of aqueous cargo solution inside an eductor-nozzle system utilizing Bernoulli's principle. The expansion of SC-CO_2 initiates the atomized nucleation of phospholipids; and to attain maximum stability the phospholipid molecules self-assemble around miniscule water droplets in a bilayer arrangement, and thus form liposomes.[30]

Conventional liposomes made up of just phospholipids and cholesterol demonstrate limited efficacy in oral delivery applications owing to the susceptibility of its phospholipid bilayer membrane towards the combined deleterious effects of digestive enzymes (i.e., phospholipases, pancreatic lipase, and cholesterol esterase), gastric acid, and bile salts.[31] Several polymers, such as natural and modified carbohydrates, have been substantially used to enterically coat the liposomal surface. These materials act as a shell around the liposomal core to prevent the disintegration of liposomes in the stomach; consequently, a higher proportion of liposomes are carried forward and delivered to the small intestine, which results in an enhanced absorption of encapsulated bioactives.[32-36]

In this work, we propose surface coating of liposomal vesicles with a dual layer of chitosan and β-lactoglobulin. They have been shown to undergo a strong electrostatic interaction in the presence of a cationic bridging agent at acidic conditions.[37] Liposomes coated with a double wall of chitosan and β-lactoglobulin will have the ability to protect the core load until reaching the target site in the gastrointestinal (GI) tract followed by degradation of the outer membrane and subsequent site-specific release of the encapsulated bioactives. Chitosan is a non-toxic, biocompatible, and biodegradable linear polysaccharide and is soluble in acidic medium.[38] In addition, chitosan also possesses mucoadhesive characteristics: it adheres to a specific site of intestinal lining and develops as a patch on its surface, which facilitates enhanced penetration of encapsulated bioactives into the epithelium cells along with reducing dilution effects by preventing premature release.[39] However, if chitosan is solely used as a wall material, it may not be an efficient delivery system owing to its solubility in acidic gastric environment which would cause premature release of encapsulated payload.[40] This limitation can potentially be overcome by an additional coating with an acid-resistant material such as β-lactoglobulin, which acts as a protective layer against degradation in the stomach. β-lactoglobulin is a major component of whey protein in bovine milk, and with an isoelectric point around 5.2 it is not easily digested in the stomach.[41,42]

We have also used Eudragit® S100 to coat the liposomes to compare the performance of chitosan and β-lactoglobulin dual layer. Eudragit® S100 is a commercially available polyanionic block co-polymer constituted by methyl methacrylate–methacrylic acid. It is insoluble in acidic gastric pH and dissolves only above pH 7.0; thus, coating liposomes with this polymer protects them from acidic conditions in the GI tract and enables site-specific release in the higher pH (> 7.0) region of small intestine.[43] In this work, we propose to coat the bioactive loaded liposomes with Eudragit® S100 through a modified solvent displacement method by using polyethylene glycol (PEG) as a non-toxic, non-immunogenic solvent that is considered as GRAS.[44,45] The purpose of this study is to elaborate

the synthesis of liposomes through a novel one-step, environmentally benign technology that allows simultaneous encapsulation of both lipophilic and hydrophilic bioactives; followed by surface decoration of generated liposomes by two different enteric coating strategies (i.e., dual layer of chitosan and β-lactoglobulin, and Eudragit® S100) to perform a comparative analysis between their efficacy to facilitate target specific release of protected payload in the GI tract.

2. Materials and Methods

2.1. Materials

Nile Red, vitamin E (α-tocopherol, 95.5 %), cholesterol (92.5 %), sodium tripolyphosphate (TPP), dimethyl sulfoxide (DMSO) (99.9 %), pancreatin (4 × USP specifications), lipase (Type II) and bile extract from porcine pancreas, and pepsin from porcine gastric mucosa (≥400 units/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sunflower phosphatidylcholine (SFPC) (Sunlipion® 90, 99 %), extracted from non-genetically modified sunflower lecithin was donated by Perimondo (Florida, NY, USA). β-lactoglobulin (BiPRO® 9500) was donated by Agropur Inc. (Eden Prairie, MN, USA). Eudragit® S100 and polyethylene glycol 400NF (PEG-400) was donated by Evonik (Piscataway, NY, USA) and Dow Chemical Company (Midland, MI, USA), respectively. Carbon dioxide (CO_2) (99.99 %) was purchased from Airgas (Ithaca, NY, USA). Vitamin C (L-ascorbic acid, 99 %) was purchased from TCI America (Portland, OR, USA). Calcein and Tris(hydroxylmethyl) aminomethane (TRIS) were purchased from Acros Organics (Morris, NJ, USA) and Bio-Rad (Hercules, CA, USA), respectively.

2.2. Liposome synthesis

SFPC was used to synthesize bioactive loaded liposomes (SL) by using the Vent-RESS system (Fig. 1 (a)). The operating mechanism of Vent-RESS system has been explained in our previous publications.[28,46] To determine the efficacy of bioactive encapsulation in the synthesized liposomes, vitamins E and C were used as model lipophilic and hydrophilic bioactives, respectively. The lipophilic cargo was prepared by blending SFPC, cholesterol, and vitamin E (5:1:1 wt. ratio) into a homogenous mixture, and the aqueous cargo was a 0.125 M vitamin C solution in 0.02 M TRIS buffer (pH = 7.4). The three major parts of Vent-RESS system are: a high-pressure pump, a mixing vessel equipped with

a stirrer, and an eductor-nozzle assembly consisting of an expansion nozzle (I.D. 1.5 mm). The lipophilic cargo was loaded into the mixing vessel followed by solubilization into SC-CO_2 at an optimized pressure and temperature of 17.2 MPa and 45 °C, respectively. After equilibration for 1 h under continuous stirring, the lipophilic cargo laden SC-CO_2 was moved to the nozzle, and it was expanded through a solenoid valve for a duration of 0.5 sec. The aqueous cargo was introduced into the expansion nozzle by a stainless-steel tube (I.D. = 1.3 mm) mounted at an angle of 45° with respect to the SC-CO_2 flow. The pressure release through the solenoid valve resulted in rapid expansion of the lipophilic cargo laden SC-CO_2 followed by substantial increase in its velocity based on Bernoulli's principle. This sudden increase in velocity due to conversion of potential energy into kinetic energy created a vacuum inside the educator-nozzle assembly (i.e., *vena contracta*). This vacuum acted as a driving force and facilitated suction of aqueous cargo inside the eductor-nozzle assembly; where the aqueous stream collided against the expanding CO_2 stream and was converted into fragmented submicron droplets. At this stage the CO_2 lost its supercritical properties, and nucleation of the dissolved phospholipids began; to attain stability, phospholipid molecules self-assembled around miniscule water droplets into a bilayer arrangement resulting into formation of liposomes. During the whole expansion process, the eductor-nozzle assembly was maintained at 80 °C to prevent precipitation of lipophilic cargo owing to cooling by the Joule-Thompson effect and CO_2 expansion. The resulting liposomes were collected in 10 mL of TRIS buffer solution (pH = 7.4).

2.3. Double wall coating of liposomes with chitosan and β-lactoglobulin

The combined influence of chitosan and β-lactoglobulin on the diameter of coated liposome was studied by means of a full factorial design of experiments. Four levels were studied for each of the two independent variables: chitosan and β-lactoglobulin's concentration within the range of 0.05 – 2.0 % w/v. Final dimeter of βlg-Cs-SL was lowest at a chitosan and β-lactoglobulin concentrations

of 0.15 and 0.05 % w/v, respectively, and was used as the optimum concentrations for coating liposomes with the pH-responsive dual wall. Synthesized liposomes were coated with chitosan first: chitosan solution (0.15 % w/v) was prepared, and the pH of the solution was maintained at 4.5 by the addition of 1 % acetic acid solution. Concentrated liposomal solution was then added dropwise to the polymer solution under continuous stirring to achieve an equivolumetric mixture (1:1 volume ratio) at pH 4.5. The suspension was equilibrated overnight at 4 ^{o}C. The excess chitosan was separated by using VIVASPIN 500 filtration membrane (100 kDa MW cutoff) and concentrated chitosan coated liposomes (Cs-SL) were separated.[47] Next, Cs-SL were coated with a secondary wall of native β-lactoglobulin to produce double wall coated liposomes (βlg-Cs-SL). β-lactoglobulin demonstrates a positive charge below its isoelectric point (~5.2) and Cs-SL was coated with β-lactoglobulin (0.05 % w/v) through ionic gelation by the aid of sodium tripolyphosphate (TPP) at pH 4.5. Native β-lactoglobulin solution (0.05 % w/v) was prepared by adding β-lactoglobulin in deionized water with thorough mixing at room temperature for 1 h.[48] The solution was then equilibrated for 2 h before further treatment to allow adequate protein hydration. Concentrated solution of Cs-SL was then dropwise added to an equal volume of native β-lactoglobulin solution followed by addition of a TPP solution (1 mg/mL) at pH 4.5 under continuous stirring. The suspension was equilibrated overnight at 4 ^{o}C followed by removal of excess native β-lactoglobulin by using ultrafiltration. Concentrated βlg-Cs-SL were separated in a 6 mM acetic acid-sodium acetate buffer (pH 4.5).

2.4. Coating of liposomes with Eudragit® S100

Synthesized SFPC-liposomes were coated with a commercially available pH-responsive polymer, Eudragit® S100 (Eu-SL), to compare the efficacy of proposed double wall coated liposomes (βlg-Cs-SL). To coat bioactive loaded liposomes with Eudragit® S100, PEG-400 was used as a non-toxic solvent. SLs were coated with Eudragit® using a nanoprecipitation method developed by Ali and Lamprecht with modifications.[49] The nanoprecipitation method was first developed by Fessi et al.,

which requires two solvents that are miscible with each-other and a polymer that dissolves in one solvent and is insoluble in the other one (i.e., non-solvent).[50] Eudragit® S100 is soluble in PEG-400 at low concentration and is insoluble in acidic buffer. Thus, PEG-400 and 6-mM acetic acid-sodium acetate buffer (pH 4.5) were used as solvent and non-solvent, respectively. Concentrated liposomal dispersion was added to an Eudragit® S100 and PEG-400 solution (50 mg/ 3 mL). After thorough mixing, the resultant solution was added dropwise into the acetic acid-sodium acetate buffer (pH 4.5) under magnetic stirring and was kept at 4 °C for 4 h. Eu-SLs were then separated by centrifugation and, after being washed three times with the acetic acid-sodium acetate buffer (pH 4.5), were kept in the same buffer.

2.5. Liposomal characterization

Confocal laser scanning microscopy (CLSM) was used to morphologically characterize SLs and Eu-SLs. Micrographs were obtained by using a Zeiss LSM 710 confocal microscope equipped with a 63× oil-phase objective lens. Synthesized liposomes were stained by the method described by Tsai and Rizvi.[51] Calcein was mixed with the aqueous cargo (2 mg/mL) and was used to stain the hydrophilic core, whereas Nile red was used to stain the lipophilic phospholipid bilayer and the Eudragit® S100 layer encompassing the liposome's bilayer. 10 µL of Nile red solution in ethanol (0.2 % w/v) was added to 1 mL of Calcein-loaded liposomes and mildly agitated by hand for 1 min. The fluorescence emission spectra of Calcein and Nile red were set between 496–535 nm and 558–635 nm, respectively and the stained liposomal suspensions were used for CLSM.

Furthermore, to elucidate the chitosan and β-lactoglobulin dual coating on liposomal surface, uncoated SLs and coated βlg-Cs-SLs were analyzed by using freeze-fracture cryogenic scanning electron microscopy (ff-SEM) by improvising the method described by Manna et al.[52] 5 µL of concentrated liposomal sample was pipetted into both sides of the freeze-fracture rivets. The rivets

were then sandwiched together followed by vitrification in liquid nitrogen (-196 °C); this was quickly attached to an SEM stub by using a colloidal graphite/TissueTek mixture. The frozen stub was transferred to the preparation chamber of Quorum PP3010t cryo-SEM transfer system (East Sussex, UK). In precooled preparation chamber the rivet was fractured followed by sublimation at -100 °C for 1 min. The exposed surface was coated with a gold/palladium (Au/Pd) mixture for 15 s at 20 mA. The sample was then imaged by a pre-cooled (−160 °C) FEI Strata 400s Dual-Beam (Hillsboro, OR, USA) operated at 5 kV.

A Brookhaven 90 PLUS particle size analyzer (Holtsville, NY, USA) equipped with BI-zeta extension was be used to determine the size distribution and zeta potential of coated and uncoated liposomes.

2.6. Encapsulation efficiency (EE) measurement

The EE of vitamins E and C in uncoated SLs was measured by the method described by Sharifi et al.[46] To measure the EE of vitamin C in Eu-SL and βlg-Cs-SL, 1.5 mL of liposomal dispersion was centrifuged at 4 °C for 20 minutes at 2000xg, followed by separation of supernatant from the concentrated liposomes. For both fractions, 200 μL of 10 % (w/v) Triton X-100 was added followed by agitation for 5 minutes to rupture the coated liposomal vesicles and thus releasing vitamin C into the solution. The solutions were then diluted to a final volume of 3 mL with additional TRIS buffer and concentration of vitamin C was determined by measuring absorbance at 265 nm using a UV/Vis spectrophotometer (UV1900, Shimadzu Scientific Instruments, Marlborough, MA, USA). For vitamin E, 1.5 mL of liposomal dispersion was centrifuged at 4 °C for 20 minutes at 2000xg, and the supernatant was decanted, leaving behind the concentrated liposomes. For both fractions, 0.2 mL DMSO was added to solubilize the coating materials along with vitamin E. The solutions were then diluted to a final volume of 3 mL with additional TRIS buffer and the absorbance of vitamin E at 295

nm was measured using a UV/Vis Spectrophotometer. The EE of vitamin E and C were calculated using equation 1:

$$\text{EE for vitamin C and E (\%)} = \frac{\text{Vitamin content in concentrated liposomes}}{(\text{Vitamin content in concentrated liposomes} + \text{Vitamin content in supernatant})} \times 100. \quad (1)$$

2.7. Study of core release under simulated gastrointestinal conditions

The stability of SL, Eu-SL, and βlg-Cs-SL under simulated gastrointestinal conditions was evaluated by the method of Minekus et al.[53] with modifications and has been precisely explained in our previous publication.[54] 5 mL of a specific liposomal dispersion was added to a 25 mL of 20 mM phosphate buffer solution (pH 6.8). 1 M hydrochloric acid was used to adjust the final pH of the solution to 2 and 1 mL of porcine pepsin (0.8 % solution) from porcine gastric mucosa was added to it. The resultant solution was placed in a shaking water bath at 200 rpm, and temperature was maintained at 37 °C. At predetermined incubation times (i.e., 0, 15, 30, 60, 90, and 120 min), samples were collected for analysis followed by centrifugation at 10,000xg for 30 min; to measure vitamin E content collected sample was passed through an ultrafiltration membrane (100 kDa MW cutoff). After 2 h treatment in SGF, a 25 mM sodium bicarbonate solution was used to adjust the pH of the solution to 5.3. A 1.5 mL multi-enzyme solution prepared in 20 mM phosphate buffer containing 2.4 mg bile extract, 0.2 mg lipase and 0.4 mg pancreatin from porcine pancreas was added to it. The final pH of the simulated intestinal fluid (SIF) was maintained at 7.2 by using a 1 M NaOH solution. The samples were again placed in a shaking water bath at 200 rpm at 37 °C. At predetermined incubation times (i.e., 120, 150, 180, 210, 240, 300, 330, and 360 min), samples were collected for analysis followed by centrifugation at 10,000xg for 30 min. The concentration of released cargo was determined using UV/Vis spectrophotometry for both vitamins. The amount of released cargo was represented as a

percentage of the total cargo released from coated or uncoated liposomes, where the minimum and the maximum concentrations have been normalized to 0 and 100 %, respectively.

A thorough understanding of the bioactive release kinetics is necessary to optimize the application of these surface-coated liposomes as potential bioactive delivery vehicles.[55] Releases of vitamin C and E in SIF from Eu-SL and βlg-Cs-SL were modelled by Higuchi (Eq. 2),[56] Sahlin-Peppas (Eq. 3),[57] and Hixson-Crowell (Eq. 4)[58] equations.

$$\frac{M_t}{M_\infty} = k_H \sqrt{t} \quad (2)$$

$$\frac{M_t}{M_\infty} = k_{SP1}\, t^n + k_{SP2}\, t^{2n} \quad (3)$$

$$M_0^{\frac{1}{3}} - M_t^{\frac{1}{3}} = K_{HC} t \quad (4)$$

Where M_t and M_∞ are amount of bioactive released at time t and at infinite time, respectively. M_0 is the initial amount of bioactive. k_H, $k_{SP\,(1 and\,2)}$, k_{HC}, are the Higuchi, Sahlin-Peppas, and Hixson-Crowell constant, respectively. The fitness of a model was determined by using Eq. 8, which measures the absolute relative deviation (ARD) between the predicted amount of released bioactive (M_p) and the experimentally obtained value of released bioactive (M_t):

$$\text{ARD } \% = \frac{|M_t - M_p|}{M_t} \text{ x } 100 \quad (5)$$

2.7. Storage stability

The storage stability of βlg-Cs-SL was determined by measuring its diameter and ability to retain encapsulated lipophilic and hydrophilic payloads after storage at 4 °C for 30 days in an acetic acid-sodium acetate buffer (pH 4.5). The samples were measured for their diameter and EE every week.

2.8. Statistical analysis

All treatments and analyses were performed in triplicate for each sample and statistical analysis was performed in R (Version 3.6.3., R Foundation for Statistical Computing, Vienna, Austria). One-way ANOVA with Tukey's honestly significant difference test with a 95% confidence interval was conducted to determine the statistical significance of differences in means.

3. Result and Discussion

Multivitamin-loaded liposomes from SFPC-phospholipids (SLs) were synthesized by using a SC-CO_2 based Vent-RESS system without the aid of any organic solvents (Fig. 1 (a)). Synthesized liposomes demonstrated an average diameter of 389.5 nm and ζ-potential of -34.53 mV (Fig. 2 (a) and (b)). The negative ζ-potential of SL is contributed by the anionic phosphate headgroups of SFPC,[59] a higher absolute value of ζ-potential indicates liposomes' ability to resist aggregation and thus overall stability of the colloidal system.[60] The presence of unilamellar vesicular type of liposomes were observed as exhibited by their CLSM images in Fig. 3 (a). The lipophilic dye Nile red was used to represent the phospholipid bilayer (Fig. 3 (a_1)), whereas the hydrophilic dye Calcein, which fluoresces bright green, was used to dye the aqueous core (Fig. 3 (a_2)). After submerging two channels, the liposomal bilayer and core demonstrated red and yellow color, respectively (Figure 2 (a_3)). SLs were also visualized through freeze-fracture (ff)-SEM (Fig. 4 (a_1)) and the presence of their unilamellar structure was confirmed. The encapsulation efficiency of model lipophilic (i.e., vitamin E) and hydrophilic bioactive (i.e., vitamin C) in SLs were around 92 and 70 %, respectively.

Synthesized SLs were coated with a dual layer of chitosan and β-lactoglobulin through ionic-gelation in the presence of a cationic bridging agent TPP. Coating with chitosan increased SL's dimeter by a factor of 2.18, and synthesized Cs-SL demonstrated an average diameter of 849.56 nm. Cs-SL was synthesized by self-assembly of chitosan on the spherical liposome surface through electrostatic interaction. Chitosan has positively charged amine side groups along its backbone (chitosan's pKa ~ pH 6.5).[38] Thus, the electrostatic interaction between the negatively charged liposomal surface and the positively charged amine side groups of chitosan promoted chitosan's enteric coating around SL. Cs-SL demonstrated an average ζ-potential around 33.69 mV (Fig. 2 (b)); the positive surface charge is attributed to the presence of positively charged amine groups of chitosan. The presence of chitosan coating around SL was also established through ff-SEM, and the micrograph of Cs-SL demonstrated

an increased diameter and wall thickness (Fig. 4 (b)). However, chitosan is highly soluble in acidic medium through protonation of the amino group; thus, a chitosan layer by itself would not be able to protect the payload in the stomach and would cause premature release. Thus, a secondary layer of β-lactoglobulin was added to Cs-SL to provide protection against acid hydrolysis and enzymatic degradation in the gastric environment. Digestion of β-lactoglobulin in the stomach is hindered owing to its resistance towards pepsin and acid hydrolysis. In acidic conditions, β-lactoglobulin conceals its hydrophobic amino groups by forming a globular structure through self-rearrangement of either disulfide or other non-covalent bonds.[61,62] Dual coating of chitosan and β-lactoglobulin increased SL's diameter by a factor of 4.09 and synthesized βlg-Cs-SL demonstrated an average diameter of 1595.56 nm. A polyvalent cation TPP was used to non-covalently dock β-lactoglobulin on the positively charged surface of Cs-SL. TPP carries five negatively charged ionizable groups with different pKa values (pKa_1 = 1, pKa_2 = 2, pKa_3 =2.79, pKa_4 = 6.47, and pKa_5 = 9.24), and its net charge is dictated by the pH. [63] β-lactoglobulin carries positive charge below its isoelectric point, which is around pH 5.0.[64] Thus, when a concentrated solution of Cs-SL was dropwise added to a native β-lactoglobulin solution (pH 4.5) followed by addition of TPP, negatively charged TPP acts as an anchor and creates a secondary layer of β-lactoglobulin coating on top of Cs-SL by acting as a non-covalent bridge between positively charged chitosan and β-lactoglobulin. The ζ-potential of βlg-Cs-SL was observed to be 16 mV; the reduction in ζ-potential compared to Cs-SL indicates the attachment of positively charged β-lactoglobulin on top of the cationic chitosan surface through TPP bridging. The ff-SEM image of βlg-Cs-SL demonstrated an increase in diameter and presence of multilayers.

SLs were coated with Eudragit® S100 (Eu-SL) by nanoprecipitation through a modified solvent displacement method as explained in section 2.3 (Fig. 1 (c)). Coating of liposomes with Eudragit® S100 increased the diameter significantly by a factor of 3.33 to give a final diameter of 1300.72 nm (Fig. 2 (a)). When stored in an acetic acid-sodium acetate buffer (pH 4.5), Eu-SLs

demonstrated a ζ-potential of −45.79 mV (Fig. 2 (b)). The negative surface charge of Eu-SL is attributed to the anionic nature of the said polymer, this value is in line with previous research by Barbosa et al. [65] The enteric coating of Eudragit® S100 around the liposomal spherical surface was also visualized in their CLSM micrographs. For Eu-SL, a substantial increase in dimeter and wall thickness was observed when compared to SL (Fig. 3 (b)). To coat SLs with Eudragit®, 6-mM acetic acid-sodium acetate buffer (pH 4.5) and PEG-400 were used as non-solvent and solvent, respectively. When SL-loaded Eudragit®-PEG-400 solution was extruded into the non-solvent phase; the solvent (i.e., PEG-400) undergoes a continuous disintegration process owing to its miscibility with non-solvent. This interfacial relocation of PEG facilitates nanoprecipitation of the liposome-loaded polymer aggregates in the shape of miniscule particulates.[66] Precipitation of the polymer particulates from the solvent and non-solvent mixture (i.e., PEG-400 and acetic acid-sodium acetate buffer) through centrifugation followed by repeated washing allowed separation of Eu-MLs.[67] PEG-400 is a non-toxic and non-immunogenic compound and its use as a solvent enabled us to avoid using any toxic organic solvents, which are frequently used in traditional nano-precipitation methods. The characteristics of synthesized particles formed through nanoprecipitation depends on the interfacial interaction between these two liquid phases, which is dictated by the Marangoni effect.[68] We suspect that PEG-400's surface-active properties help to stabilize the interface that is formed between the polymer solution and the water. PEG-400's ability to interact with other hydrogen bond donors promotes its strong interaction with the carboxyl group of Eudragit® S100. Thus, in this nanoprecipitation process, PEG-400 is not only acting as a solvent, but also as a stabilizer.[69,70]

The stability of both coated and uncoated liposomes was evaluated under simulated gastric and intestinal conditions (Fig. 5). For uncoated SLs, 41 and 28 % of vitamins C and E were respectively released during the first 2 h of initial incubation in SGF, whereas for βlg-Cs-SLs in SGF after 2 h of incubation, around 95 % of the encapsulated vitamins C and E remained intact. For Eu-SLs, the

amount of payload retention was 97 and 98 % for vitamin C and E after 2 h of treatment in the SGF condition. After 2 h of incubation in the SGF, coated and uncoated liposomes were transferred to SIF. For SL, within the first hour of incubation in the SIF, a substantial amount of the encapsulated cargo was released. For βlg-Cs-SL, during treatment in the SIF, both vitamins demonstrated a burst release pattern resulting in the release of around 90 % of the encapsulated lipophilic and hydrophilic cargos. Eu-SL demonstrated a gradual release of vitamin E and C in the SIF and released more than 90 % of the encapsulated payload during 4 h of incubation (Fig. 5 (A) and (B)). From the comparison between developed chitosan and β-lactoglobulin dual coating with respect to commercially available Eudragit® S100 coating, it could be concluded that this dual coating is equally effective in shielding payload from degradation in the stomach and facilitating target-specific release in the intestinal environment. In their work, Shalaby et al. explored chitosan's ability to protect liposomal payload in the gastric environment.[71] They encapsulated recombinant human insulin (Humilin-N®) in unilamellar vesicular type liposomes synthesized through THF and observed an insulin EE of 85.7 %. Liposomal surface charge was modulated by incorporating N-[1-(2, 3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) in the phospholipid bilayer. DOTAP provides liposomes with positive surface charge, which increases insulin entrapment along with enhancing residence time of liposomes in the endothelial tract. Furthermore, liposomes were coated with chitosan to provide protection against enzymatic and acid-mediated degradation in the stomach, along with increasing liposomes' residence time in the small intestine by exploiting chitosan's mucoadhesive properties. Cationic chitosan-coated liposomes demonstrated 18.9 and 73.3 % release of loaded insulin after 48 h of incubation in SGF and SIF, respectively. In *ex vivo* intestinal mucoadhesion test, chitosan-coated cationic liposomes' tissue residence time was substantially higher compared to that of the uncoated liposomes. When orally administered in streptozotocin-induced diabetic mice, a significant reduction in blood glucose level was observed within 1 h of oral administration, and the effect was

sustained for 8 h after administration.[71] Shao et al. used the solvent injection method to synthesize liposomes loaded with Coenzyme Q10, a lipophilic benzoquinone.[72] Liposomes were coated with chitosan and d-a-tocopheryl polyethylene glycol 1000 succinate (TPGS) to enhances drug stability, cellular uptake, and for prolonging circulation time. For chitosan and TPGS coated liposomes, excellent storage stability was observed, in addition to good stability in acidic pH. Furthermore, coated liposomes demonstrated significantly higher mucin penetration ability compared to their uncoated counterparts. TPGS and chitosan-coated liposomes increased cellular uptake of CoQ10 in Caco-2 cells by around 30-fold when compared to the untreated drug. When orally administered in rats, coated liposomes demonstrated an extended and sustained CoQ10 release profile for up to 24 h and caused a 3.4-fold increase in systemic exposure of CoQ10 when compared to untreated drug. However, most of these works involve the application of toxic organic solvents in liposome synthesis and/or during their surface coating. Against this backdrop, in this work we have synthesized liposomes through a novel one-step, environmentally benign technology, followed by their surface decoration by two different enteric coating strategies (i.e., dual layer of chitosan and β-lactoglobulin or a layer of Eudragit® S100) without the use of any organic solvent throughout the process.

To further illustrate the bioactive release kinetics from coated liposomes, the release of vitamins E and C from Eu-SL and βlg-Cs-SL in the SIF was modelled using three different approaches discussed in section 2.8. The goodness of fit for a specific model was determined by measuring the ARD (Eq. 5) between experimentally obtained value of released-bioactive with respect to the predicted amount and has been mentioned in Table 1 along with the predicted values of other reaction constants. For both βlg-Cs-SL and Eu-SL, the poor fit of the Higuchi equation, as observed by the high ARD values (Table 1 (a) and (b)), indicates that the diffusion of vitamin C from liposomal core is not completely governed by Fickian diffusion. Thus, it was postulated that bioactive release from surface coated liposome is rather governed by a multistep process which involves the initial dissolution of the

liposome's surface coating in small intestine, followed by collapse of the liposome's bilayer structure. This has been validated by the improved prediction of bioactive release from βlg-Cs-SL and Eu-SL by the Hixon Crowell and Sahlin-Peppas equations, respectively (Table 1). Hixon Crowell equation explains the release of bioactive from a spherical particle when there is a change in its surface area and volume because of the decrease in its diameter during dissolution phase, while the spherical shape of the particle remains intact. Blg-Cs-SL undergoes structural disintegration in the high-pH SIF condition due to change in electrostatic interaction between the chitosan and β-lactoglobulin layers. Thus, the better prediction of bioactive release from βlg-Cs-SL by the Hixon Crowell equation could be attributed to the structural disintegration process of βlg-Cs-SL in the SIF, which happens through initial pH-triggered shedding of the outer biopolymer layer while retaining spherical shape of the liposomal core followed by complete dissolution of the structure resulting in burst release of the encapsulated bioactives. [73] For Eu-SLs, a better prediction of bioactive release by Sahlin-Peppas equation indicates that the release of vitamins from Eu-SLs is indeed happening through a complex process that involves release of encapsulated vitamins from the hydrated liposomal core and through the relaxation of polymer chains in the Eudragit® coating, representing the Fickian and non-Fickian types of diffusion, respectively. [74]

The storage stability of βlg-Cs-SL in an acetic acid-sodium acetate buffer (pH 4.5), while stored at 4 ^{o}C, was determined by measuring the change in its diameter and ability to retain encapsulated payloads for a duration of 30 days. The diameter of βlg-Cs-SL increased significantly by a factor of 1.3 ($p < 0.05$) (Fig. 7 (a)). The measured EE of vitamin E decreased by 10 % after storing the dispersion at 4 ^{o}C for 30 days; however, this change was not significant (Fig. 7 (b)). During storage, the retention of vitamin C decreased by 24 %. Several factors could have played a role in the reduced EE of vitamin C during storage: (i) leakage of aqueous cargo over time, (ii) osmotic swelling of βlg-Cs-SL during storage. This osmotic swelling can be attributed to permeation of storage buffer into the

liposomal aqueous core enabled by an increased permeability of chitosan and β-lactoglobulin dual-coating during storage. The liposomal aqueous core is a solution of vitamin C in TRIS buffer, and it possesses a higher osmotic pressure than the storage buffer. Consequentially storage buffer migrates into liposomal aqueous core; resulting in an increase in diameter and decrease in EE of vitamin C owing to dilution.

4. Conclusion

This study developed a novel and green technology amenable to scaleup for the synthesis and industrial production of multi-bioactive-loaded and surface-decorated liposomal microcapsules tailored for site-specific intestinal delivery. Liposomes were synthesized from SFPC (SL) through a one-step, eco-friendly, environmentally benign liposome synthesis technology that allows simultaneous encapsulation of both hydrophobic and hydrophilic bioactives without the aid of any toxic solvents. Synthesized SLs were decorated with a dual wall of chitosan and β-lactoglobulin (βlg-Cs-SL) to protect the encapsulated payload until reaching the targeted site in the gastrointestinal tract. The efficacy of this newly developed dual coating was compared to coating SLs with a commercially available pH responsive polymer Eudragit® S100 (Eu-SL). SLs demonstrated an average diameter of 390 nm for both enteric coatings. Similar values were also obtained for the diameter of the final product. Blg-Cs-SL and Eu-SL had average diameters of 1595 and 1300 nm, respectively. To evaluate the effectiveness of SL for bioactive encapsulation, vitamins E and C were used as model lipophilic and hydrophilic bioactives, with encapsulation efficiencies of 92 and 70 %, respectively. The stability of coated and uncoated SFPC liposomes was determined in simulated gastrointestinal fluids (SIF). For βlg-Cs-SL and Eu-SL, in SGF after 2 h of incubation, less than 5 % of the encapsulated vitamin C was released, whereas for uncoated liposomes, 41 % of vitamin C was released within 2 h of incubation period. In SIF, coated liposomes released most of their remaining payload when incubated for 4 h. The results of this investigation indicate that the chitosan and β-lactoglobulin dual coating provided substantial stability to liposomes by protecting their encapsulated payload from harsh simulated gastric conditions and facilitated their site-specific delivery in an intestine-like environment. The performance of this dual coating proved to be equally effective when compared to coating with a commercially available counterpart, Eudragit® S100. In conclusion, this study developed a novel, safe, and scalable method to synthesize surface-decorated liposomes, simultaneously co-encapsulating both lipophilic

and hydrophilic cargos. Their ability to maintain structural integrity in gastric conditions followed by site-specific, triggered release of encapsulated cargo in the intestine will make them highly suitable for targeted oral delivery of bioactives in pharmaceutical and food applications.

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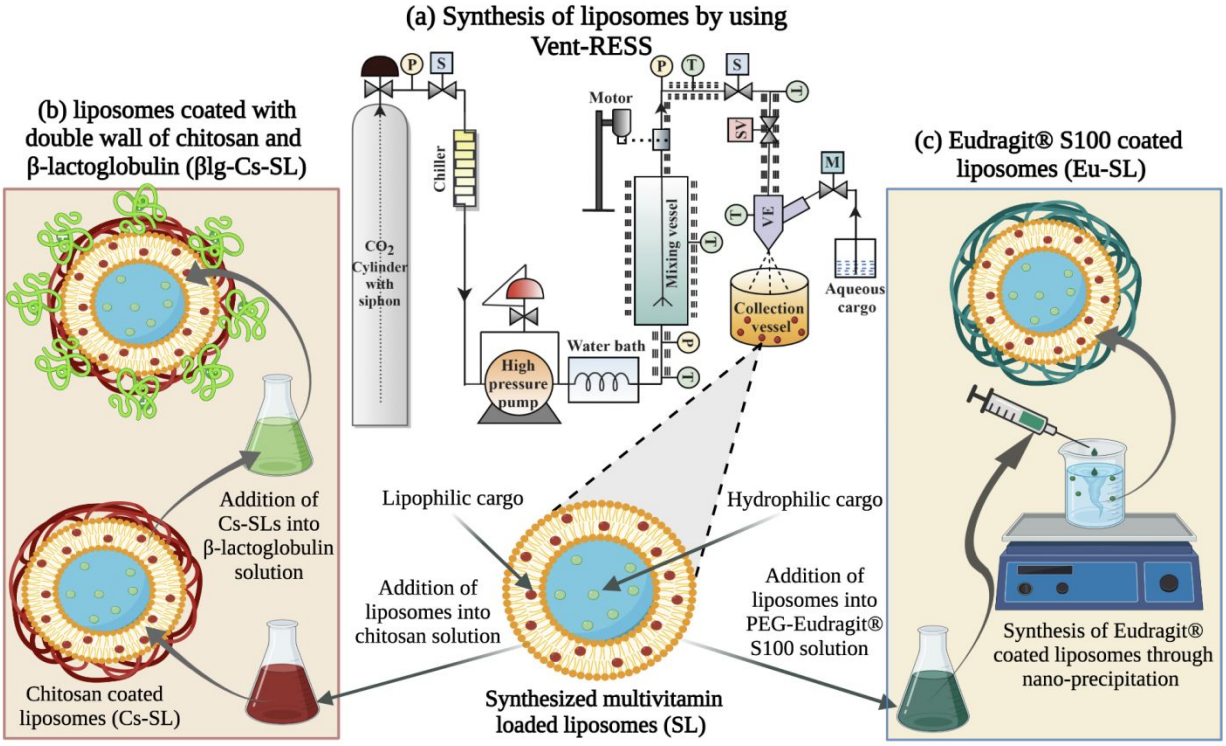

Fig. 1. Illustrative representation of (a) synthesis SFPC-liposomes (SL) through Vent-RESS system; and consecutive coating of SL with (b) dual layer of β-lactoglobulin and chitosan (βlg-Cs-SL), and (c) Eudragit® S100 (Eu-SL).

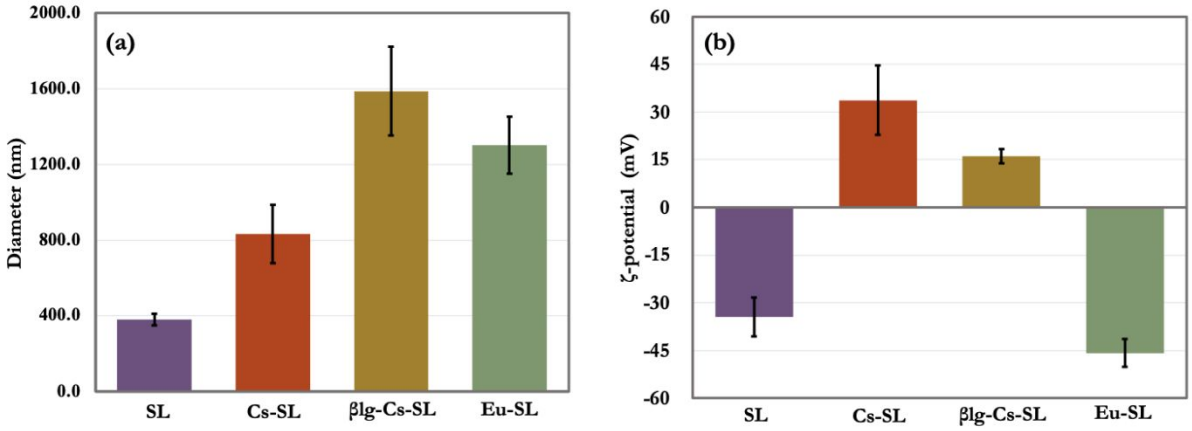

Fig. 2. (a) Average diameter and (b) ζ-potential of SFPC- liposomes (SL), chitosan coated liposomes (Cs-SL), β-lactoglobulin and chitosan coated liposomes (βlg-Cs-SL), and Eudragit® S100 coated SFPC liposomes (Eu-SL).

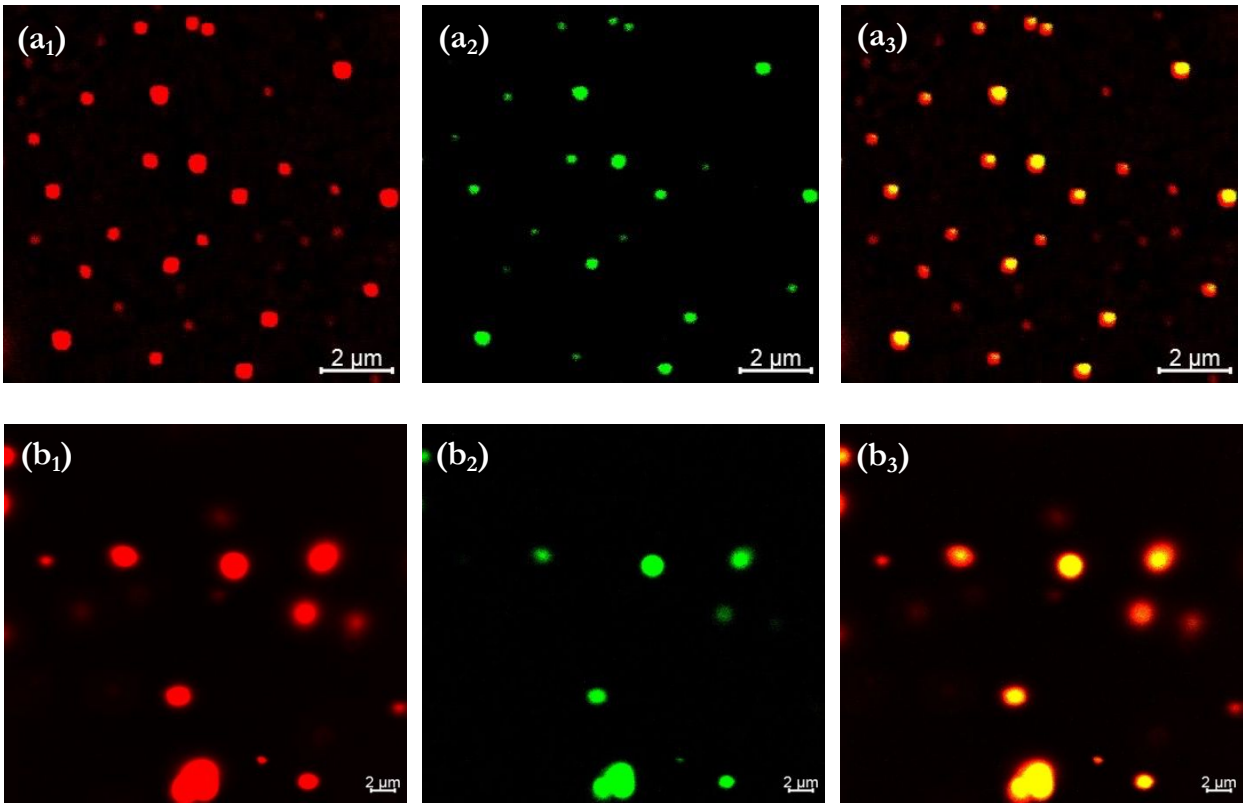

Fig. 3. Confocal laser scanning microscopy (CLSM) images of (a) SFPC liposomes (SL) and (b) Eudragit® S100 coated SFPC liposomes (Eu-SL).

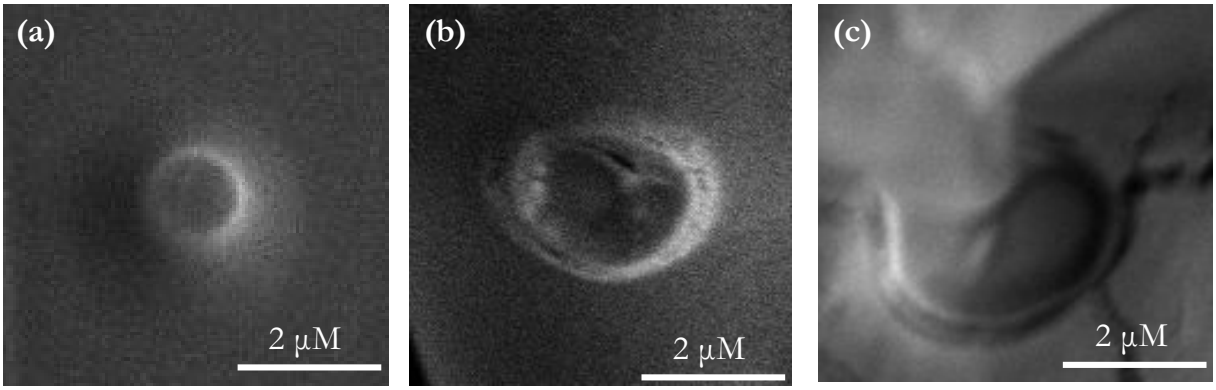

Fig. 4. Freeze fracture scanning electron microscopy (FF-SEM) images of (a) SFPC- liposomes (SL), (b) chitosan coated liposomes (Cs-SL) (c) β-lactoglobulin and chitosan coated liposomes (βlg-Cs-SL).

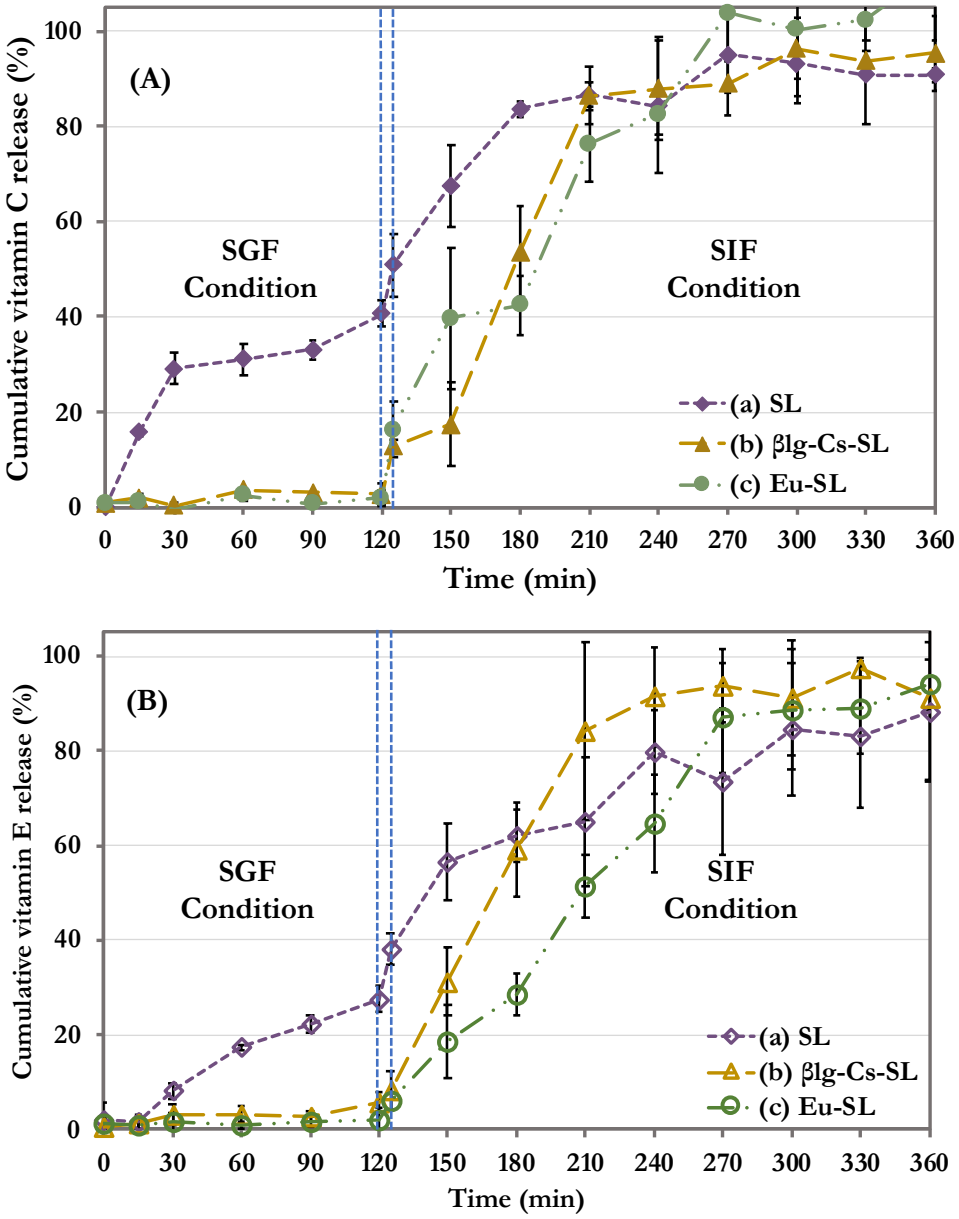

Fig. 5. Percentage release of (A) vitamin C and (B) vitamin E from (a) SFPC- liposomes (SL), (b) β-lactoglobulin and chitosan coated liposomes (βlg-Cs-SL), and (c) Eudragit® S100 coated SFPC liposomes (Eu-SL) during treatment in simulated gastric fluid (SGF) for 2 hours followed by treatment in simulated intestinal fluid (SIF) for an additional 4 hours.

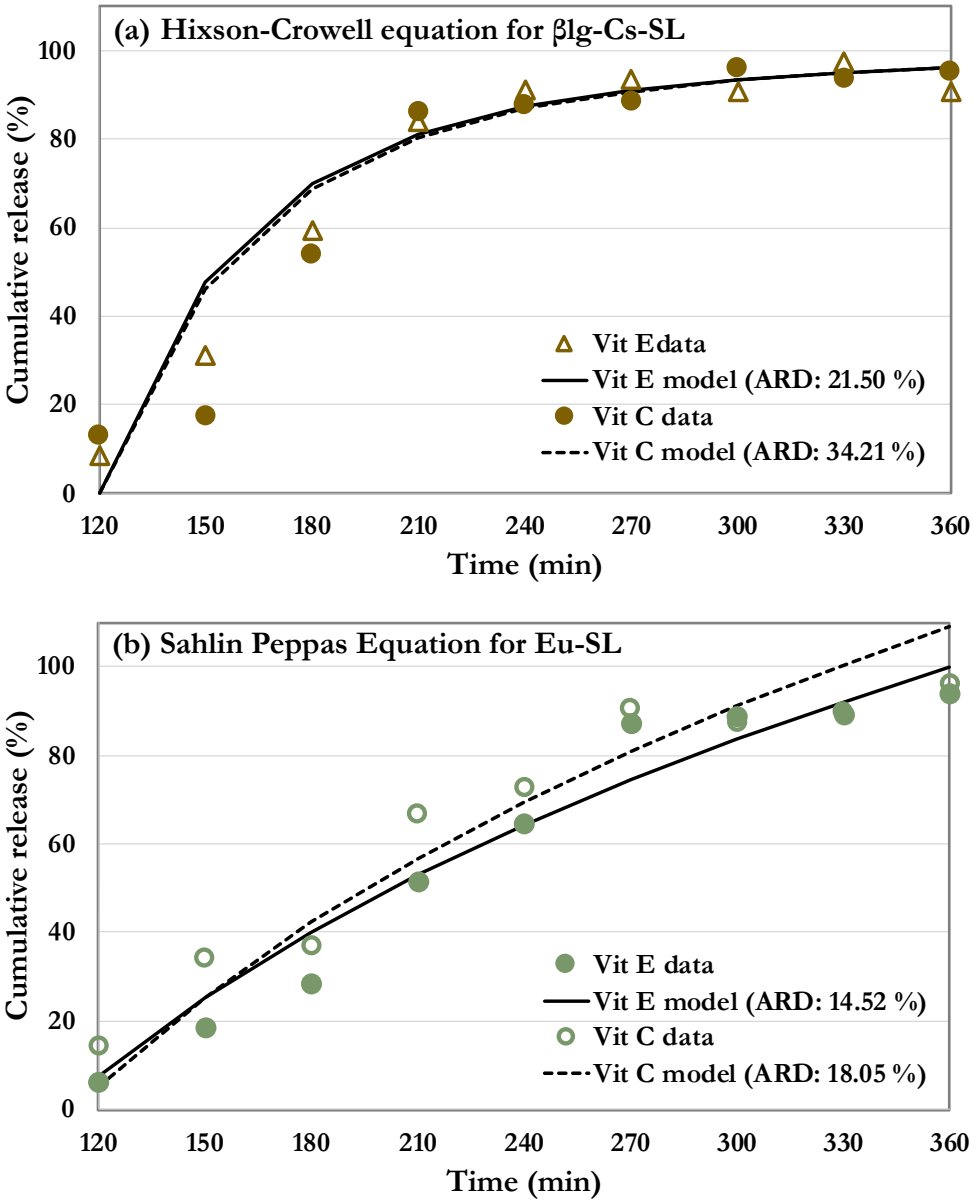

Fig. 6. The percent release of vitamins C and E from (a) β-lactoglobulin and chitosan coated liposomes (βlg-Cs-SL) in simulated intestinal fluid for 4 h has been modeled by using Hixon Crowell equation and (b) bioactive release from Eudragit® S100 coated SFPC liposomes (Eu-SL) in simulated intestinal fluid for 4 h has been modeled by using Sahlin Peppas equations. The fitness of the model has been represented by the absolute relative deviation (ARD) value and a lower ARD value represents better fit.

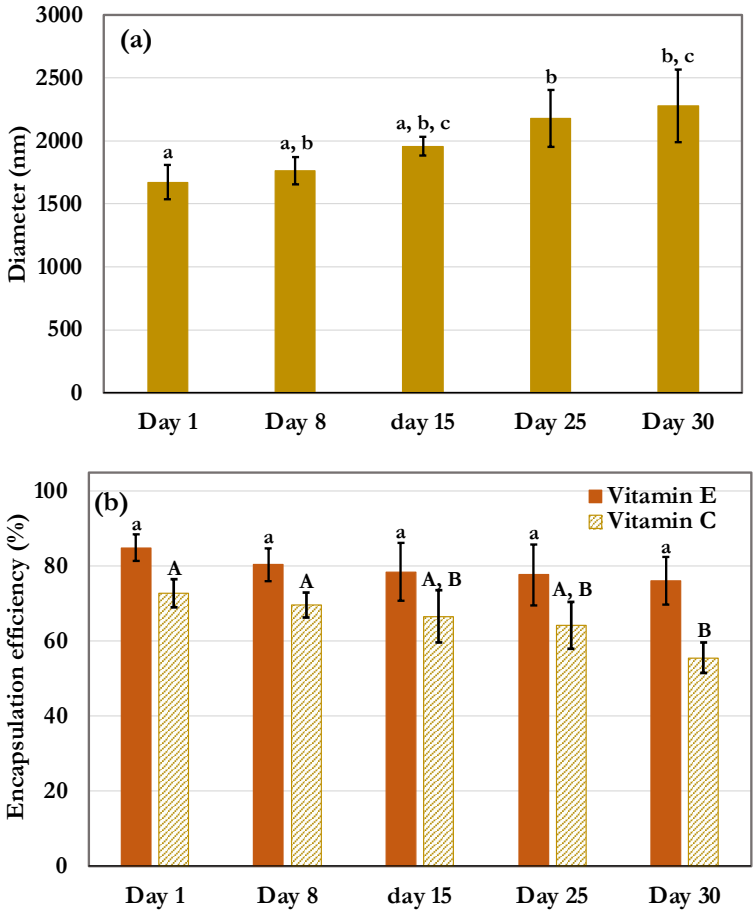

Fig. 7 Change in (a) diameter and (b) encapsulation efficiencies of vitamins E and C in β-lactoglobulin and chitosan coated liposomes (βlg-Cs-SL) during storage at 4 °C for 30 days. Alphabetical labels indicate statistically significant differences between treatments ($p < 0.05$).

Table 1. Predicted reaction constants obtained during modeling of percent release of vitamins C and E in simulated intestinal fluid from (a) β-lactoglobulin and chitosan coated liposomes (βlg-Cs-SL) and (b) Eudragit® S100 coated liposomes (Eu-SL) by using three different equations as mentioned in Section 2.8. The fitness of a model has been represented by the R^2 and the absolute relative deviation (ARD) values.

(a)

Vitamin type / **Equations**	**Vitamin C**			**Vitamin E**		
	Constants	R^2	ARD (%)	Constants	R^2	ARD (%)
Higuchi equation	$k_H = 4.78\ \text{min}^{-0.5}$	0.54	70.64	$k_H = 4.87\ \text{min}^{-0.5}$	0.56	78.34
Sahlin-Peppas equation	$k_{SP1} = -7108\ \text{min}^{-0.03}$ $k_{SP2} = 6551\ \text{min}^{-0.06}$ $n = 0.02$	0.58	42.47	$k_{SP1} = -1108\ \text{min}^{-0.05}$ $k_{SP2} = 889.2\ \text{min}^{-0.10}$ $n = 0.05$	0.84	32.68
Hixon Crowell equation	$k_{HC} = 0.01\ \%^{0.33}.\text{min}^{-1}$	0.84	34.21	$k_{HC} = 0.01\ \%^{0.33}.\text{min}^{-1}$	0.94	21.50

(b)

Vitamin type / **Equations**	**Vitamin C**			**Vitamin E**		
	Constants	R^2	ARD (%)	Constants	R^2	ARD (%)
Higuchi equation	$k_H = 4.45\ \text{min}^{-0.5}$	0.61	47.26	$k_H = 5.02\ \text{min}^{-0.5}$	0.52	109.25
Sahlin-Peppas equation	$k_{SP1} = -2417\ \text{min}^{-0.03}$ $k_{SP2} = 2080\ \text{min}^{-0.06}$ $n = 0.03$	0.90	18.05	$k_{SP1} = -1473\ \text{min}^{-0.04}$ $k_{SP2} = 1204\ \text{min}^{-0.08}$ $n = 0.04$	0.96	14.52
Hixon Crowell equation	$k_{HC} = 0.01\ \%^{0.33}.\text{min}^{-1}$	0.72	34.68	$k_{HC} = 0.01\ \%^{0.33}.\text{min}^{-1}$	0.62	46.61

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