Supplementary Material

1. Experimental Design

Table A1. ANOVA sheet of sequential sum of squares method to screen appropriate model for each response of CS-PLHNs.

Response	Source	Sum of Squares	df	Mean Square	F value	*p*-value Prob>F	Remark
Particle size (Y_1)	Mean vs Total	2.241E+006	1	2.241E+006			
	Linear vs Mean	16531.15	4	4132.79	4.36	0.0087	
	2FI vs Linear	1087.27	6	181.21	0.15	0.9865	
	Quadratic vs 2FI	21024.29	4	5256.07	111.40	< 0.0001	Suggested
	Cubic vs Quadratic	518.07	8	64.76	2.73	0.1191	
	Residual	142.48	6	23.75			
	Total	2.280E+006	29	78633.53			
Encapsulation efficiency (Y_2)	Mean vs Total	96095.27	1	96095.27			
	Linear vs Mean	336.74	4	84.19	26.04	< 0.0001	
	2FI vs Linear	19.62	6	3.27	1.02	0.4462	
	Quadratic vs 2FI	43.58	4	10.90	10.60	0.0004	Suggested
	Cubic vs Quadratic	8.83	8	1.10	1.19	0.4268	
	Residual	5.55	6	0.93			
	Total	96509.60	29	3327.92			
PDI (Y_3)	Mean vs Total	1.89	1	1.89			
	Linear vs Mean	0.088	4	0.022	6.18	0.0014	
	2FI vs Linear	7.068E-003	6	1.178E-003	0.27	0.9435	
	Quadratic vs 2FI	0.076	4	0.019	110.40	< 0.0001	Suggested
	Cubic vs Quadratic	1.831E-003	8	2.289E-004	2.38	0.1530	
	Residual	5.763E-004	6	9.606E-005			
	Total	2.07	29	0.071			

df- degree of freedom; Prob>F is the significance level, *significant values at $p<0.05$

Table A2. ANOVA data for lack of fit tests applied on different response models of CS-PLHNs.

Response	Source	Sum of Squares	df	Mean Square	F value	*p*-value Prob>F	Remark
Particle size (Y_1)	Linear	22727.02	20	1136.35	100.80	0.0002	Suggested
	2FI	21639.75	14	1545.70	137.11	0.0001	
	Quadratic	615.46	10	61.55	5.46	0.0581	
	Cubic	97.39	2	48.70	111.40	0.1002	
	Pure Error	45.09	4	11.27	4.32		
Encapsulation efficiency (Y_2)	Linear	74.40	20	3.72	4.67	0.0726	Suggested
	2FI	54.78	14	3.91	4.91	0.0679	
	Quadratic	11.20	10	1.12	1.40	0.3977	
	Cubic	2.37	2	1.18	1.48	0.3296	
	Pure Error	3.19	4	0.80			
PDI (Y_3)	Linear	0.085	20	4.262E-003	106.56	0.0002	Suggested
	2FI	0.078	14	5.584E-003	139.61	0.0001	
	Quadratic	2.247E-003	10	2.247E-004	5.62	0.0554	
	Cubic	4.163E-004	2	2.082E-004	5.20	0.0771	
	Pure Error	1.600E-004	4	4.000E-005			

Note: *p*-value>0.05 in each of the screened model suggests non-significant lack of fit.

Table A3 Statistical analysis of particle size (Y_1), encapsulation efficiency (Y_2) and PDI (Y_3) of CS-PLHNs in the Box-Behnken experimental design

Term	Particle size (Y_1)		Encapsulation efficiency (Y_2)		PDI (Y_3)	
	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value
Constant	+220.56	-	+29.7	-	+0.15	
X_1	+28.35	<0.0001	+4.21	<0.0001	+0.066	<0.0001
X_2	-20.58	<0.0001	-2.80	<0.0001	-0.049	<0.0001
X_3	-6.55	0.0052	-0.69	0.0342	-0.0082	0.0469
X_4	+10.38	0.0001	+1.43	0.0002	+0.021	<0.0001
X_1X_2	-1.50	0.6690	-0.25	0.6295	+0.002	0.7648
X_1X_3	-4.05	0.2580	-0.25	0.6295	-0.0045	0.5037
X_1X_4	+3.20	0.3673	+0.025	0.9614	+0.0017	0.7934
X_2X_3	+7.35	0.0504	+0.85	0.1157	+0.017	0.0197
X_2X_4	-13.57	0.0014	-2.00	0.0015	-0.037	<0.0001
X_3X_4	+2.15	0.5414	-0.24	0.6431	+0.006	0.3756
X_1^2	+44.09	<0.0001	-0.38	0.3559	+0.085	<0.0001
X_2^2	+28.53	<0.0001	-2.24	<0.0001	+0.052	<0.0001
X_3^2	31.39	<0.0001	-1.40	0.0035	+0.061	<0.0001
X_4^2	34.78	<0.0001	-1.34	0.0047	+0.064	<0.0001

Table A4 Model summary statistics of the quadratic response surface models for particle size (Y_1), encapsulation efficiency (Y_2) and PDI (Y_3) of CS-PLHNs

Response Variable	Quadratic Model							
	F-value	Prob>F[$]	R^2	Adj. R^2	Pred. R^2	Adeq. Prec.	C.V. (%)	Lack of Fit
Y_1	58.50	<0.0001***	0.9832	0.9664	0.9080	24.907	2.47	0.0581[#]
Y_2	27.80	<0.0001***	0.9653	0.9306	0.8323	19.227	1.76	0.3977[#]
Y_2	71.04	<0.0001***	0.9861	0.9722	0.9239	26.579	5.13	0.0554[#]

Adj. R^2, Adjusted R^2; Pred. R^2, Predicted R^2; Adeq. Prec., Adequate Precision; C.V., Coefficient of Variation
[$]Prob>F is the significance level, *significant values at $p<0.05$, **significant values at $p<0.001$, *** significant values at $p<0.0001$, [#]not significant

2. *In vitro* hemolytic assay

Method

Blood compatibility of CS-PLHNs was evaluated by *in vitro* hemolytic assay. Fresh rat blood was used in this study. About 1.5 ml acid citrate dextrose (ACD) was added to 10 ml of fresh blood to avoid the activation of coagulating factors. Different concentrations of reconstituted lyophilized CS-PLHNs ranging from 1 to 20 mg/ml were prepared. To 1 ml of the blood sample, 1 ml of above samples were added separately. The whole samples were incubated for 48 hr with shaking in an incubator shaker at 37 ºC. The samples were centrifuged at 6000 rpm for 10 min to obtain the plasma (Plasma will be red in color if hemolysis occured). The plasma were collected and analyzed by UV spectroscopy at 540 nm for measurement of released hemoglobin content. The obtained sample values were compared with that of positive control (blood + 1% triton X) and negative control (blood + normal saline). The %hemolysis was calculated by following equation.

$$\%\ Hemolysis = \frac{(sample\ absorbance - negative\ control\ absorbance)}{(positive\ control\ absorbance - negative\ control\ absorbance)} \times 100$$

Results

Hemolytic assay was carried out to evaluate the blood compatibility of CS-PLHNs as shown in Figure 1A. The blood compatibility is a significant index for biomaterials. The biomaterial or formed CS-PLHNs upon systemic exposure in blood environment might damage the erythrocytes in certain degree or cause the formation of thrombus. In order to ensure the safety of the developed CS-PLHNs, *in-vitro* hemolytic assay was performed. The results indicates that the % hemolysis of the sample was within the range of less than 5%, the critical safe hemolytic ratio

for biomaterials according to ISO guideline (ISO/TR 7406), which indicated that the damage of the sample on the erythrocytes was negligible. Hence, based on the results, it was proved that the CS-PLHNs were biocompatible and safe for the systemic consumption. Hence, it can be used for further *in-vivo* studies. Moreover, it also suggested indirectly that CS-PLHNs were lack of any serious toxicity issue. However, further detailed toxicity studies are necessary for establishing its complete safety profile.

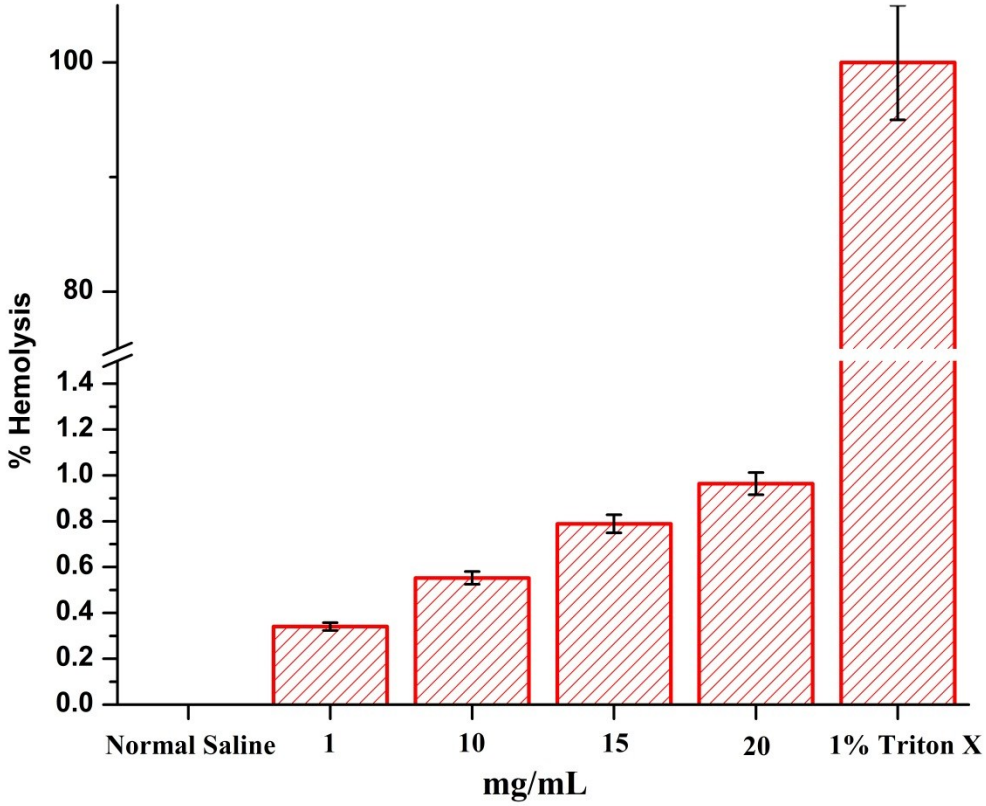

Figure 1A. *In-vitro* hemolytic assay of CS-PLHNs