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Improved Stability and Antidiabetic Potential of Insulin Containing Folic Acid Functionalized Polymer Stabilized Multilayered Liposomes Following Oral Administration

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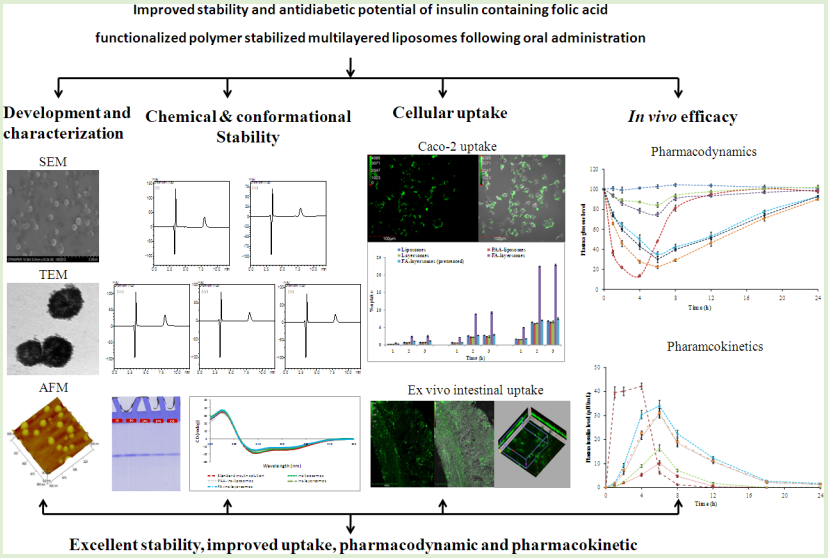
Supporting Information



ABSTRACT: The present study reports the folic acid (FA) functionalized insulin loaded stable liposomes with improved bioavailability following oral administration. Liposomes were stabilized by alternating coating of negatively charged poly(acrylic acid) (PAA) and positively charged poly(allyl amine) hydrochloride (PAH) over liposomes. Furthermore, folic acid was appended as targeting ligand by synthesizing folic acid−poly(allyl amine) hydrochloride conjugate. The insulin entrapped within the freeze-dried formulation was found stable both chemically as well as conformationally and developed formulation exhibited excellent stability in simulated biological fluids. Caco-2 cell and ex vivo intestinal uptake studies revealed higher uptake of folic acid functionalized layersomes in comparison with their plain counterparts. In vivo pharmacodynamic and pharmacokinetic studies further revealed almost double hypoglycemia and approximately 20% relative bioavailability in comparison with subcutaneously administered standard insulin solution. Overall the proposed strategy is expected to contribute significantly in the field of designing ligand-anchored, polyelectrolyte-based stable systems in drug delivery.

1. INTRODUCTION

Among the different proteins and peptides of therapeutic potential, insulin has been investigated extensively, which plays a crucial role in carbohydrate and fat metabolism and has been emerged as a principal therapy to treat certain forms of diabetes mellitus. Since the pioneering discovery of insulin, it is delivered via subcutaneous administration; however, lots of newer formulations with variable pharmacokinetic and sophisticated semiautomatic devices with lesser pain have emerged as a result of intensive research. Nevertheless, poor patient compliance, hypoglycemia, and peripheral hyperinsulinemia are the associated drawbacks of current therapy that need to be addressed.[1,2]

The quest to eliminate the needle phobia and associated problems with subcutaneous insulin has led to various alternative routes, namely, pulmonary, nasal, transdermal, buccal, and oral have been explored extensively.[3] Among these, the oral route seems to be most promising, offering better patient compliance and avoidance of major problems like peripheral hyperinsulinemia, transient hypoglycemia, and weight gain with the current therapy.[4,5] Although the oral route has been recognized as a better alternative to current therapy, enzymatic degradation in the gastrointestinal tract

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(GIT) and poor intestinal permeability are the major hurdles posing a challenge to the scientific community to make the insulin orally deliverable.[6]

With a view to find a way around, a variety of nanocarrier-based approaches, namely, polymeric nanoparticles,[7−9] liposomes,[10] emulsions,[11] and hydrogels,[12−14] have been reported to overcome the enzymatic barrier by protecting the entrapped insulin within the system, simultaneously conquering the poor permeability by additionally following the specialized uptake through intestinal M cells.[15,16] One step ahead, ligand-mediated active targeting emerged as a novel strategy to further fortify the effectiveness of these nanocarriers and improving the deliverability of therapeutic agents. Among the different receptors expressed over the GI tract, folic acid (FA) receptors have been reported to be present in sufficient quantity and fortified response by improving uptake and transport of bioactives or vesicular systems across the GI tract.[17,18] These findings were supported in our previous observations, in which insulin loaded FA-PLGA nanoparticles demonstrated strikingly higher bioavailability,[19] yet relatively low entrapment and poor scalability due to the high cost of PLGA that restricts their further commercial development.

Among various nanocarrier-based approaches, liposomes can be considered the most prospective candidate due to biocompatibility, biodegradability, and high drug loading for both hydrophilic and hydrophobic drugs and well established technology for industrial scalability. However, instability due to aggregation in the gastric environment and degradation in the presence of bile salts and pancreatin lipase are the major hurdles that have to be overcome to make the liposomes orally deliverable.[20]

In the present report, to make the liposomes orally deliverable, layer-by-layer coating of oppositely charged polyelectrolytes over the liposomes core was applied which ultimately resulted in the formation of robust structure "Layersomes" which demonstrated the advantages of both particulate systems (increasing storage stability and robustness) as well as vesicular system (high drug load). Besides the stabilization by using layer-by-layer approach, FA was also appended as a ligand to explore the targeting potential of the developed system to deliver the bioactives in a rational way. We also speculated the commercial feasibility after successful exploration of the concept, as well established scale-up technology is already available for liposomes. Therefore, it was interesting to investigate the efficacy of layersomes in providing stability to one of the most sensitive drug, that is, insulin, simultaneously investigating its targeting potential toward the FA receptors widely distributed across the GI tract. As far as the present literature is concerned, this is the first report that explores the unique combination of layer-by-layer strategy with targeting potential in the delivery of therapeutic moieties.

2. MATERIALS AND METHODS

2.1. Materials. Phosphotidylcholine (Epikuron 200, >90% PC) was provided as a generous gift from Cargill Corporation, Germany. Insulin (recombinant human), cholesterol (CH), stearylamine (SA), poly(allylamine hydrochloride) (PAH, MW ∼ 15000 D), poly(acrylic acid) (PAA, MW ∼ 5100 D) streptozotocine, acrylamide, *N*,*N*′-methylenebisacrylamide, ammonium persulpahte (APS), tetramethylethylenediamine (TEMED), and insulin-FITC (>1 mol of FITC in 1 mol of insulin) were procured from Sigma Aldrich, St. Louis, MO. Folic acid (FA) was purchased from Sisco Research Lab. Pvt. Ltd., (Mumbai, India). Trifluoroacetic acid, pancreatin, and pepsin were obtained from Loba Chemie (Mumbai, India). Ultrapure water (LaboStar ultrapure water systems, Germany) was prepared in house and used for all the experiments. All other reagents used were of analytical grade.

2.2. Synthesis and Characterization of FA-PEG-PAH Conjugate. In order to design the system with target specificity, the FA-PEG-PAH conjugate was synthesized in two steps. Briefly, folic acid (5 mg) was dissolved in water by making the water slightly alkaline with dropwise addition of NaOH solution followed by activation of carboxyl groups of folic acid with EDC/NHS for 4 h. PEG (20 mg, MW 2000) was added to the above solution and stirred for 12 h to get the reaction complete. The resulting intermediate (folate-PEG-COOH) was further activated with EDC/NHS for 4 h in water. The activated intermediate was added dropwise to previously neutralized PAH (10 mg, MW 15000 D). The reaction mixture was stirred for 12 h and resulting product was dialyzed for 24 h to remove unreacted reactants and freeze-dried. The synthesized conjugate was characterized by proton NMR.

2.3. Preparation of Insulin-Loaded Liposomes (Ins-Liposomes). Insulin liposomes were prepared by a thin film hydration method.[21] Briefly PC, CH, and SA were dissolved in 10 mL of a choloroform and methanol mixture (1:1 v/v) and the thin film was formed in a rotary evaporator under vacuum at 70 rpm and 37 °C. Following film formation, the film was hydrated with insulin solution (25 mL) and subsequently sonicated in a probe sonicator. Insulin solution was prepared by dissolving a weighed quantity of insulin in a minimum quantity (500 μL) of 0.01 M HCl and diluted up to 100 mL with water. The final pH of the insulin solution was adjusted to 7.4 by dropwise addition of 0.01 N NaOH solution. Exhaustive optimization of different process variables, such as PC/CH mole ratio, time for hydration, amount of SA, sonication time, and loading efficiency, was carried out. By considering reader's interest, detailed optimization has been provided in Supporting Information.

2.4. Preparation of Insulin-Loaded Layersomes. Insulin-loaded layersomes were prepared by alternating electrostatic deposition of PAA and PAH over the liposomes core as polyanion and polycation, respectively.[21,22] Briefly, cationic Ins-liposomes were taken as the core and anionic polyelectrolyte, PAA, was deposited over the cationic liposome core as the first coating layer. These negatively charged PAA-Ins-liposomes were further taken as a template for the deposition of cationic PAH as second layer, which ultimately resulted in the formation of layersomes. Folic acid anchored layersomes were prepared by using FA-PEG-PAH instead of PAH. Exhaustive optimization in terms of concentration and volume of polyelectrolytes solutions, stirring speed, and time was carried out, which has been provided in Supporting Information.

2.5. In Vitro Characterization. *2.5.1. Size and Zeta Potential Measurement.* Size and PDI of Ins-liposomes, PAA-Ins-liposomes, Ins-layersomes, and FA-Ins-layersomes were measured by dynamic light scattering, while zeta potential was determined on the basis of electrophoretic mobility under an electric field by using zetasizer (Nano ZS, Malvern, U.K.).[22]

2.5.2. Encapsulation Efficiency. The percentage of insulin entrapped within the system was determined by indirect method by measuring the free insulin concentration in supernatant. Briefly, formulation dispersion was centrifuged at 45000 rpm for 30 min at 4 °C by using ultracentrifuge (Hitachi WX Series, Rotor Model No. P50AT4−0092), and supernatant was collected and analyzed for insulin concentration by validated HPLC method.[23]

2.5.3. Shape and Morphology. Shape and morphology of the developed formulation was determined using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM). For SEM analysis, a drop of colloidal dispersion of different formulations was dropped on a glass coverslip previously adhered to a metallic stub by a biadhesive carbon tape. The drop was air-dried, coated with gold, and analyzed by SEM (S-3400N, Hitachi, Japan).[24,25]

Sample preparation for TEM analysis was done by placing a drop of each formulation over the Formvar-coated grid, dried, stained with

phosphotungstic acid (1% w/v) solution, and analyzed using TEM (Morgagni 268D, Fei Electron Optics) operated at 80 kV.[22]

AFM analysis was performed by placing a drop of different formulations on the silicon wafer with the help of a pipet and allowed to dry in air. The microscope is vibration damped and measurements were made using commercial pyramidal silicon nitride (Si_3N_4) tips (Veeco's CA, U.S.A.). The cantilever used for scanning was having a length of 325 mm and a width of 26 mm with a nominal force constant 0.1 N/m. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded.[26]

2.6. Freeze-Drying. Developed formulations were freeze-dried (Vir Tis, Wizard 2.0, New York, U.S.A.) by our previously patented stepwise freeze-drying cycle with slight modification.[27] Freeze-drying cycle comprised of freezing at −60 °C for 8 h, primary drying at −60 to 20 °C for 42 h, and secondary drying at 25 °C for 2 h. A constant pressure of 200 Torr was applied in each step. A preliminary screening of different cryoprotectants, namely, dextrose, sucrose, lactose, trehalose, mannitol, glycine, and inulin was carried out at 5% w/v concentration. Trehalose was finalized based on preliminary screening and further optimized for varying concentration of 2.5−10% w/v. Freeze-dried formulations were examined for appearance of the cake, redispersibility index, and redispersibility score.

2.7. Stability Studies. *2.7.1. In Process Stability. 2.7.1.1. Chemical Stability.* The chemical integrity of the insulin entrapped within the freeze-dried formulations was analyzed by RP-HPLC and native gel electrophoresis.[19] Freeze-dried formulations were resuspended in water (1 mL) and entrapped insulin was extracted by disrupting the vesicles using Triton X-100 (100 μL, 1% v/v) in case of liposomes, while using sonication for 1 h in case of PAA-Ins-liposomes, Ins-layersomes, and FA-Ins-layersomes.[22] After extraction, the formulations were centrifuged at 45000 rpm for 30 min, supernatant was collected and analyzed for chemical stability. In RP-HPLC analysis chromatograms obtained in case of different samples were compared for change in retention time or the presence of additional peaks, if any, due to chemical degradation. In gel electrophoresis, standard insulin along with insulin samples from different formulations were loaded into the wells (stacking gel 5%, resolving gel 15%) and subjected to electrophoresis at 20 mA and 200 V. Finally, resolved bands were visualized after staining with coomassie brilliant blue followed by thorough destaining.[28]

2.7.1.2. Conformation Stability. Approximately 350 μL of different samples (as in section 2.7.1.1) were placed in rectangular quartz cuvettes (path length 0.1 cm) and analyzed in triplicate at 25 °C using 1 nm bandwidth and 0.1 nm step size in the wavelength range of 250−190 nm[19] using circular dichroism (CD, J-815, JASCO Apparatus, Japan).

2.7.2. Stability in Simulated Biological Milieu. Stability of different formulations was determined in presence of simulated gastric fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 6.8) and phosphate buffer saline (PBS, pH 7.4). Briefly, 200 μL of different formulations were diluted up to 10 mL with different media followed by incubation at 37 °C for 2 h in SGF, 4 h in SIF and 6 h in PBS[22] and overall effect on size, PDI, zeta potential, and entrapment efficiency was measured. The drug retained within the formulations was extracted as mentioned in section 2.7.1.1.

2.8. In Vitro Release. In vitro release was performed in three different media SGF (pH 1.2), SIF (pH 6.8), and PBS (pH 7.4) to simulate almost all the physiological conditions encountered during the journey of a delivery system following oral administration. The composition of SGF and SIF was almost the same as used in section 2.7.2, except pepsin and pancreatin, which were excluded from the composition of SGF and SIF, respectively. Different formulations (20 μL) were diluted up to 1 mL with corresponding media and incubated at 37 °C with continuous shaking at 80 rpm in a shaker bath up to 2 h in the case of SGF, up to 4 h in the case of SIF, and up to 24 h in the case of PBS in separate micro centrifuge tubes. Contents of the micro centrifuge tubes corresponding to each time point were ultracentrifuged at 45000 rpm, and supernatant was collected and analyzed for insulin content by RP-HPLC method.

2.9. Caco-2 Cell Uptake. Caco-2 cells (American Type Culture Collection) were grown and cultured in a 96-well plate (Costars, Corning Incorporated) at a density of 5×10^4 cells/well by following our previous protocol.[26] Cultured cells were incubated with free insulin-FITC conjugate at 10 μg/mL and insulin-FITC-loaded formulations at different concentrations of 1, 3, and 10 μg/mL for 1, 2, and 3 h. HBS solution was used for all the dilutions, and blank HBS solution was used as negative control. Following incubation cells were thoroughly washed with PBS and visualized under a confocal microscope (Model Olympus FV 1000). For quantitative determination, cells were lysed by adding 200 μL of 0.1% Triton X-100 and fluorescence was measured at 490 and 525 nm, excitation and emission wavelength, respectively. Additionally, to study any contribution of receptor mediated pathway in the cellular uptake of FA-layersomes, Caco-2 cells were preincubated with excess of FA to block this additional path of cellular uptake before incubating the cells with FA-layersomes.

2.10. Ex Vivo Intestinal Uptake. Confocal laser scanning microscopy (CLSM) was performed to access the uptake and localization of the different formulations into intestinal region. Briefly, free Insulin-FITC (10 μg/mL) and insulin-FITC-loaded formulations (in equivalent concentration) were administered orally in overnight fasted Sprague−Dawley rats. After 4 h of administration, rats were sacrificed and duodenal region was dissected and washed thoroughly with Ringer's solution. Circular tissue sections were prepared, mounted on slide, and visualized using CLSM.[29]

2.11. In Vivo Studies. *2.11.1. Animals.* Adult male Sprague−Dawley rats (250 ± 30 g) were procured from the central animal facility of the NIPER, India, and used for pharmacodynamics and pharmacokinetics evaluation of developed formulations. Animals were kept in plastic cages and had free access to water and food and maintained at temperature of 25 ± 2 °C and relative humidity of 50−60% under 12 h light/dark cycles. All the experimental protocols were dually approved by Institutional Animal Ethics Committee (IAEC), NIPER, India. All the experiments were performed in accordance with guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

2.11.2. Antidiabetic Activity. Diabetes was induced in animals by intraperitoneal administration of STZ at a single dose of 55 mg/kg by following our previous protocol.[19] Diabetic animals were randomly distributed into seven groups containing 6 animals in each group and kept on overnight fasting prior to initiation of study. Groups 1−4 received Ins-liposomes, PAA-Ins-liposomes, Ins-layersomes, and FA-Ins-layersomes orally at a dose equivalent to 50 IU/kg of insulin. Group 5 was treated with oral standard insulin solution at a dose of 50 IU/kg and considered as negative control, while group 6 received standard insulin solution subcutaneously at a dose of 5 IU/kg and considered as positive control. To further verify the receptor-mediated uptake of FA-Ins-layersomes, group 7 was pretreated with excess of FA (saturated solution of FA) to saturate the receptors prior to oral administration of FA-Ins-layersomes. Blood samples (~300 μL) at different time points (0, 1, 2, 4, 6, 8, 12, 18, and 24 h) were collected through retro orbital plexus under mild anesthesia and further centrifuged at 10000 rpm for 10 min at 4 °C. Plasma samples were collected and divided in two parts, out of which one was used for glucose estimation while the other was used for insulin estimation and stored at −80 °C till further analysis. Plasma glucose level was determined by glucose oxidase-peroxidase method using commercially available kit (LiquiMAX Glucose, Avecon Healthcare Pvt. Ltd.) by strictly following the manufacturer protocol. The cumulative hypoglycemic effect following oral administration was determined by comparing AUC value of individual treatment group with negative control group by using following equation:

$$\text{cumulative hypoglycemia}(\%) = \frac{\text{AUC}_{(\text{oral insulin})} - \text{AUC}_{(\text{treatment})}}{\text{AUC}_{(\text{oral insulin})}} \times 100$$

Pharmacological availability (PA) following oral administration of different formulations was determined by comparing AAC following

oral administration with AAC upon SC administration of insulin solution with dose correction as follows:[19]

$$\mathrm{PA}(\%) = \frac{\mathrm{AAC}_{(\mathrm{oral})} \times \mathrm{dose}_{(\mathrm{SC})}}{\mathrm{AAC}_{(\mathrm{SC})} \times \mathrm{dose}_{(\mathrm{oral})}} \times 100$$

where oral and SC denotes route of administration through oral and subcutaneous routes, respectively.

2.11.3. Pharmacokinetics. Plasma insulin concentrations were determined using insulin ELISA kit (ELH-Insulin-001, RayBiotech Inc. Norcross, U.S.A.) by following the manufacturer's instructions supplied with the kit. Plasma insulin concentration (μIU/mL) versus time profile was created and relative bioavailability ($\mathrm{BA_R}$) was calculated using the following formula:

$$\mathrm{BA_R}(\%) = \frac{\mathrm{AUC}_{(\mathrm{oral})} \times \mathrm{dose}_{(\mathrm{SC})}}{\mathrm{AUC}_{(\mathrm{SC})} \times \mathrm{dose}_{(\mathrm{oral})}} \times 100$$

2.12. Statistical Analysis. All in vitro results have been represented as mean ± standard deviation (SD), while all in vivo results have been represented as mean ± standard error of mean (SEM). Statistical analysis was performed by applying one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test; $p < 0.05$ was considered as statistically significant.

3. RESULTS

3.1. Synthesis and Characterization of FA-PEG-PAH Conjugate. Overlay NMR spectra of synthesized conjugate and individual components is shown in Figure 1. The chemical shift values at δ 3.6, 2.6, 1.9, 1.5, 6.8, 7.5, and 8.2 ppm was observed in the synthesized conjugate.

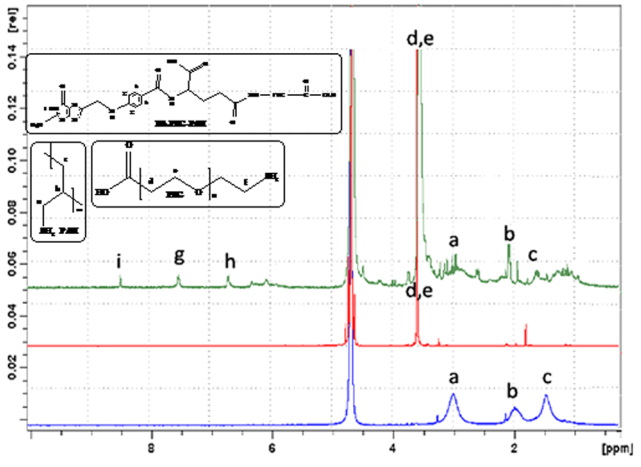

Figure 1. ^{1}H NMR spectra of FA-PEG-PAH.

3.2. Preparation and Optimization of Insulin-Loaded Formulations. Exhaustive optimization of various preparative variables resulted in the formation of different formulations with desired quality attributes (Supporting Information). The size, PDI, zeta potential, and entrapment efficiency (EE) of different formulations is shown in Table 1.

Morphology determination by SEM, TEM, and AFM analysis further confirmed the formation of almost spherical shape structures. The results obtained in all the techniques were also in good correlation with the results of DLS analysis (Figure 2).

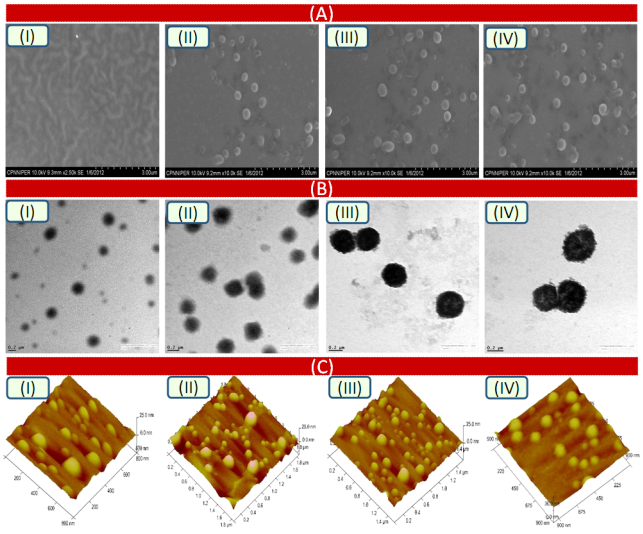

Figure 2. Morphology determination using (A) SEM, (B) TEM, and (C) AFM. I, II, III, and IV represent Ins-liposomes, Ins-PAA-liposomes, Ins-layersomes, and FA-Ins-layersomes, respectively.

3.3. Freeze-Drying. Different formulations were freeze-dried to convert them into dried form and provide long-term stability. Effect of freeze-drying on quality attributes of different formulations is shown in Table 2. On the basis of the redispersibility index with different cryoprotectants, trehalose was finalized as it resulted in the formation of voluminous, easy to redisperse cake with redispersibility index close to one. However, other cryoprotectants resulted in either collapsed or hard to redisperse cake, which upon reconstitution resulted in aggregation, as is evident by a high redispersibility index. Therefore, trehalose was selected and further optimized for suitable concentration. Trehalose (5% w/v) resulted in a redispersibility index close to 1. At lower concentration (2.5%), trehalose exhibited significant increase ($p < 0.01$) in redispersibility index in comparison with 5% w/v trehalose, while the difference was insignificant ($p > 0.05$) at higher concentrations (Table 3).

3.4. Stability Studies. *3.4.1. In Process Stability. 3.4.1.1. Chemical Stability.* The RP-HPLC chromatograms obtained in case of standard insulin and insulin extracted from different formulations is shown in Figure 3. The entrapped insulin in freeze-dried formulation was found chemically stable

Table 1. Quality Attributes of Optimized Formulations[a]

parameters	size (nm)	PDI	ZP (mV)	EE (%)
Ins-liposomes	228.6 ± 15.7	0.253 ± 0.04	+34.9 ± 2.5	88.2 ± 2.3
Ins-PAA-liposomes	241.3 ± 12.3	0.235 ± 0.02	−36.2 ± 1.9	91.2 ± 1.8
Ins-layersomes	254.6 ± 8.6	0.231 ± 0.03	+33.6 ± 2.1	93.6 ± 2.6
FA-Ins-layersomes	266.2 ± 10.4	0.246 ± 0.06	+25.4 ± 2.6	92.9 ± 1.4

[a]Values are presented as mean ± SD ($n = 6$).

Table 2. Preliminary Screening of Different Cryoprotectants Based on Redispersibility Index and Reconstitution Score for Lyophilization of Ins-Liposomes, PAA-Ins-Liposomes, Ins-Layersomes, and FA-Ins-Layersomes[a]

	Ins-liposomes			PAA-Ins-liposomes			Ins-layersomes			FA-Ins-layersomes		
	PS (nm)	Ri = (Sf/Si)	RS	PS (nm)	Ri = (Sf/Si)	RS	PS (nm)	Ri = (Sf/Si)	RS	PS (nm)	Ri = (Sf/Si)	RS
IS	228 ± 15			241 ± 12			254 ± 8			266 ± 10		
D	624 ± 30	2.73 ± 0.13	**	684 ± 25	2.68 ± 0.10	**	679 ± 28	2.81 ± 0.11	**	644 ± 28	2.42 ± 0.10	**
S	554 ± 28	2.42 ± 0.12	**	609 ± 16	2.39 ± 0.06	**	576 ± 44	2.39 ± 0.18	**	604 ± 23	2.27 ± 0.08	**
L	458 ± 27	2.01 ± 0.12	**	461 ± 14	1.81 ± 0.05	**	471 ± 31	1.95 ± 0.12	**	442 ± 19	1.66 ± 0.07	**
T	**301 ± 21**	**1.31 ± 0.09**	***	**275 ± 9**	**1.08 ± 0.03**	***	**291 ± 15**	**1.22 ± 0.04**	***	**283 ± 9**	**1.06 ± 0.03**	***
M	387 ± 25	1.69 ± 0.11	**	397 ± 13	1.56 ± 0.05	**	395 ± 18	1.64 ± 0.07	**	406 ± 22	1.52 ± 0.08	**
G	1169 ± 36	5.11 ± 0.15	*	1250 ± 26	4.90 ± 0.10	*	1165 ± 39	4.83 ± 0.16	*	1254 ± 33	4.71 ± 0.12	*
I	1122 ± 58	4.91 ± 0.25	*	1252 ± 33	4.91 ± 0.13	*	1198 ± 38	4.96 ± 0.15	*	1249 ± 33	4.69 ± 0.12	*

[a]PS, particle size; Ri, redispersibility index; RS, reconstitution score; IS, initial size; D, dextrose; S, sucrose; L, lactose; T, trehalose; M, mannitol; G, glycine; I, inulin (***redispersible within 20 s with mere mixing, **redispersible within 1 min, *reconstitution requires high shear vortexing for 2 min, but the cake was not completely redispersed). Bold portion indicates the optimized one. Values are presented as mean ± SD (n = 6).

Table 3. Optimization of Trehalose Concentration Based on Redispersibility Index and Reconstitution Score for Lyophilization of Ins-Liposomes, PAA-Ins-Liposomes, Ins-Layersomes, and FA-Ins-Layersomes[a]

	Ins-liposomes			PAA-Ins-liposomes			Ins-layersomes			FA-Ins-layersomes		
	PS (nm)	Ri = (Sf/Si)	RS	PS (nm)	Ri = (Sf/Si)	RS	PS (nm)	Ri = (Sf/Si)	RS	PS (nm)	Ri = (Sf/Si)	RS
IS	228 ± 15			241 ± 12			254 ± 8			266 ± 10		
2.5%	345 ± 20	1.51 ± 0.08	**	356 ± 19	1.48 ± 0.07	**	325 ± 11	1.27 ± 0.05	**	318 ± 14	1.19 ± 0.05	**
5%	**301 ± 21**	**1.31 ± 0.09**	***	**291 ± 15**	**1.22 ± 0.04**	***	**275 ± 9**	**1.08 ± 0.03**	***	**283 ± 9**	**1.06 ± 0.03**	***
7.5%	301 ± 27	1.31 ± 0.11	***	287 ± 12	1.19 ± 0.04	***	278 ± 10	1.09 ± 0.04	***	285 ± 13	1.07 ± 0.05	***
10%	310 ± 22	1.35 ± 0.09	***	292 ± 14	1.20 ± 0.05	***	283 ± 10	1.11 ± 0.05	***	293 ± 12	1.10 ± 0.04	***

[a]PS, particle size; Ri, redispersibility index; RS, reconstitution score; IS, initial size (***redispersible within 20 s with mere mixing, **redispersible within 1 min). Bold portion indicates the optimized concentration. Values are presented as mean ± SD (n = 6).

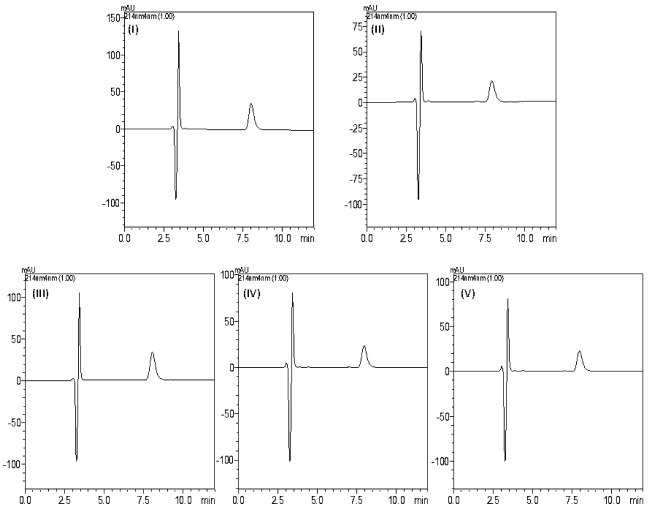

Figure 3. RP-HPLC chromatogram of (A) standard insulin solution and insulin extracted from (B) Ins-liposomes, (C) PAA-Ins-liposomes, (D) Ins-layersomes, and (E) FA-Ins-layersomes.

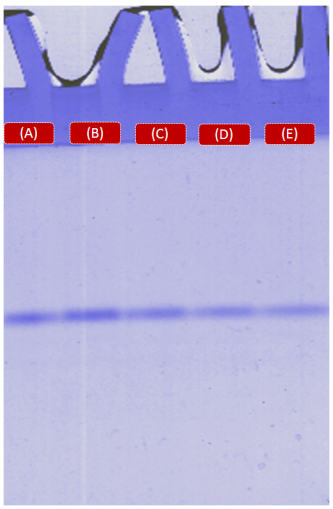

Figure 4. Resolved bands obtained in gel electrophoresis for (A) standard insulin solution and insulin extracted from (B) Ins-liposomes, (C) PAA-Ins-liposomes, (D) Ins-layersomes, and (E) FA-Ins-layersomes.

as no change in retention time and additional peaks were observed in RP-HPLC chromatograms obtained for different samples of insulin. Further chemical stability of the entrapped insulin was confirmed by native gel electrophoresis. Same level of bands (Figure 4) for standard insulin and insulin extracted from Ins-liposomes, PAA-Ins-liposomes, Ins-layersomes, and FA-Ins-layersomes confirmed the chemical stability of the entrapped insulin throughout the process.

3.4.1.2. Conformational Stability. The overlay circular dichroism (CD) spectra of standard insulin and insulin extracted from different formulations are shown in Figure 5. Different samples of insulin extracted from formulations were found to maintain their native conformation, as is evident from the superimposed CD spectrum obtained in all the cases.

3.4.2. Stability in Simulated Biological Milieu. The effect of different simulated biological fluids on formulation parameters is shown in Table 4. Ins-liposomes and PAA-Ins-liposomes were found unstable as significant change ($p < 0.05$) in all the formulation quality attributes was observed, while Ins-layersomes and FA-Ins-layersomes were quite stable as insignificant change ($p > 0.05$) in formulation quality attributes was observed after incubation in simulated biological fluids.

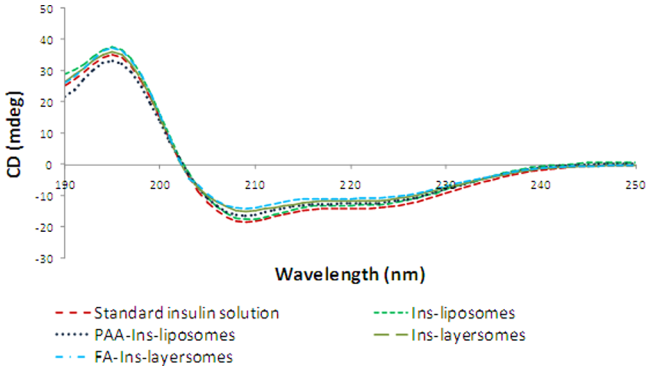

Figure 5. Overlay CD spectra of different insulin samples.

3.5. In Vitro Release. The in vitro release profile obtained with different insulin-loaded formulations is shown in Figure 6. An almost similar release profile was observed in all the fluids, irrespective of the pH of the medium. Cumulative percentage of drug released within 24 h approached to 72% in the case of Ins-layersomes, while it was approximately 96 and 92% in the case of Ins-liposomes and PAA-Ins-liposomes. Although FA modification did not affect the release profile significantly yet it was slightly increased in PBS while slightly decreased in SGF and SIF. Model fitting further revealed Baker-Lonsdale model of release in the case of Ins-liposomes and PAA-Ins-liposomes, while the Higuchi model was revealed in the case of Ins-layersomes and FA-Ins-layersomes (Table 5).

3.6. Caco-2 Cell Uptake. All the formulations exhibited higher uptake over free insulin-FITC, as evident by higher green fluorescence observed in CLSM analysis (Figure 7). Quantitative determination by spectrofluorimetry revealed time- and concentration-dependent cellular uptake of different formulations (Figure 8). The cellular uptake of free insulin-FITC was almost negligible. At all the concentrations, liposomes exhibited slightly higher, although insignificant, uptake in comparison to PAA-liposomes and layersomes. FA modification had a significant impact on cellular uptake and resulted in a 3.33-fold higher cellular uptake in comparison with liposomes at 10 μg/mL after 3 h of incubation. Pretreatment with excess of FA significantly reduced the cellular uptake, yet it was slightly higher in comparison to liposomes.

3.7. Ex Vivo Intestinal Uptake. Ex vivo intestinal uptake study further revealed higher uptake of insulin-FITC loaded formulations in comparison with free insulin-FITC (Figure 9). Visually distinguished higher fluorescence was observed in the case of FA-modified layersomes as compared to plain layersomes, as well as liposomes and PAA-liposomes. Apical 3D view confirmed the deeper uptake of FA-modified layersomes. A uniform high fluorescence was observed throughout the intestinal region, however, any specific confined area of uptake could not be identified.

3.8. Antidiabetic Activity. Blood glucose level (% of initial value) verses time profiles following administration of different formulations is depicted in Figure 10. Subcutaneous insulin exhibited severe transient hypoglycemia as up to 87% reduction in blood glucose level was observed within 4 h. Oral insulin solution was practically inactive as no blood glucose reduction was observed, rather somewhat of an increase in basal glucose value was observed. Although a slight fall in blood glucose level was observed in the case of Ins-liposomes and PAA-Ins-liposomes, yet it was insignificant ($p > 0.05$) in comparison with oral insulin solution. The blood glucose reduction observed in the case of Ins-layersomes was significantly higher ($p < 0.001$) in comparison with oral-insulin, Ins-liposomes, and PAA-Ins-liposomes. It exhibited up to 65% blood glucose reduction in 6 h, which reached the basal value during 24 h. In support of our hypothesis FA-Ins-layersomes exhibited highest hypoglycemic response among all the groups. The blood glucose level reduced up to 78% in 6 h which was maintained below 50% up to 12 h. Performance in terms of cumulative hypoglycemia and pharmacological availability of FA-Ins-layersomes revealed almost double (1.92-fold) cumulative hypoglycemia (Table 6) and 19.2 ± 0.5 BA_R in comparison with subcutaneously administered standard insulin solution (Table 7). Pretreatment with excess of FA exhibited slightly higher, although insignificant, hypoglycemic response in comparison with Ins-layersomes.

Table 4. Effect of Simulated Biological Fluids on Formulation Quality Attributes of Ins-Liposomes, PAA-Ins-Liposomes, Ins-Layersomes, and FA-Ins-Layersomes[a]

	size (nm)		PDI		zeta potential (mV)		drug entrapped (%)	
parameters	initial	final	initial	final	initial	final	initial	final
				Ins-Liposomes				
SGF pH 1.2	228.6 ± 15.7	120.9 ± 7.0	0.253 ± 0.040	0.349 ± 0.032	+34.9 ± 2.5	+16.5 ± 2.1	88.2 ± 2.3	28.6 ± 3.1
SIF pH 6.8	228.6 ± 15.7	202.8 ± 8.9	0.253 ± 0.040	0.335 ± 0.037	+34.9 ± 2.5	−13.8 ± 3.0	88.2 ± 2.3	32.4 ± 2.9
PBS pH 7.4	228.6 ± 15.7	240.1 ± 8.6	0.253 ± 0.040	0.294 ± 0.033	+34.9 ± 2.5	+6.7 ± 2.4	88.2 ± 2.3	31.6 ± 3.2
				PAA-Ins-Liposomes				
SGF pH 1.2	241.3 ± 12.3	-	0.235 ± 0.020	-	−36.2 ± 1.9	-	91.2 ± 1.8	-
SIF pH 6.8	241.3 ± 12.3	276.1 ± 12.2	0.235 ± 0.020	0.291 ± 0.030	−36.2 ± 1.9	−10.1 ± 2.5	91.2 ± 1.8	38.4 ± 3.6
PBS pH 7.4	241.3 ± 12.3	257.4 ± 9.3	0.235 ± 0.020	0.264 ± 0.024	−36.2 ± 1.9	−16.0 ± 2.1	91.2 ± 1.8	36.2 ± 2.8
				Ins-Layersomes				
SGF pH 1.2	254.6 ± 8.6	258.9 ± 5.4	0.231 ± 0.030	0.234 ± 0.017	+33.6 ± 2.1	+32.7 ± 3.8	93.6 ± 2.6	76.2 ± 2.4
SIF pH 6.8	254.6 ± 8.6	262.5 ± 9.8	0.231 ± 0.030	0.236 ± 0.010	+33.6 ± 2.1	+31.1 ± 3.4	93.6 ± 2.6	71.4 ± 3.1
PBS pH 7.4	254.6 ± 8.6	260.9 ± 7.2	0.231 ± 0.030	0.238 ± 0.011	+33.6 ± 2.1	+32.2 ± 3.1	93.6 ± 2.6	72.6 ± 2.6
				FA-Ins-Layersomes				
SGF pH 1.2	266.2 ± 10.4	271.7 ± 1.4	0.246 ± 0.060	0.249 ± 0.010	+25.4 ± 2.6	+23.2 ± 3.0	92.9 ± 1.4	78.4 ± 2.8
SIF pH 6.8	266.2 ± 10.4	278.4 ± 5.4	0.246 ± 0.060	0.255 ± 0.006	+25.4 ± 2.6	+23.7 ± 2.6	92.9 ± 1.4	72.8 ± 3.4
PBS pH 7.4	266.2 ± 10.4	273.6 ± 2.7	0.246 ± 0.060	0.234 ± 0.017	+25.4 ± 2.6	+24.6 ± 2.3	92.9 ± 1.4	70.3 ± 3.2

[a]Sign (-) represent precipitation. Values are presented as mean ± SD ($n = 6$).

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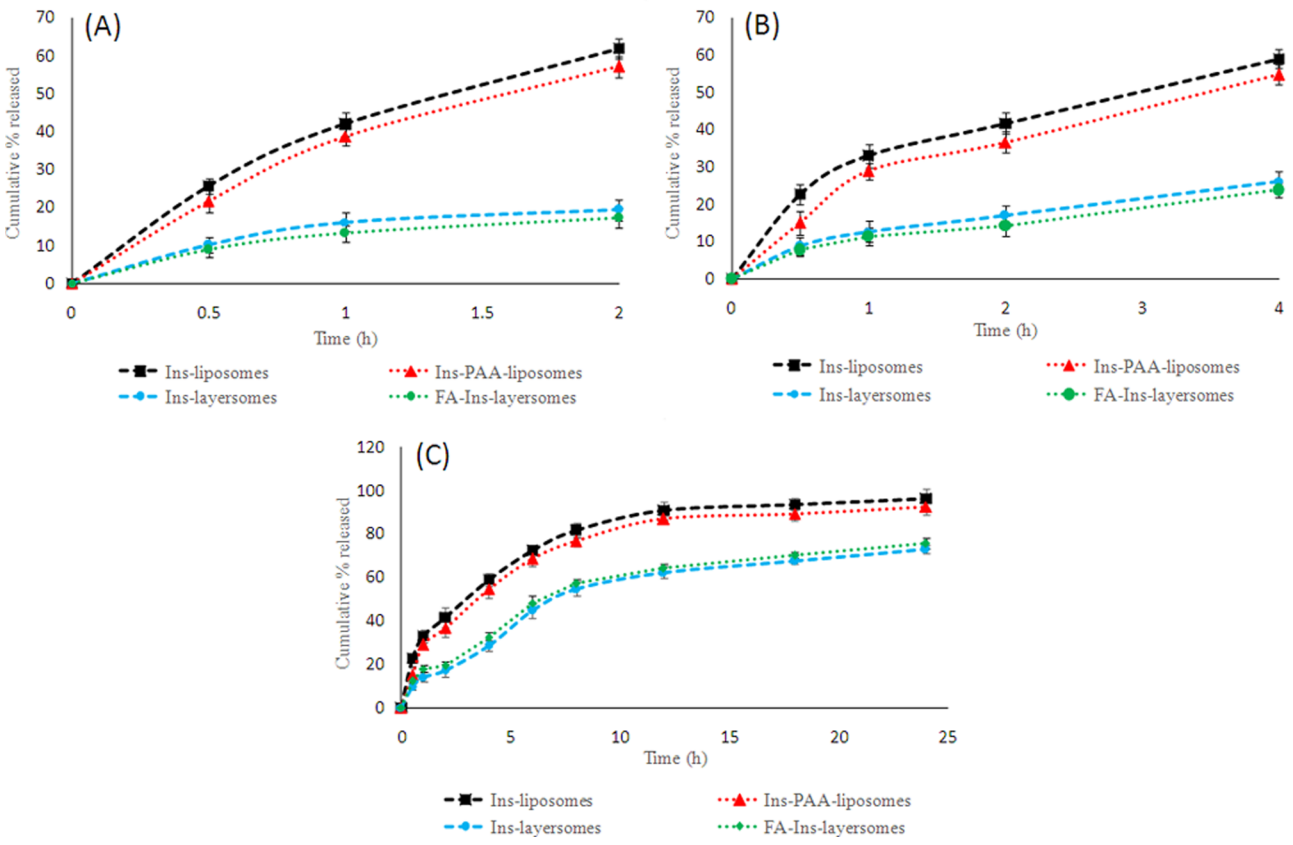

Figure 6. Comparative release profile of Ins-liposomes, PAA-Ins-liposomes, Ins-layersomes, and FA-Ins-layersomes in different simulated biological fluids: (A) SGF (pH 1.2), (B) SIF (pH 6.8), and (C) PBS (pH 7.4). Values are presented as mean ± SD (n = 6).

Table 5. Kinetic Models Used for Analysis of Insulin Release from Different Formulations and Overall Correlation Coefficient (R^2)

	correlation coefficient (R^2)			
release model	Ins-liposomes	PAA-Ins-liposomes	Ins-layersomes	FA-Ins-layersomes
zero order	0.821	0.850	0.841	0.830
first order	0.980	0.971	0.928	0.930
Hixson–Crowell	0.942	0.939	0.901	0.963
Weibull	0.968	0.973	0.962	0.963
Higuchi	0.970	0.976	0.974	0.972
Baker–Lonsdale	0.992	0.988	0.966	0.969
Korsmeyer–Peppas (n = 0.5)	0.970	0.976	0.962	0.963
Korsmeyer–Peppas (n = 0.75)	0.898	0.921	0.912	0.905
Korsmeyer–Peppas (n = 1.25)	0.750	0.781	0.769	0.757

3.9. Pharmacokinetics. Pharmacokintic profile obtained in case of different treatment groups is shown in Figure 11. Subcutaneously administered insulin exhibited highest concentration with a steep increase in one hour, followed by an insignificant increase up to 4 h, and a subsequent decrease to basal value after 8 h. In contrast, a gradual increase up to 6 h and a subsequent decrease was observed in the case of different formulations. FA-Ins-layersomes were able to maintain the insulin level above 12 μIU/mL up to 12 h and exhibited almost 2-fold higher AUC in comparison with insulin administered subcutaneously (Table 7). FA pretreatment saturated the FA receptors and came down the insulin level comparable to Ins-layersomes.

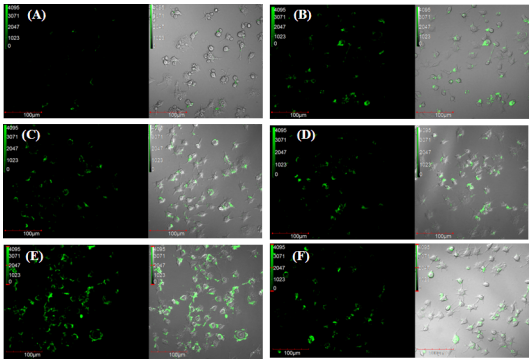

Figure 7. Representative confocal images of Caco-2 cell monolayers coincubated for 3 h with (A) free insulin-FITC and insulin-FITC loaded (B) liposomes, (C) PAA-liposomes, (D) layersomes, (E) FA-layersomes, and (F) FA-layersomes pretreated with excess of FA.

4. DISCUSSION

Liposomes have been investigated extensively in the field of drug delivery and a large number of products have already been approved for clinical investigation, however, delicate nature and susceptibility to degradation at low pH environment of gastric fluid and lipase and bile salts[20] restricted their use through parenteral route only. In the present work, layer-by-layer coating of oppositely charged polyelectrolytes over the liposomes was investigated to stabilize the liposomes against harsh gastrointestinal environment and also exploited their

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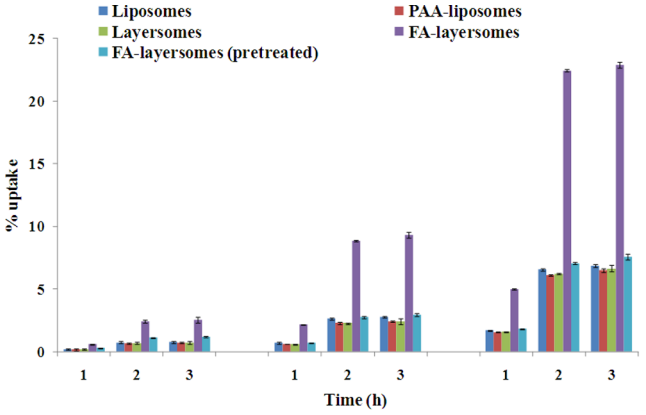

Figure 8. Quantitative determination of cellular uptake by Caco-2 cell monolayers coincubated with insulin-FITC loaded liposomes, PAA-liposomes, layersomes, FA-layersomes, and FA-layersomes pretreated with an excess of FA for 1, 2, and 3 h at 1, 3, and 10 μg/mL. Values are presented as mean ± SD (n = 3).

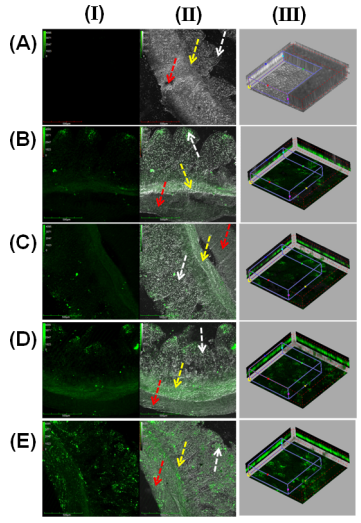

Figure 9. Representative confocal images of intestinal cross sections following oral administration of (A) free insulin-FITC and insulin-FITC loaded (B) liposomes, (C) PAA-liposomes, (D) layersomes, and (E) FA-layersomes. Panels I, II, and III represent fluorescent images, overlay images, and an apical 3D view of fluorescent images, respectively. White, yellow, and red arrows indicate the mucosal, submucosal, and muscular regions, respectively.

potential in the area of active targeting by anchoring FA as targeting ligand. The chemical shift values in the synthesized conjugate (FA-PEG-PAH) at δ 3.6 ppm could be ascribed to the PEG backbone (d,e) protons, while the chemical shifts at δ 2.6, 1.9, and 1.5 ppm could be assigned to PAH (a, b, and c) protons, respectively. Appearance of aromatic (g,h) and hetroaromatic (i) protons of folic acid at chemical shift δ 6.8, 7.5, and 8.2 ppm further confirmed the synthesis of hypothesized conjugate.

Extensive optimization of different preparative variables was carried out, which resulted in different formulations of desired quality attributes. As a formulation component, stearyl amine was used strategically to provide positive charge over the liposomes, which were taken as a template and alternatingly coated with negatively charged PAA and positively charged PAH/FA-PEG-PAH to finally obtain a robust structure layersomes/FA-layersomes. The developed formulations were

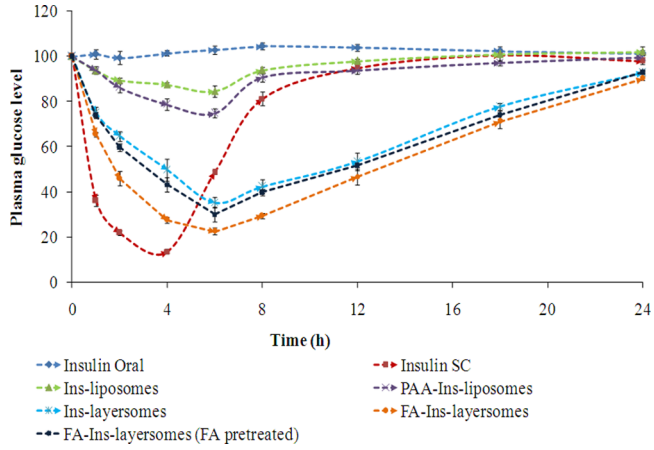

Figure 10. Antidiabetic activity of standard insulin and different formulations. Graph indicates plasma glucose level versus time profile following subcutaneous (5 IU/kg) and oral (50 IU/kg) administration of standard insulin solution and oral administration of insulin loaded Ins-liposomes, PAA-Ins-liposomes, Ins-layersomes, FA-Ins-layersomes, and FA-Ins-layersomes (at a dose equivalent to 50 IU/kg) following pretreatment with excess of folic acid. Plasma glucose level was calculated as % of initial value. Values are presented as mean ± SEM (n = 6).

spherical in shape, which was confirmed by morphological studies. No visible spherical structures were observed in SEM analysis of liposomes, which could be ascribed to disorientation of molecular arrangement due to the high voltage electron beam. In support of our hypothesis, subsequent coatings of polyelectrolytes over liposomes resulted in formation of spherical particulate structures that were robust enough to withstand molecular disorientation of lipid molecules due to high voltage focused beam of electrons. TEM analysis further confirmed the formation of spherical structures in all the formulations. Loosely held polyelectrolyte layer was visualized in TEM image of FA-Ins-layersomes and could be attributed to the long chain length of PEG spacer, which might result in the protruding out of the FA molecules at the surface. The spherical shape was further confirmed by AFM analysis, and observed particle size was in good correlation with that obtained from dynamic light scattering analysis.

Despite discrete advantages, poor storage stability in dispersion form is the major drawback of liposomes, which can be obviated by converting them into solid dispersible form, which is one of the most challenging task. For this purpose, freeze-drying was performed and trehalose was finalized in preliminary screening based on the formation of cake elegancy and excellent redispersibility. Although the elegant cake was also observed (data not shown) in the case of mannitol, glycine, and inulin, yet the redispersibility index was quite higher, which might be the consequence of providing bulk rather cryoprotection in the case of mannitol and glycine, while adsorption and charge reversal in the case of inulin. Poor redispersibility observed in the case of dextrose, sucrose, and lactose could be ascribed to the formation of collapsed network, which was not able to provide cryoprotection. Lower concentration (2.5% w/v) of trehalose was found less effective and could be attributed to the formation of a poor network, which is the prime requirement to provide cryoprotection against undue stress of freeze-drying.

Physical or chemical stress may lead to the chemical degradation and loss of conformation of insulin, which is the

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Table 6. Pharmacodynamic Parameters Following Subcutaneous Administration of Standard Insulin Solution (5 IU/kg) and Oral Administration of Standard Insulin Solution, Ins-Liposomes, PAA-Ins-Liposomes, Ins-Layersomes, FA-Ins-Layersomes, and FA-Ins-Layersomes (50 IU/kg) Following Pretreatment with Excess of Folic Acid[a]

treatment groups	dose (IU/kg)	AUC	cumulative hypoglycemia (%)	PA (%)	T_{max} (h)	C_{min} (% of initial value)
insulin solution oral	50	2457.9 ± 29.3				94.9 ± 2.0
insulin solution (SC)	5	1859.7 ± 16.7	24.3 ± 0.6	100	4	13.7 ± 1.1
Ins-liposomes	50	2303.6 ± 19.6	6.2 ± 0.8	2.5 ± 0.3	6	83.7 ± 1.6
PAA-Ins-liposomes	50	2201.3 ± 33.9	10.4 ± 1.3	4.2 ± 0.5	6	74.9 ± 2.0
Ins-layersomes	50	1532.5 ± 43.7	37.6 ± 1.7	15.4 ± 0.7	6	33.8 ± 1.3
FA-Ins-layersomes	50	1305.4 ± 35.3	46.8 ± 1.4	19.2 ± 0.5	6	22.3 ± 1.2
FA-Ins-layersomes (FA pretreated)	50	1461.2 ± 23.9	40.5 ± 0.9	16.6 ± 0.4	6	30.0 ± 3.2

[a] T_{max}: time at which minimum relative glucose level was observed; C_{min}: minimum basal glucose value. Values are presented as mean ± SEM (n = 6).

Table 7. Pharmacokinetic Parameters of Different Treatment Groups[a]

treatment group	dose (IU/kg)	AUC (μIU·h/mL)	BA_R (%)	T_{max} (h)	C_{max} (μIU/mL)
insulin SC	5	200.8 ± 9.0	100	4	42.3 ± 1.3
Ins-liposomes	50	51.4 ± 6.6	3.1 ± 2.6	6	10.2 ± 0.9
PAA-Ins-liposomes	50	84.8 ± 5.8	5.2 ± 3.3	6	16.5 ± 1.3
Ins-layersomes	50	247.1 ± 8.7	15.1 ± 2.1	6	30.9 ± 2.0
FA-Ins-layersomes	50	315.7 ± 9.9	19.3 ± 1.6	6	34.1 ± 2.2
FA-Ins-layersomes (FA pretreated)	50	268.1 ± 10.3	16.4 ± 1.4	6	31.1 ± 1.4

[a] AUC, area under the curve; BA_R, relative bioavailability. Results are represented as mean ± SEM (n = 6).

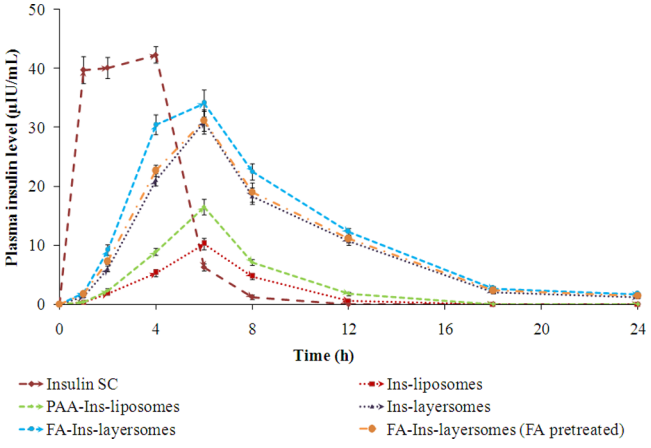

Figure 11. Pharmacokinetic profile following subcutaneous administration of standard insulin solution and oral administration of Ins-liposomes, PAA-Ins-liposomes, Ins-layersomes, and FA-Ins-layersomes. Results are represented as mean ± SEM (n = 6).

prime requirement to keep the insulin biologically active. Therefore, it was of upmost importance to ascertain the chemical and conformational stability of insulin as it was exposed to a variable level of physical and chemical stress during the entire process of formulation development. Insulin extracted from different samples was found quite stable, and no sign of chemical degradation and conformation destabilization was observed, indicating the suitability of the formulation and method in dealing with sensitive drugs like insulin. Besides the chemical and conformational stability of insulin entrapped within the delivery vehicle, stability of this ferrying cargo in gastrointestinal conditions plays a crucial role in the therapeutic performance. Therefore, to evaluate the protective effect of layer-by-layer coating of polyelectrolytes, the developed formulations were challenged against gastrointestinal conditions in vitro by incubating them in simulated biological fluids. In support of our hypothesis, strong electrostatic attraction between oppositely charged polyelectrolytes layers could result into the formation of robust structure in case of layersomes/FA-layersomes, which could eliminate the degradation of lipid molecules by preventing direct exposure of phospholipids to external environment. In contrast to layersomes/FA-layersomes, Ins-liposomes, and PAA-Ins-liposomes were found quite unstable and could be attributed to the disruption and destabilization of the Ins-liposomes due to diffusion of excess of hydrogen ions and aggregation and destabilization of negatively charged PAA-Ins-liposomes induced by adsorption of oppositely charged ions from the surrounding media.

Significant retardation in drug release (Figure 6) in case of layersomes/FA-layersomes could be attributed to the barrier provided by layer-by-layer coating of polyelectrolytes. Further release kinetics suggested Baker-Lonsdale model of release in case of Ins-liposomes and PAA-Ins-liposomes indicative of diffusion and erosion based drug release from the system while Higuchi model in case of Ins-layersomes and FA-Ins-layersomes indicative of diffusion of drug from a matrix system (Table 5). These findings are in line with our previous observation and further supported our concept of formation of solid particles with liposomes in the core. Slightly lower, although insignificant, release observed in FA-Ins-layersomes in comparison with plain Ins-layersomes at pH 2.0 and 6.8 might be the consequence of lower solubility of FA-PEG-PAH conjugate at low pH values.

In vitro cellular uptake study was performed in Caco-2 cells because of their unique property to spontaneously differentiate into monolayers of polarized cells that resemble intestinal enterocytes.[30] The fluorescence observed in the case of insulin-FITC was almost nondetectable, which might be ascribed to the obvious reason of poor permeability due to high molecular weight. Higher uptake, although insignificant in comparison with PAA-liposomes and layersomes, observed in the case of liposomes, could be ascribed to relatively more hydrophobic surface which got modified toward more hydrophilic after polyelectrolytes coatings. The phenomenon was in line with our previous findings in which hydrophobicity of the nanocarriers has been reported to play a crucial role in cellular uptake.[21,22] In line with our hypothesis, FA-layersomes exhibited significantly higher uptake in comparison with all the formulations, supporting the fact that targeting to FA

receptors could be exploited as a better targeting strategy for oral delivery of bioactives. Although saturation of FA receptors by pretreatment with FA could diminish the uptake, it was slightly higher in comparison with plain layersomes, which might be correlated with the obvious reason that saturation of all the receptors is not practically possible. On the similar line, an ex vivo intestinal uptake study also demonstrated uniform fluorescence throughout the intestinal region, indicative of cellular uptake through all the possible routes, that is, transcellular as well as paracellular, rather than following the single uptake mechanism. Interestingly, FA modification in FA-layersomes could able to make them available in the deeper region (submucosal and muscular reason; Figure 9) and attributed to the obvious reason of additional mechanism of cellular uptake following the receptor-mediated endocytosis.

To further evaluate the effect of FA modification on oral absorption and subsequent therapeutic potential, hypoglycemic activity following oral administration was determined. Orally administered standard insulin solution exhibited no hypoglycemic effect; rather, an increase was observed, which might be the consequence of enzymatic degradation, poor permeability across the GI tract, and stress of dosing and blood sample collection. A bit fall in glucose level observed in case of Ins-liposomes and PAA-Ins-liposomes could possibly be ascribed to the somewhat protection to the entrapped insulin against gastrointestinal degradation. Interestingly, Ins-layersomes exhibited significantly higher ($P < 0.001$) hypoglycemia in comparison to Ins-liposomes and PAA-Ins-liposomes. Encapsulation of insulin within the robust and stable particles, that is, "layersomes" could prevent its gastrointestinal degradation. This observation can be better correlated with our in vitro stability results in which Ins-liposomes and PAA-Ins-liposomes were found unstable in simulated biological fluids, while Ins-layersomes exhibited excellent stability in stability studies. There are certain reports in which nano formulations have been reported to follow additional uptake mechanism through a specialized region of the GI tract, that is, M-cells in Peyer's patches.[28] Thus, better hypoglycemic response observed in the case of Ins-layersomes in comparison with control and Ins-liposomes could be ascribed to the combined effect of better protection and enhanced uptake through specialized mechanisms. In line with our hypothesis, further surface modification with FA (FA-Ins-layersomes) fortified the hypoglycemic response among all the treatment groups, which could be attributed to the apparent reason of enhanced uptake following additional uptake mechanism. In order to prove the presence of additional uptake mechanism in case of FA appended systems an additional treatment group was taken and pretreated with excess of FA to saturate the FA receptors prior to oral administration of FA-Ins-layersomes. Interestingly, hypoglycemia observed in this group was significantly higher ($p < 0.05$) in comparison to plain Ins-layersomes, which clearly corroborate the presence of FA receptor mediated uptake for FA-modified systems. Although we speculated the saturation of receptors following excess dose of FA yet, complete saturation of FA receptors is practically impossible and this might be the possible explanation why a bit higher hypoglycemic response was observed in case of FA-Ins-layersomes even after presaturation with FA in comparison with plain Ins-layersomes. This observation is also in line with our observation in Caco-2 cell uptake studies. In the array of experimentation to prove the efficacy of the proposed system, pharmacokinetic studies were performed in which the plasma insulin level was quantified in response to the hypoglycemia observed. Insulin could not be detected in the case of standard insulin solution following oral administration. FA-Ins-layersomes demonstrated C_{max} (34.1 ± 2.2 μIU/mL) within six hours and the highest BA_R (19.3 ± 1.6) among all the treatment groups, which further supported the suitability of the proposed system as a ferrying cargo for oral insulin delivery. The proposed system exhibited quite higher relative bioavailability in comparison with previous reports.[31−33] FA-Ins-layersomes exhibited excellent PK/PD correlation as evident by PA of antidiabetic activity and BA_R of pharmacokinetics. Also, the results were comparable to our previous observation with FA-PEG-PLGA nanoparticles.[19]

5. CONCLUSIONS

In the current report, liposomes were stabilized by layer-by-layer coating of polyelectrolytes, which finally converted to a robust structure "layersomes". Along with stabilization, targeting potential of the system was also explored by appending FA as a ligand to have the advantages of FA transporters in the GI tract. The developed FA-Ins-layersomes exhibited excellent stability in simulated biological fluids and hypoglycemic response in diabetic rats. The developed formulation exhibited prolonged hypoglycemia up to 18 h, indicative of easy-to-administer, patient-friendly oral formulation that can combat diabetes with improved therapeutic profile. Well documented technology for large scale production of liposomes is another hidden advantage that can make this approach a clinical reality. In the current scientific panorama, when oral delivery of insulin is still the dream for the scientific community, the current findings may add some novel tools in designing the polyelectrolyte-based systems with target specificity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional experimental details and analytical results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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