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Development and *in vitro* evaluation of polar lipid based lipospheres for oral delivery of peptide drugs

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Abstract

A 3^2 factorial design was employed to produce oral sustained release lipospheres prepared by modified double emulsion solvent evaporation technique for Serratiopeptidase (acid-labile enzyme) using wax and polar lipid combination as retardants. The effects of formulation variables selected through preliminary trials namely peptide and stabilizer (Tween® 80) concentration was evaluated by F-test on the drug content and size of lipospheres. The results of analysis of variance tests for both effects indicated that the test is significant ($p < 0.05$). The effect of Tween® 80 concentration (SSY_1- 41.66; SSY_2 – 25.30) was found to be higher than peptide amount (SSY_1- 3.94; SSY_2 – 4.03) on the size and drug content of lipospheres. Characterization was carried out through photomicroscopy, scanning electron microscopy, particle size analysis and i*n vitro* drug release study. The effect of formulation variables on the integrity of enzyme was confirmed by *in vitro* proteolytic activity. The drug release from lipospheres followed first-order kinetics and was characterized by the Higuchi diffusion and Ritger-Peppas model. Lipospheres having maximum drug content (11.93±0.89) released 3-4% enzyme at pH 1.2 in 4 h. In phosphate buffer, lipospheres showed an initial burst release of 20.89±1.87% to 27.89±2.03% in one hour with additional 73.22±2.36% to 94.75±2.78% in next 12 hours. Thus, peptide loaded lipospheres with desirable characters in terms of maximum peptide content and diffusion release pattern were successfully prepared with formulation optimization approach.

Keywords: Cetyl alcohol, Enzyme, factorial design, Lipospheres; Peptide, Serratiopeptidase

Introduction

Proteins and polypeptides are important classes of bioactive agents that play key roles in controlling various body functions required for good health when administered in the correct quantities at the appropriate body site and at the correct time. Proteins and peptides have many attractive properties but they also have disadvantages that limit their widespread acceptance by patients and physicians such as numerous chemical and physical instability mechanisms, rapid enzymatic degradation which is responsible for low oral and transdermal bioavailability and short *in vivo* half lives,

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which often necessitate their frequent delivery [1]. The development of delivery systems for this rapidly expanding class of therapeutic agents is the major challenge.

Polymeric delivery systems represent an interesting strategy particularly oral multiparticulate systems that show more predictable gastric emptying, high degree of dispersion in the digestive tract, lesser risk of dose dumping and reduced local irritation [2,3]. But, the use of synthetic polymer matrix materials often goes along with detrimental effects on incorporated peptides during manufacturing of the formulations or during the erosion of the polymers after application [4]. Moreover, the degradation of polymer might possibly cause systemic toxic effects through the impairment of reticuloendothelial system (RES) or after phagocytosis of particles by human macrophages and granlocytes [5]. Therefore, alternative carrier substances have been investigated; among them lipidic materials have garnered growing attention. Numerous lipid based delivery systems such as liposomes, solid lipid nanoparticles, oily suspensions, submicron lipid emulsions, lipid implants, lipid microtubules and microcylinders, lipid microbubbles and lipid microspheres (Lipospheres) have been investigated for proteins and peptides [6].

The lipospheres carrier system has several advantages over other delivery systems in term of physical stability, low cost of ingredients, ease of preparation and scale-up, high dispersability in an aqueous medium, high entrapment of hydrophobic drugs, controlled particle size and extended release of entrapped drug [7]. The liposphere drug delivery system is an aqueous microdispersion of solid water insoluble spherical microparticles of a particle size between 0.2 and 100 μm. The lipospheres are made of solid hydrophobic triglycerides having a monolayer of phospholipids embedded on the surface of the particle. The solid core contains the bioactive compound dissolved or dispersed in a solid fat matrix. These are generally used as carrier vehicle for hydrophobic drugs. These exhibit low entrapment of hydrophilic drugs which could be improved by using polar lipids like cetyl alcohol, stearyl alcohol and cetostearyl alcohol etc [8].

Method of preparation has much influence on the properties of lipospheres and therefore the desired properties were kept in mind during the selection of a particular method of preparation. For the formulation of lipospheres certain requirements were optimal protein loading, high yield, high encapsulation efficiency, stability of the encapsulated protein, batch uniformity and inter-batch reproducibility, adjustable release profiles, low burst effect and free-flowing lipospheres.

Polypeptides and proteins like proteolytic enzymes (e.g., Serratiopeptidase, STP) offer a powerful treatment for pain and inflammation with widespread use in arthritis, fibrocystic breast disease, chronic bronchitis, sinusitis, atherosclerosis, wound debridement, and carpal tunnel syndrome [9]. These produce pharmacological effects by absorption through the intestines into the blood stream [10]. But, oral bioavailability of these peptide drugs is generally very low, owing to the acidic conditions of the stomach and poor permeability across intestinal mucosa [11]. Enteric coated preparations are available, but they release the drug with a burst, leading to other gastrointestinal tract–related disorders thus aggravating the inflammatory conditions. In the present study, efforts have been made to prepare a biocompatible sustained release system for proteins and peptides with high biocompatibility and physico-chemical stability. Furthermore, lipid matrix materials might provide a less detrimental environment for peptides and proteins during the application.

Use of the response surface methodology has been proven to be useful tool in the development and optimization of controlled release preparations [12].

The objective of the investigation was to develop a sustained-release lipospheres of STP using wax and polar lipid combination which due to the gastro resistant lipid matrix used and prolonged release properties, may hinder drug discharge in the stomach, minimize unwanted effects of the drug on G.I.T and liver.

A 3^2 factorial design is an established method to study the effect of selected parameters. The drug amount (X_1) and Tween® 80 concentration (X_2) were selected as independent variables while the mean diameter and the percent peptide content of lipospheres were chosen as the dependent variables in the present investigation. The levels for these 2 parameters were determined from the preliminary trials. Furthermore, formulations were evaluated for *in-vitro* release and proteolytic activity.

Materials and Methods

Materials

Serratiopeptidase (MW 52kDa) was received as gift sample from Advanced Enzyme Technologies Ltd, Nasik, India. Paraffin wax was purchased from Himedia labs. Cetyl alcohol, Tween® 80, potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate and sodium hydroxide were purchased from S.D. Fine Chemicals Ltd. (India). All other chemicals used were of analytical grade.

Preparation of Lipospheres

Lipospheres were prepared by a method based on the water-in-oil-in-water double emulsion (w/o/w) method reported by Reithmeier et al (2001) and Cortesi et al (2002) with few modifications [4,13]. STP was solubilized in the 100µl internal aqueous phase of a w/o/w double emulsion containing Tween®20 (3% w/v) as stabilizer to prevent loss of STP to the external phase during solvent evaporation [14]. This aqueous solution of peptide was emulsified in 300mg of Paraffin wax and cetyl alcohol dissolved in 1.0ml of methylene chloride under vigorous vortex-mixing for 10 seconds. The obtained primary emulsion was further emulsified into a small volume (3.0ml) of a stabilizer (0.2%v/v Tween®80, 37°C) containing aqueous phase. Hardening of the oily internal phase resulting in encapsulation of the peptide was accomplished by pouring emulsion into 100ml of ice cold water maintained at 4 °C and stirred at 300 rpm. After 3–5 hours, microparticles were isolated by filtration, washed with ice cold water and dried at room temperature (25°C) for 24 hours. Preliminary studies were carried out to identify the process/formulation variables in order to select key variables (Table 1). Different values of ratios of paraffin wax: cetyl alcohol, Tween® 80 concentration, peptide amount and stirring speed at elevated temperature prior to cooling were studied in the preliminary studies to select the best formulations (to obtain discrete and free flowing LS with high yields and drug content) for further studies. Key variables were further optimized as per 3^2 full factorial design and a total of 9 batches (SLS1 to SLS9) of STP lipospheres were produced (Table 2). The other formulation and processing variables were maintained constant during the process.

Characterization

Particle size

Particle size analysis of STP-loaded lipospheres was performed by optical microscopy using a compound microscope (Erma, Tokyo, Japan) [9]. A small amount of dry lipospheres was suspended in purified water (10mL). The suspension was ultrasonicated for 5 seconds. A small drop of suspension thus obtained was placed on a clean glass slide. The slide containing lipospheres was mounted on the stage of the microscope and 300 particles were measured using a calibrated ocular micrometer. The process was repeated for each batch prepared.

Morphology

The surface morphology and shape of the lipospheres were analyzed by scanning electron microscopy for selected batches (Leo, VP-435, Cambridge, UK). Photomicrographs were observed at ×303 magnification operated with an acceleration voltage of 15kV and working distance of 10mm was maintained. Microspheres were mounted on the standard specimen-mounting stubs and were coated with a thin layer (20nm) of gold by a sputter-coater unit (VG Microtech, Uckfield, UK).

Drug content

Twenty milligrams of the dried lipospheres were accurately weighed. They were added to 5ml of ethyl acetate. After the lipospheres dissolved completely, 5ml of phosphate buffer (pH-7.4) was added to this solution and mixed thoroughly. The resulting solution was filtered using a Whattman filter (0.45µm pore size) and analyzed for STP content by measuring absorbance in UV-spectrophotometer (Shimadzu UV-1700, Pharmaspec, Tokyo, Japan) at 229.5nm by the first-derivative spectrophotometric method using phosphate buffer (pH-7.4) and ethanol mixture (1:1) as blank [15]. Results were expressed as mean (±SD) of 3 experiments. The measured responses are shown in Table 3.

In vitro release

In vitro release of STP from lipospheres was evaluated in both acidic buffer (pH-1.2) and phosphate buffer (pH-7.4). Amount of lipospheres equivalent to 20mg of STP were transferred to the prewarmed dissolution media (20ml) and maintained at 37±0.5°C under stirring at 50rpm. Samples were withdrawn every hour

up to 12hrs and the volume was replaced immediately by fresh phosphate buffer. The sample withdrawn was centrifuged (3000rpm, 15min). The supernatant solution was filtered and analyzed for STP content by measuring absorbance in UV-spectrophotometer (Shimadzu UV-1700, Pharmaspec, Tokyo, Japan) at 229.5nm by first derivative spectrophotometric method using phosphate buffer (pH-7.4) as blank. Results were expressed as mean (±SD) of 3 experiments.

In vitro proteolytic activity

Prepared lipospheres and plain STP solution were placed separately in 5ml of HCl buffer (pH-1.2) or phosphate buffer (pH-7.4) maintained at 37±0.5ºC and stirred constantly at 100rpm. After 2hrs, samples were recovered by centrifugation at 3000rpm for 15min at room temperature (n=3).

The proteolytic activity was determined as per the method reported in Food Chemical Codex (2003)**.** The assay was based on a 30min proteolytic hydrolysis of casein at 37 ºC and pH 7.0. Unhydrolyzed casein was removed by filtration and the solubilized casein was determined spectrophotometrically at wavelength of 275nm. In this method, the protease activity is expressed as PU units of preparation derived from *Bacillus subtilis* var. and *Bacillus licheniformis* var. One bacterial protease unit (PU) is defined as quantity

$$\frac{PC}{g} = \left(\frac{A_u}{A_s}\right)\left(\frac{1.89}{30\ w}\right) \quad (1)$$

of enzyme that produces 1.5µg/ml equivalent of L-tyrosine per minute under the condition of the assay.

Activity of enzyme was calculated by equation:

Where A_u is the value obtained by subtracting blank reading from test reading, A_s absorption of standard solution, 1.89 the final volume in ml of reaction mixture, 30 the time of the reaction in minutes and w

$$Yi = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad (2)$$

the weight of the original sample in 'g'.

Response Surface Analysis

The results are expressed as second order polynomial equation of the following form (Eq 1): where, b_i is the estimated coefficient for the factor X_i, while Y_i is the measured response. The coefficients corresponding linear effects (b_1 and b_2), interaction (b_{12}) and the quadratic effects (b_{11} and b_{12}) were determined from the results of the experiment (STAT-EASE, design expert, 7.0.3). To assess the reliability of the model, a comparison between the experimental and predicted

$$\%\,Bias = \frac{Predicted\ value - Experimental\ value}{Predicted\ vaue} \times 100 \quad (3)$$

values of the responses is also presented in terms of %Bias in Table 3.

Bias was calculated by equation 2:

Results and Discussion

Peptide loaded lipospheres (SLS) were prepared by w/o/w double emulsion method using wax (Paraffin wax) and polar lipid (cetyl alcohol) with Tween® 80 for improved entrapment of hydrophilic drugs [16]. Lipid based carriers were selected to eliminate the toxic effects associated with the use of polymers as carriers. Melt dispersion technique is commonly used for preparation of lipospheres but w/o/w double emulsion method was considered with the aim to possibly reduce the exposure to high temperature of thermolabile compounds, such as proteins and peptides.

When the lipid solution in methylene chloride was used, the aqueous phase coalesced rapidly, especially when the emulsion was prepared by vortex-mixing. May be the use of additional stabilizers could improve the emulsion stability and the encapsulation efficiency in case of the w/o/w-solvent evaporation method. Tween® 20 and Tween® 80 were used as stabilizers in inner and outer aqueous phase respectively for liposphere formation and emulsion stabilization. Serratiopeptidase is a neutral protease with isoelectric point of 6.1 [17]. Drug loading has been reported to improve by adjusting the pH of the aqueous phase by suppression of ionization [18]. The solubility of STP and hence leaching into the outer aqueous phase increases as the pH of the solution increases above its isoelectric point. The pH of Tween® 80 aqueous solution is in the range of 6.0 to 6.5 and hence did not warrant pH adjustment [17].

The Paraffin wax due to its physical properties and behaviour in the intestinal lumen was used to prepare gastro-resistant SLS formulations using the adopted technique [19]. Since lipospheres produced with paraffin wax alone resulted in poor drug entrapment and release, efforts were made to enhance drug release from the lipospheres by incorporating a polar wax modifier like cetyl alcohol. Literature citations reveal that cetyl alcohol has been successfully employed as a

wax modifier to modulate drug release from wax microspheres [20]. Tween® 80 was used to stabilize the oil in water emulsion by reducing the interfacial tension between the hydrophobic wax dispersion and the external aqueous phase, producing an emulsified oily dispersion, which resulted in drug loaded lipospheres on cooling. Fatty alcohols like cetyl alcohol and stearyl alcohol have been reported to improve release and entrapment of hydrophilic peptide due to their polar hydrophilic nature [21].

Different formulation variables have been found to affect the peptide loading, entrapment as well as release profile of lipospheres (Table 1). The preliminary LS formulations consisting of blends of paraffin wax and cetyl alcohol at 1:1 ratio, where a 1:2 (peptide: lipid) ratio was used, are given in Table 1. The production yield of SLS prepared from all formulations were high (>85%). Cetyl alcohol itself exhibits emulsifying capability further stabilizing the primary emulsion [22]. Moreover it also imparts sphericity with smooth surface and modifies the release of the entrapped drug. As being polar lipid, it improves the entrapment of hydrophilic drugs [20]. The slight loss of solids could be attributed to the losses occurring during various steps of processing such as sticking of the lipid solution, adsorption on the glass wall during solidification or loss of lipospheres during the washing step etc.

Table 1. Preliminary STP loaded LS formulations

S.No	Variables	Code	Values	MD (μm)	% Drug content	% EE
1.	Stirring speed (rpm)	LA	500	69.34±1.43	7.50±0.34	78.92±2.32
		LB	1000	30.05±1.52	11.69±0.90	69.05±2.76
		LC	1500	64.54±1.66	3.63±0.42	35.80±1.90
2.	Wax:Wax modifier (Paraffin wax:Ct.A)(w/w)	LD	1:2	Aggregates	3.89±0.47	42.08±1.90
		LE	1:1	19.67±1.03	11.53±1.22	66.06±1.73
		LF	2:1	32.15±0.78	6.79±0.98	78.50±1.89
3.	Peptide amount (mg)	LG	50	28.54±1.23	8.56±1.22	74.34±2.09
		LH	100	32.98±1.22	10.34±0.96	70.98±2.42
		LI	150	38.14±1.90	6.56±0.91	34.67±1.76

*MD-Mean Diameter; EE-Entrapment efficiency; EAP-External aqueous phase; Cet.A-Cetyl alcohol

Increase in stirring speed during second emulsification significantly affected the size. By adjusting the stirring speed during the emulsification process, it was possible to modify the size of the particles. Increasing the stirring speed from 500 to 1000 rpm, the mean diameter of particles progressively decreased (Table 1). But on further increase in stirring speed from 1000rpm, larger aggregates were formed due to increase in the surface free energy of smaller particles. Loading also increased with increase in stirring might be due to better dispersion of lipid particles [12]. Particles obtained at 1000rpm presented a spherical geometry with absence of interaction phenomenon. So, 1000rpm speed was selected for further studies.

Incorporation of cetyl alcohol significantly affected the % drug loading of the formed SLS. At 1:1 ratio of paraffin wax: cetyl alcohol maximum loading was obtained due to polar hydrophilic nature of cetyl alcohol [12]. Cetyl alcohol effected encapsulation because it might decrease the hydrophobicity of the wall matrix and served as a good wetting agent for the drug allowing it to be finely dispersed. With increase in paraffin wax content decreased loading was observed due to hydrophobicity of the wax matrix decreasing the amount of hydrophilic drug.

By inspection of Table 1, it is obvious that by increasing the amount of peptide used in the preparation of lipospheres from 50mg to 150mg, size and loading of LS increased and the entrapment efficiency decreased significantly ($P<0.05$). This may be explained by the fact that as the ratio of drug to the lipid matrix increases, a reduced space becomes available for the drug to be entrapped. Inefficient encapsulation of drug due to inadequate dispersion makes it possible for the drug to remain on the outer surface of the particles. During the washing stage of the encapsulation process the drug on the outside appeared to have been removed, resulting in low drug yield. Similar results were obtained with verapamil solid lipid nanoparticles [23]. But after increasing the peptide amount to 150mg there was significant decrease in loading might be due to insufficient amount of lipid to surround the peptide.

In order to gain further insight into the effects of peptide amount (factor X_1) and other important process variables such as stabilizer (Tween® 80) concentration in outer aqueous phase (factor X_2) on the properties of LS formulations, a 3^2 factorial design model was used to optimize the formulations (Table 2). Formulation LE prepared with paraffin wax: cetyl alcohol (1:1) with stirring speed of 1000rpm can offer a starting basis for

this purpose since it represented a more meaningful STP content in the formulation with uniform spherical discrete lipospheres.

Table 2. Full factorial experimental design layout with coded levels and actual values of variables

F. code	Variable X_1 Peptide amount (mg)	Variable X_2 Tween® 80 (%v/v)
SLS1	30 (-1)*	0.1(-1)
SLS2	60(0)	0.1(-1)
SLS3	90(+1)	0.1(-1)
SLS4	30(-1)	0.15(0)
SLS5	60(0)	0.15(0)
SLS6	90(+1)	0.15(0)
SLS7	30(-1)	0.2(+1)
SLS8	60(0)	0.2(+1)
SLS9	90(+1)	0.2(+1)

*Values in parenthesis indicates coded values

All the formulations prepared within the experimental design yielded smooth spherical lipospheres as evident from optical and SEM photographs (Fig 1, 2; SLS 6; Table 3). The mean diameter of prepared lipospheres ranged from 19.69-26.07µm.

These were visible as dense spherical structure due to solid lipid matrix. In case of SEM photographs aggregates were visible might be due to some wax matrix clumping after drying.

The mean diameter (Y_1) and drug content (Y_2) of lipospheres from the nine experiments were used to generate predictor equations for lipospheres with independent variables as peptide amount (X_1) and Tween® 80 concentration (X_2). Limit for these variables were selected from preliminary trials. The results of multiple regression analysis and analysis of variance (ANOVA) are summarized in Table 4.

For estimation of coefficients in the approximating polynomial function (equation 2) applying uncoded values of factor levels, the least square regression method was used. A suitable polynomial equation involving the individual main effects and interaction factors was selected based on the estimation of several statistical parameters such as the multiple correlation coefficient (R^2), adjusted multiple correlation coefficient (adjusted R^2) and the predicted residual sum of squares (PRESS) were calculated by the design expert software 7.0.3.

As presented in Table 4, the quadratic model was selected as a suitable statistical model for optimized formulation with maximum loading because it had the smallest value of PRESS (4.38 for Y_2). PRESS is a measure of the fit of the model to the points in the design. The smaller the PRESS statistic is, the better the model fits to the data points [24].

Table 3. Experimental and predicted responses obtained for the studied parameters

F. code	% Yield	MD (µm) (Y_1)			Drug content (%)Y_2			% EE
		Exp	Pred	% Bias	Exp	Pred	% Bias	
SLS1	85.20±2.54	25.78	25.71	-0.27	3.64	3.48	-4.59	78.90±1.43
SLS2	83.36±3.41	25.98	25.38	-2.36	4.52	4.52	0	70.45±1.76
SLS3	84.58±1.89	26.07	26.74	2.50	5.80	5.96	2.68	69.06±1.55
SLS4	84.24±2.8	22.46	23.11	2.81	9.65	9.89	2.42	80.56±2.32
SLS5	86.77±2.05	22.90	23.08	0.77	10.35	10.51	1.52	79.46±1.79
SLS6	87.51±3.05	25.56	24.73	-3.35	11.93	11.53	-3.46	70.08±2.04
SLS7	88.23±2.48	20.43	19.85	-2.92	8.51	8.43	-0.94	70.77±2.33
SLS8	87.48±2.90	19.69	20.11	2.08	8.78	8.62	-1.85	69.54±1.62
SLS9	89.24±2.67	21.90	22.06	0.72	8.99	9.23	2.60	68.90±1.80

Table 4. Summary of results of a) model analysis b) lack of fit c) R-square analysis for measured responses

Source	(Mean Diameter) Y_1		(%Drug content) Y_2	
	Sum of squares	P>F	Sum of squares	P>F
Model analysis				
Mean vs total	4936.00		578.72	
Linear vs. mean	45.60	0.0007	29.33	0.1431
2FI vs. linear	0.35	0.5452	0.71	0.7513
Quadratic vs. 2FI	1.64	0.4682	31.08	0.0013
Cubic vs. quadratic	2.42	0.1689	0.32	0.3835
Residual	0.071		0.056	
Total	4986.08		640.22	
R-square analysis	Adjusted R-square	PRESS	Adjusted R-square	PRESS
Linear	0.8806	10.04	0.3026	67.92
2FI	0.8678	15.42	0.1815	115.70
Quadratic	0.8672	30.07	0.9835	4.38
Cubic	0.9886	12.96	0.9927	10.21

From the p-values presented in table 4, it can be concluded that for both responses the cross product contribution (2FI) of the model was not significant indicating the absence of interaction effects. The results of multiple regression analysis and analysis of variance test (ANOVA) are summarized in Table 5.

Table 5. Regression analysis data for measured responses

Coefficients	MD (µm)Y_1		Drug content (%) Y_2	
	FM	RM	FM	RM
b_0	23.08	23.42	10.51	8.02
b_1	0.81	0.81	0.82	0.82
b_2	-2.63	-2.63	2.05	2.05
b_{11}	0.84	-	0.20	-
b_{22}	-0.33	-	-3.94	-
b_{12}	0.29	-	-0.42	-
R^2	0.9502	0.9104	0.9938	0.4770
Significance	0.0361	0.0007	0.0016	0.1431
F	11.45	30.50	96.28	2.74

*FM-Full Model; RM-Reduced Model

The mean diameter and percent drug content of STP lipospheres showed R^2 values of 0.9502 and 0.9938 (Table 5), respectively; indicating good fit, and it was concluded that the second-order model adequately approximated the true surface. Furthermore, low value of %bias for all batches showed good agreement between predicted and experimental values as shown in Table 3.

The predictor equation generated for the mean diameter (MD) was found to be significant with an F-value of 30.50 ($p < 0.0007$) and R^2 value of 0.9104 (equation 4):

$$Y_1(MD) = 23.08 + 0.81X_1 - 2.63X_2 \quad -(4)$$

The equation generated revealed that both main factors independently exerted a significant influence on the mean diameter. The influence of the main effects on the particle size of the lipospheres was further elucidated using the response surface plot (Figure not shown).

The predictor equation generated for the drug content was found to be significant with an F-value of 86.00 ($p< 0.0001$) and R^2 value of 0.9810 (equation 5):

$$Y_2(\%\,Drug\ content) = 10.51 + 0.82X_1 + 2.05X_2 - 3.94X_2^2 - (5)$$

The equation generated revealed the absence of interaction effect with contribution of each effect especially of Tween® 80 concentration (Factor X_2).

Table 6. Release behavior of STP in phosphate buffer (pH 7.4) from SLS1-SLS9

Formulation code	First order		Higuchi		Ritger-Peppas	
	k	R^2	K	R^2	n	R^2
SLS1	0.099	0.996	21.85	0.994	0.516	0.994
SLS2	0.108	0.993	22.52	0.995	0.513	0.996
SLS3	0.135	0.968	24.42	0.993	0.515	0.996
SLS4	0.131	0.978	24.67	0.995	0.536	0.997
SLS5	0.128	0.976	24.00	0.994	0.515	0.996
SLS6	0.142	0.968	24.57	0.992	0.504	0.992
SLS7	0.154	0.948	25.83	0.991	0.542	0.994
SLS8	0.158	0.960	25.11	0.996	0.490	0.997
SLS9	0.216	0.927	26.09	0.988	0.468	0.987

*K, Release rate constant; R2, coefficient of determination; n, release exponent

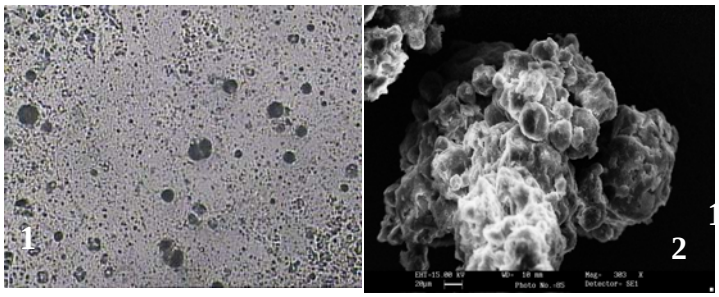

Fig 1. Photomicrographs & **Fig 2.** SEM photographs of SLS6 lipospheres

For the mean diameter (Y_1) and percent drug content (Y_2) of STP lipospheres (SLS), the calculated F values 11.45 and 96.28 respectively, were found to be greater than the critical (statistical table value) value of F 5, 3, 95% (9.01) and hence it may be concluded that one variable contributes significantly in the regression. The break up of source sum of squares (Source SS) in ANOVA indicated that the contribution of factor X_2 (Tween® concentration) ($SSY_1 = 41.66$; $SSY_2 = 25.30$) is much higher than factor X_1 (Drug concentration) ($SSY_1 = 3.94$; $SSY_2 = 4.03$) for optimizing the mean diameter as well as drug content of lipospheres. The interaction terms X_1X_2 and the polynomial terms X_1X_1 and X_2X_2 indicated insignificant values of individual source sum of squares. Interaction effect was further investigated by testing the model in portions [25].

The calculated value of F for effect on mean diameter and % drug content were found to be 0.799 and 81.48 respectively. The critical (statistical table value) value of F 3, 3, 95% is 9.28. In case of mean diameter, the calculated value of F is lower than the critical table value, we conclude that the interaction do not significantly contribute for the prediction of mean diameter in the microspheres. But, in case of drug content interaction between variables (X_1 and X_2) is significant indicating the combined role of X_1 and X_2 on determining the response Y_2, effect of X_2 being significant comparatively.

Response surface plots indicate the negative effect of Tween® 80 on MD (Y_1) of lipospheres. This result was entirely relevant to the higher dispersion property of the higher emulsifier concentration in formulation LS6 (X_2, 0), resulting in decreasing the interfacial tension and formation of smaller particles. Similar results were reported by Gibaly and Ghaffar (2005), while encapsulating allopurinol in beeswax-ceryl alcohol lipospheres prepared by melt dispersion technique [26]. STP at high level (X_1, +1) and Tween® 80 at medium level (X_2, 0) yielded microspheres with highest drug content (11.93±0.89%) which may be due to increased availability of drug at higher at high STP level and better dispersion obtained at medium level of Tween® 80 stabilizer. When X_1 was set at high level (+1) and

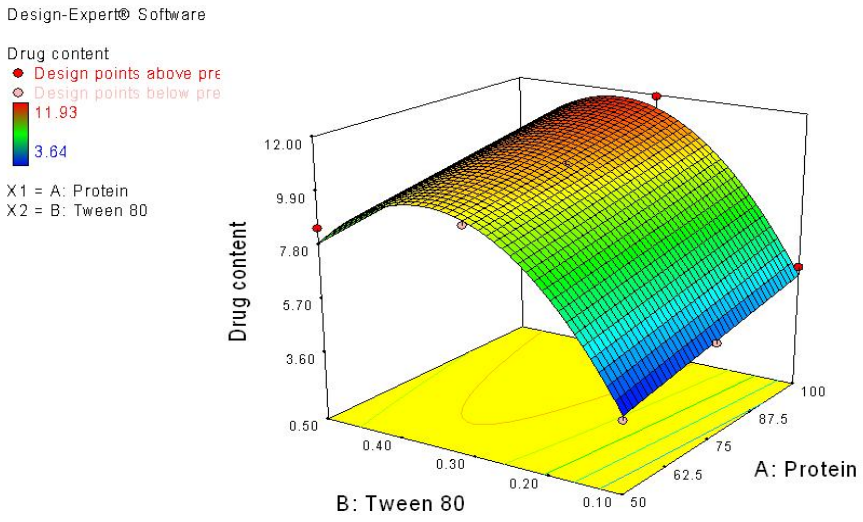

Fig 3. 3D surface curve for the effect of selected variables on the drug content of lipospheres

X_2 was set either at low (-1) or at high level (+1) less than 11.93% of the drug was loaded in the lipospheres. At both low and high level of (Tween® 80) X_2, lower drug content was found and maximum loading was found at medium level. Possible reason for decreased loading at low level of X_2 is decreased viscosity and at higher concentration of Tween® 80 in external aqueous phase leads to high water content and high wettability resulting into decreased loading [27]. Moreover, as for Tween® 80, its CMC is ~0.014mol/L whereby at a higher concentration of Tween® 80 than its Critical Micelle Concentration (CMC) sphere-shaped micelles are formed which further transform into cylinder-shaped micelle structures [28].

Figure 3 represent the response surface plot, which shows the effects of the X_1 and X_2 on the drug content of lipospheres. As can be seen through the response surface graphs, X_1 is the most significant factor effecting drug content whereas X_2 affects size as well as drug content of lipospheres significantly.

The positive coefficient of X_1 and negative coefficient of X_2 in case of Y_1 response (equation 4) refers to the positive influence of drug amount and negative influence of Tween® 80 on the size of lipospheres. Similarly, equation (equation 5) for drug content of lipospheres depicts the positive coefficient of X_1 and X_2 on the drug content of lipospheres (Fig 3).

Increase in peptide amount increased the drug loading with subsequent decrease in entrapment efficiency (Table 3). This may be explained by the fact that as the ratio of peptide to the lipid matrix increases, a reduced space becomes available for the peptide to be entrapped [29]. Inefficient encapsulation of peptide makes it possible to remain on the outer surface of the particles. During the washing stage of the encapsulation process the peptide on the outside appeared to have been removed, resulting in low entrapment.

Contour plots show that various combinations of X_1 and X_2 may satisfy any specific requirement (in this case- size in range and maximum peptide content) while taking into consideration other factors such as cost, stability etc. The results from the estimated ridge of maximum response in terms of desirability revealed that optimum peptide amount (X_1) and Tween® 80 (X_2) were 90mg and 0.16%v/v for desirable response.

Release of STP from SLS1-9 in phosphate buffer (pH-7.4) was faster than that into acidic buffer (pH-1.2) reflecting differences in extent to which the peptide

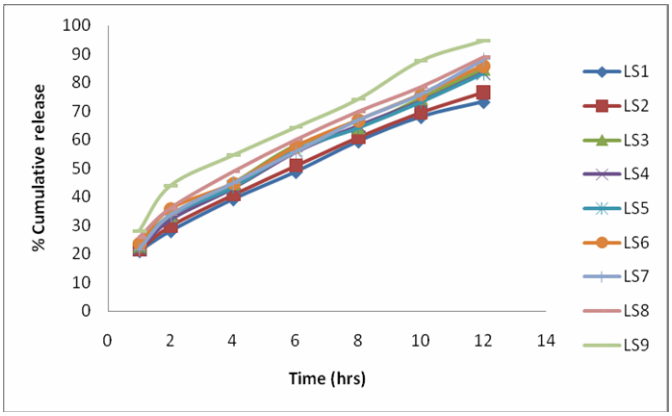

Fig 4. *In vitro* release profile of serratiopeptidase in phosphate buffer (pH-7.4) from lipospheres formulations (SLS1-9)

dissolved in the two fluids. A maximum drug release of 16.87–28.3% was observed for all the formulations in acidic buffer (pH 1.2) after 4 h (figure not shown). On the other hand, more than 80% of STP was rapidly released from these formulations within 12 h in phosphate buffer (pH-7.4) and complete release occurred in about 24 h (Fig 4). Thus, the formulation could protect the peptide from gastric degradation and would release its drug load slowly at pH 7.4. Release pattern of all formulations was similar with difference in the burst release. Burst effect was more persistent in the formulations with higher STP content due to the surface located peptides. These findings are in agreement with those of Adeyeye and Price (1994) and Giannola and De Caro (1997) who reported that rapid drug release (such as phenytoin and diclofenac sodium) from fatty acid or alcohol-wax microspheres would be expected due to the hydrophilicity and leaching characteristics [30,31].

The faster dissolution with increased drug load can be explained by peptide present outside the LS and presumable increase in channels and number of pores (produced by the drug release) within the wax matrix. This effect caused a decrease in diffusion path length through the released part region and enhanced drug release [29,30].

Drug release kinetics from the STP formulations SLS1–SLS9 were examined at pH 7.4. The release curves for STP showed a non-linear drug dissolution pattern (Fig. 6) and the drug release from the LS was affected by the drug content and the LS size, in addition to the structure of the matrix. All formulations showed an initial burst from 20.89±1.87% to 27.89±2.03% in one hour with additional 73.22±2.36% to 94.75±2.78% in next 12 hours. There was significant

effect of variable X_1 (Peptide amount) as compared to X_2 (Tween® 80) on the initial burst. Peptide amount exhibited relative positive effect on the initial burst due to increased loading and decrease in lipid to peptide ratio causing loss of drug (Formulations SLS3, 6 and 9). The cumulative percentage release of STP after 12h was found to be higher for batches with high STP and Tween® 80 content.

The release rates were analyzed by least square linear regression method. Release models such as first order model, Higuchi model and Ritger-Peppas empirical model were applied to the release data (Table 6) [32,33]. Using the Ritger–Peppas model, a plot of the logarithm of the fractional solute release versus the logarithm of time (between 1 and 12 h) yielded a straight line with a determination coefficient (R^2) of 0.992–0.997 for the nine formulations. The slope of this line determines the diffusional exponent (*n*) value, which is characteristic of the transport mechanism of diffusional release. This equation may be used only for a granular inert matrix system which maintains a constant planar surface area, where the drug diffusion coefficient is clearly concentration independent and the effect of solubility is implicit. But the value of n obtained was between 0.490-0.542 which indicates non-Fickian (anomalous) release referring to a combination of both diffusion and erosion controlled-drug release (Siepmann and Peppas, 2001). Results supported the diffusional release kinetics and also the erosion or solubilization of the wall matrix-driven phenomena. The value of coefficient of determination (R^2) in Higuchi equation was found to be >0.9 in alkaline buffer medium which again indicates the diffusion-controlled release. The result obtained is supported by the literatures reported on ibuprofen and diclofenac loaded wax microspheres [29, 30].

The quicker drug release at low wax loads can be due to increased peptide positioned on the liposphere surface that remained exposed to the dissolution fluid. At higher peptide loads, drug dissolution resulted in a subsequent increase in the number of channels and pores within the wax matrix, which decreased the diffusional path length through the drug depleted zone and enhanced drug release [30].

The lipospheres showed about 6.35-7.08% loss of proteolytic activity in acidic medium whereas retention of activity in basic medium was found to be 97.90-98.26%. Meager loss of activity in alkaline media may be due to processing steps involved in preparation of formulation. At the same time plain STP solution exhibited almost complete loss of activity in acidic medium and 86.84% activity was retained in alkaline medium. SLS exhibited much better retention of proteolytic activity as compared to plain STP solution. Possible explanation for the improved physical and chemical stability of proteolytic enzyme might be due to reduced mobility and retention of peptide in solid lipid matrix.

Conclusion

Lipospheres can be considered as a promising delivery system for oral delivery of peptide drugs like enzymes. Lipospheres were able to entrap the peptide at high levels and sustain its release over a prolonged time. STP wax LS developed by a factorial design had high drug content and showed sustained-release and enteric behavior suitable for oral use. Increase in peptide load increased the rate of drug release whereas Tween® 80 concentration decreased the size of lipospheres with improvement in peptide content. The encouraging results obtained in this study could propose lipospheres for future *in vivo* studies. These novel lipospheres were found to be promising for protection of the protein and peptides exhibiting better therapeutic effect. But, further studies in terms of pharmacokinetics, toxicology and animal studies are required for clinical utility of the formulation.

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