Electronic Supplementary Material (ESI) for RSC Advances.

Support information for

Fabrication and Characterization of DDAB/PLA-alginate Composite microcapsules System as Single-shot Vaccine

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Methods

Modified Nanoprecipitation Method for DDAB/PLA Nanoparticles Preparation

A certain amount of DDAB and PLA was completely dissolved in 15 mL oil mixture of acetone and ethanol under ultrasonic. Then, the oil phase was slowly dropped into 90 mL deionized water under magnetic stirring with speed of 400-600 rpm. The suspension was continuously stirred overnight for complete evaporation of organic solvent. The DDAB/PLA nanoparticles were finally washed and collected by centrifugation at 20000 g for 15 min for three times. Finally, the nanoparticles were lyophilized for further experiments.

Single Factor Experiment

In order to determine which factors had significant influence on alginate micorcapsules size and HBsAg encapsulation efficiency, single-factor experiment was designed first. Here, we mainly explored the effects of three factors including gas flow rate, liquid flow rate and the mass ratio of ALG to NPs on the average particle size of microcapsules and HBsAg encapsulation efficiency.

Response Surface Methodology (RSM) Experiment

According to Box-Behnken model, gas flow rate (A), liquid flow rate (B) and the mass rate of ALG to NPs (C) were employed as factors, and the particle size of alginate microcapsules and HBsAg encapsulation efficiency were used as response variables. In the previous experiments, we explored the value range of every factor. The factors and levels of RSM experiments were shown in Table S1. The different responses changed with the initial gas flow rate (450, 600, and 750 L/h), liquid flow rate (1.00, 2.00, and 3.00 mL/min) and mass ratio of ALG to NPs (C) (10:1, 15:1 and 20:1). 17 experimental runs in total decided by the 3^3 factorial Box-Behnken models were employed. The quadratic polynomial equation predicted for optimization of dependent variables(Y) was Eq. (1):

$$Y= \alpha_0+\sum_{i=1}^{3} \alpha_i X_i+ \sum_{i=1}^{3} \alpha_{ii} X_i^2 \sum_{i=1}^{3} \sum_{i<j=2}^{3} \alpha_{ij} X_i X_j \qquad (1)$$

Where α_0, α_i, α_{ii}, and α_{ij} were regression coefficients; X_i and X_j were the input variables, which affected the response variable Y. The appropriate program from Design Expert™ 8.0.6 was employed.

Table S1 The factors and levels of RSM experiments

Level	**Factor**		
	Factor A	**Factor B**	**Factor C**
	Gas flow (L/h)	Liquid flow Rate (mL/min)	The mass ratio of ALG to NPs (mg/mg)
-1	450	1.00	10:1
0	600	2.00	15:1
1	750	3.00	20:1

Results and Discussion

Single-factor Experiment

As a vaccine delivery system, the immune response *in vivo* was significantly affected by the antigen encapsulation efficiency and particle size of microcapsules. During preparation of alginate microcapsules three factors including liquid flow rate, gas flow rate and the mass ratio of ALG to NPs were employed for evaluating their influence on the HBsAg encapsulation efficiency and the particle size of microcapsules. Other preparation parameters were consistent with follows: sodium alginate concentration was 1.5 wt%, and the concentration of solidifier of $CaC1_2$ was 0.5 M, and the nozzles with 0.7 mm diameter and 15 cm away from liquid surface was employed.

The Effect of Liquid Flow Rate

The alginate solution containing HBsAg-loaded DDAB/PLA NP suspension was sprayed under liquid flow rate of 1.00, 2.00, 2.50, 3.00 mL/min, respectively. The gas flow rate was fixed at 600 L/h, and the mass ratio of ALG to NPs was 15:1. After solidification, the alginate microcapsules were employed for evaluating the antigen encapsulation efficiency using BCA assay kit and the particle size measurement using MasterSizer 2000 Laser Diffraction Particle Size Analyzer. As shown in Figure S1A, the antigen encapsulation efficiency and particle size of microcapsules increased with the increase of liquid flow rate. In order to meet the needs of vaccine formulation for injection, the particle size of alginate microcapsules should be smaller than 50 μm, and the antigen encapsulation efficiency should be high enough. The obtained microcapsules size was smaller than 50 μm and HBsAg encapsulation efficiency was 50-60% when liquid flow rate was 1.00 and 2.00 mL/min.

The Effect of Gas Flow Rate

The alginate solution containing HBsAg-DDAB/PLA NPs suspension was also sprayed under various gas flow rate of N_2 including 500, 600, 750 L/h, respectively. The liquid flow rate was fixed at 1.00 mL/min, and the mass ratio of ALG to NPs was 15:1. The evaluation results of antigen encapsulation efficiency and particle size of microcapsules were shown in Figure S1B. The antigen encapsulation efficiency and particle size of microcapsules both decreased with the increase of gas flow rate. The alginate capsules with size less than 50 μm were obtained when the gas flow rate was 600 and 750 L/h. The higher shear force led to broken of microcapsules and leakage of HBsAg-loaded NPs due to higher gas flow rate, which further resulted in lower HBsAg encapsulation efficiency.

The Effect of Mass Ratio of ALG to NPs

The alginate microcapsules containing HBsAg-loaded DDAB/PLA NPs were also fabricated using different mass ratio of ALG to NPs as 5:1, 10:1, 15:1, 20:1 and 25:1. The liquid flow rate and the gas flow rate were fixed at 1.00 mL/min and 600 L/h, respectively. The results proved that the mass ratio of ALG to NPs exhibited almost no effect on particle size of alginate microcapsules, but exhibited great influence on encapsulation efficiency and loading efficiency of antigen (Figure S1C in Support information). The results demonstrated that the antigen loading efficiency was decreased with the increase of mass ratio of ALG to NPs, while the encapsulation efficiency was increased with augment of mass ratio of ALG to NPs.

The above single-factor experiments demonstrated that the liquid flow rate, the gas flow rate, and the mass ratio of ALG to NPs had significant influence on antigen encapsulation efficiency and particle size of microcapsules. Therefore, these factors were selected for the further response surface method optimization experiments.

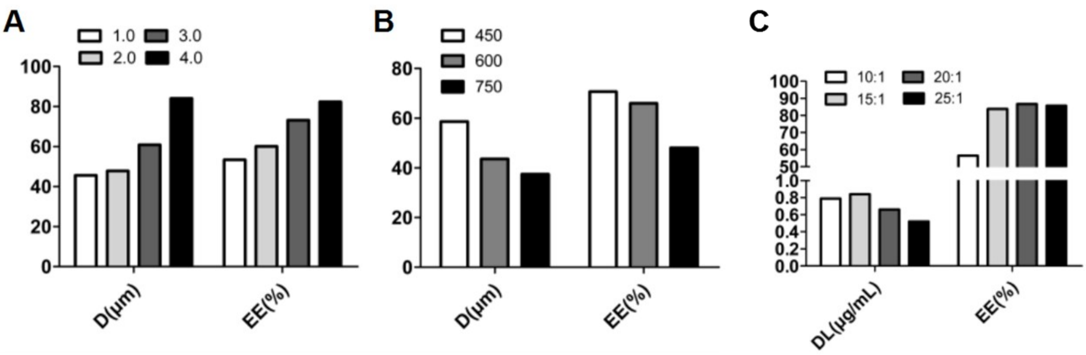


Figure S1 Single-factor experiments-the influence of the liquid flow rate (A), the gas flow rate (B), and the mass ratio of ALG to NPs (C) on the HBsAg encapsulation efficiency and the particle size of microcapsules.

Table S2 Immunization Study in Mice (n=6)

Groups	Formulation	Immunization at
A	PBS	Days 0, 14, and 28
B	PBS plus HBsAg	Days 0, 14, and 28
C	Aluminum plus HBsAg	Days 0, 14, and 28
D	Microcapsules	Day 0

Table S3 ANOVA for response surface reduced quadratic model

Std. Dev.	6.37	**R-Squared**	0.9294
Mean	58.79	**Adj R-Squared**	0.8386
C.V. %	10.84	**Pred R-Squared**	0.1290
PRESS	3509.25	**Adeq Precision**	10.905

Table S4 ANOVA for response surface reduced quadratic model

Std. Dev.	3.91	**R-Squared**	0.9592
Mean	34.28	**Adj R-Squared**	0.9068
C.V. %	11.41	**Pred R-Squared**	0.6770
PRESS	848.07	**Adeq Precision**	15.039

The effects of gas flow, liquid flow rate and the mass ratio of ALG to NPs on the microcapsules size are shown in Figure S2B-D. The factors as gas flow rate and liquid flow rate have an interaction effect on microcapsules size, and the effect of liquid flow rate on microcapsule size was greater than that of gas flow rate (Figure S2B). We found that the mass ratio of ALG to NPs had little impact on microcapsules size (Figure S2C-D). The factor of liquid flow rate has little impact on antigen encapsulation efficiency, and the factor of gas flow rate showed important influence on antigen encapsulation efficiency, and the encapsulation efficiency was decreased with increase of gas flow rate (Figure S2F). The factors as the mass ratio of ALG to NPs and gas flow rate have an interaction effect on encapsulation efficiency, and the effect of gas flow rate on encapsulation efficiency was greater than that of the mass ratio of ALG to NPs (Figure S2G). Both factors as the mass ratio of ALG to NPs and liquid flow rate showed little impact on encapsulation efficiency when the mass ratio of ALG to NPs was more than 15. However, when the mass ratio of ALG to NPs was between 10 and 15, the factors as the mass ratio of ALG to NPs and liquid flow rate demonstrated interaction effect on encapsulation efficiency, and the effect of the mass ratio of ALG to NPs on encapsulation efficiency was greater than that of liquid flow rate (Figure S2H). In addition, there is no strong evidence for the departures from the normal plots of the residuals for size and encapsulation efficiency. As seen in Figure S2A and E, all the points in the plot form a straight line. As a result, we can say that the model is fairly suitable.

The ideal composite microcapsules not only show high encapsulation efficiency, but also have smaller particle size. Based on the analysis of the response surface model, we found that the mass ratio of ALG to NPs had little impact on microcapsules size and antigen encapsulation efficiency. The mass ratio of ALG to NPs was fixed at 15:1, and the effects of gas flow rate and liquid flow rate on microcapsules size and antigen encapsulation efficiency were investigated. With multiple responses, the optimal condition where all parameters simultaneously meet the desirable criteria could be visually searched by overlaying plot (Figure S3). Graphical optimization showed the shadow region could meet the above conditions at the same time, and the encapsulation efficiency of composite microcapsules was more than 60% and the size was about 30 μm. According to overlay plot, the shadow region was determined as optimum process parameters.

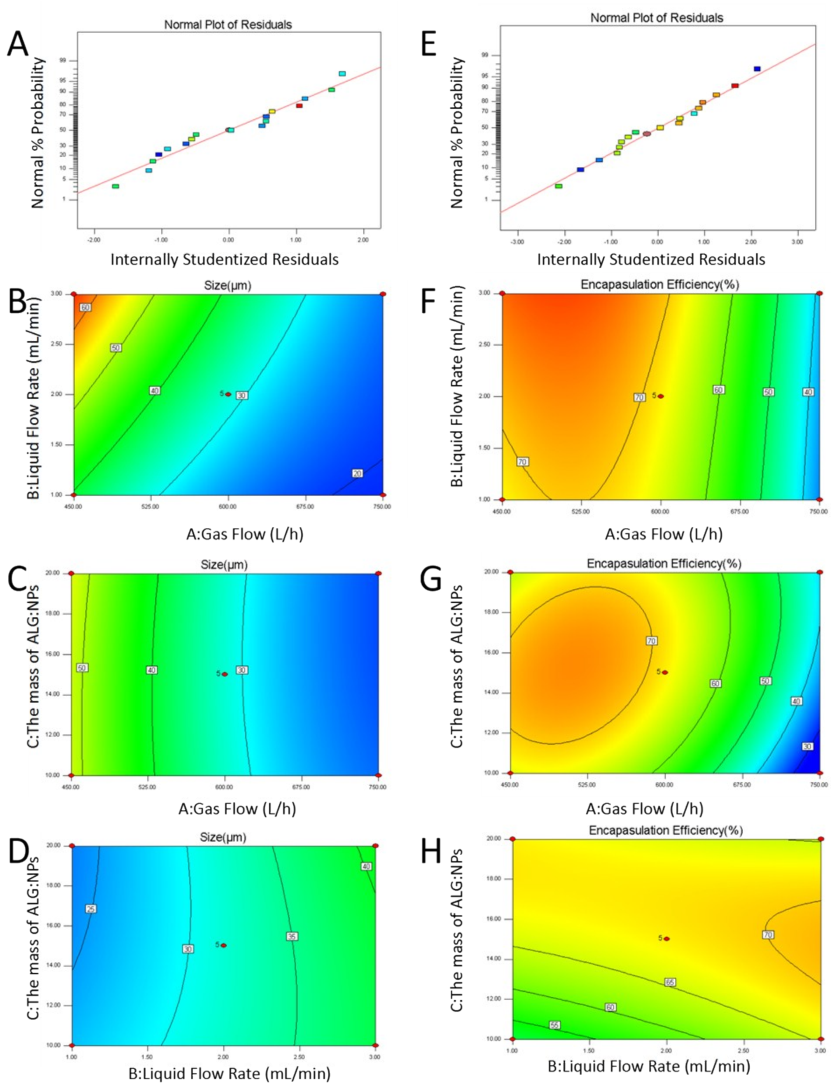


Figure S2 DOE-RSM analysis of size as a function of gas flow rate, liquid flow rate and the mass ratio of ALG to NPs (B; C; D), perturbation analyses (A); DOE-RSM analysis of final HBsAg encapsulation efficiency as a function of gas flow rate, liquid flow rate, and the mass ratio of ALG to NPs (F; G; H), perturbation analyses (B).

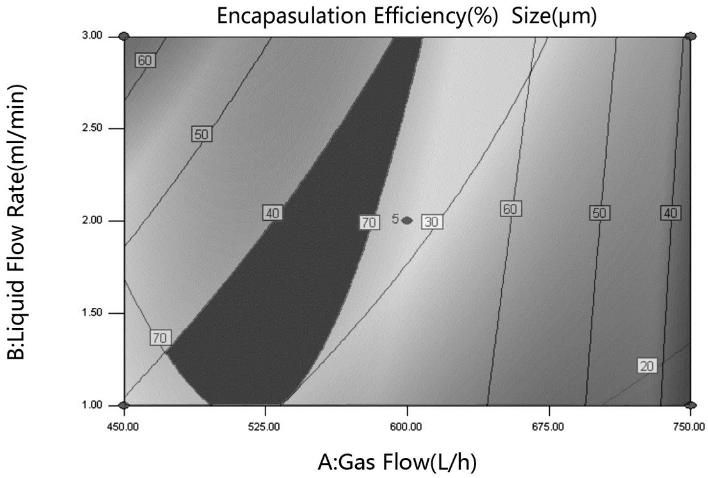


Figure S3 Overlay plot showing optimal values of gas flow rate, liquid flow rate, and the mass ratio of ALG to NPs for achieving an optimal compromise between size and encapsulation efficiency

Table S5 Verification test of RSM (Encapsulation efficiency)

No.	**A/L/h**	**B/ml/min**	**C/mg/mg**	**Predicted value/ %**	**Actual value/ %**	**Error/ %**
1	591.5	1	18.68:1	69.18	70.52	1.9
2	591.5	1	18.68:1	69.18	64.64	6.5
3	591.5	1	18.68:1	69.18	63.7	7.9

Table S6 Verification test of RSM (Size)

No.	**A/L/h**	**B/ml/min**	**C/mg/mg**	**Predicted value/ μm**	**Actual value/ μm**	**Error/ %**
1	591.5	1	18.68:1	24.249	27.060	11.5
2	591.5	1	18.68:1	24.249	20.473	15.5
3	591.5	1	18.68:1	24.249	26.780	10.4

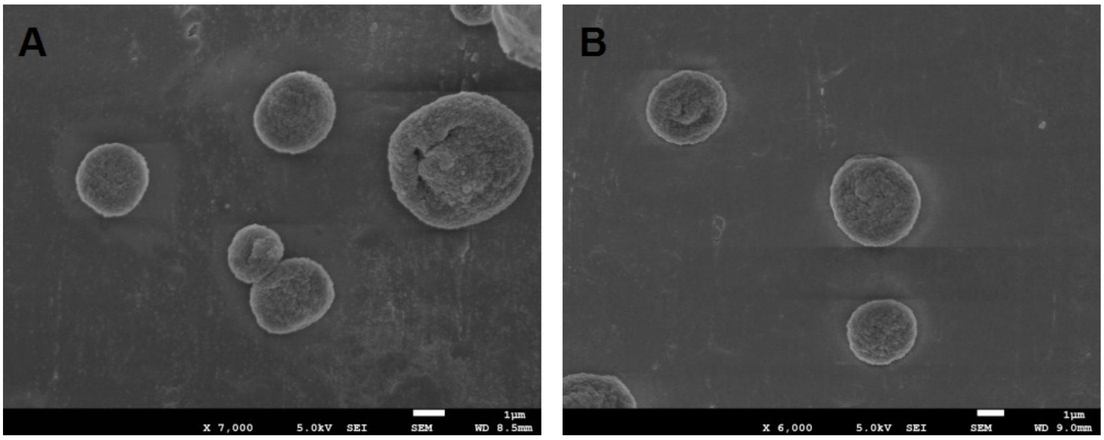
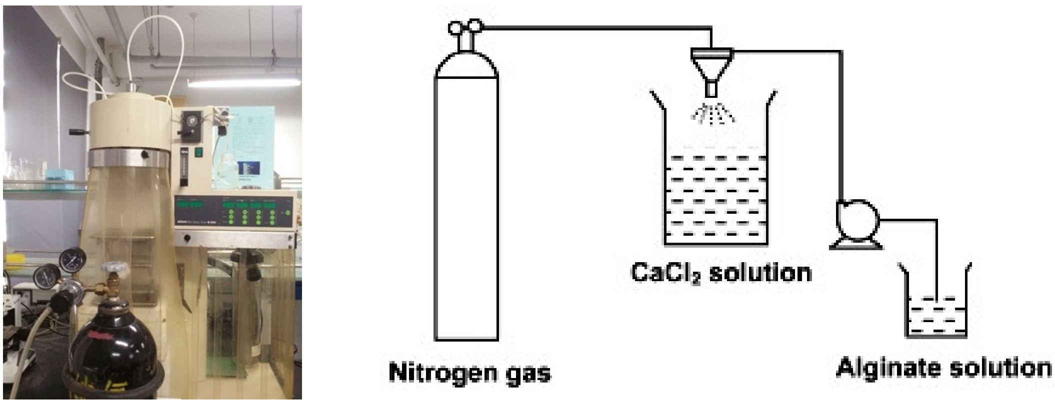


Figure S4 The SEM images of alginate microcapsules containing HBsAg-loaded DDAB/PLA nanoparticles solidified by $CaCl_2$ with concentrations of 0.5 M (A) and 1.0 M (B)

The microcapsules were prepared employing the equipment as shown in Figure S5, which could continuously prepare microcapsules to meet large scale productions. We had prepared three batches microcapsules and measured the encapsulation efficiency of HBsAg, particle size and morphology of the microcapsules. The measurements suggested that three batches microcapsules showed similar particle size, morphology and antigen encapsulation efficiency as shown in Figure S6 and Table S7.



A B

Figure S5 The photograph (A) and schematic diagram (B) of equipment for microcapsules preparation

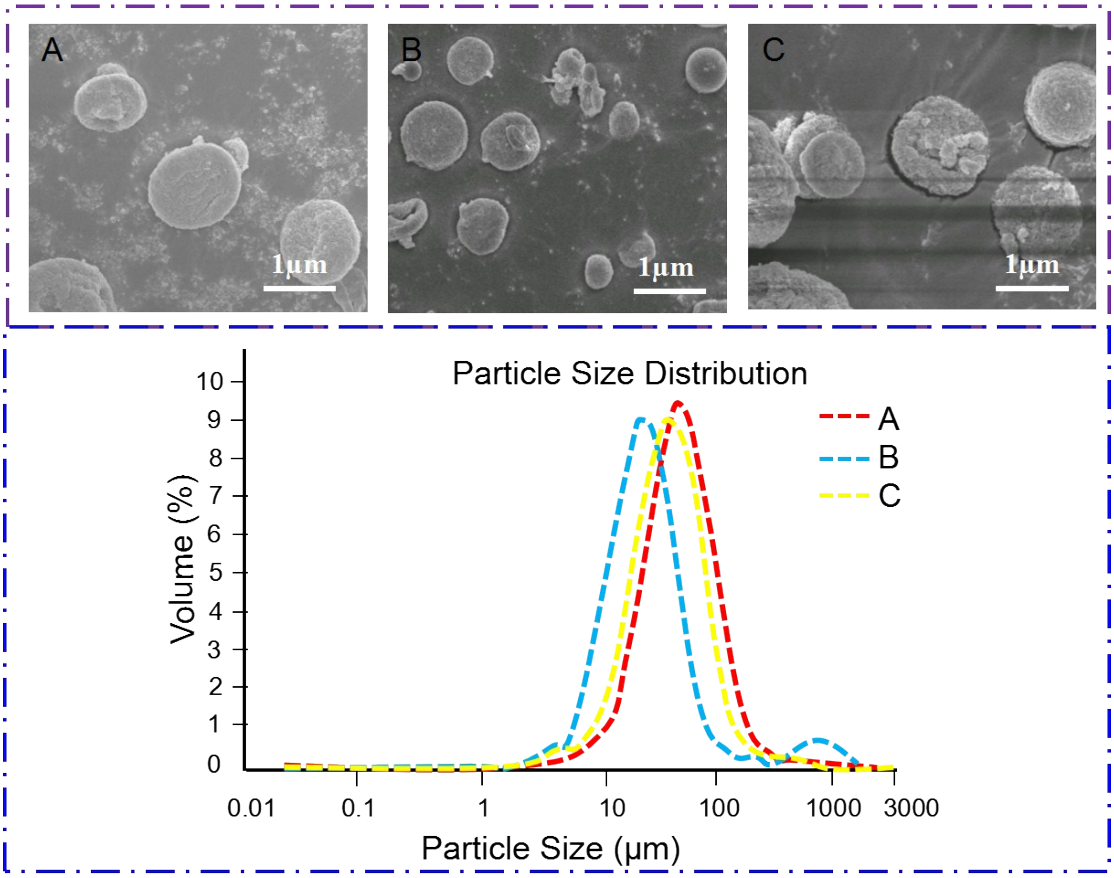


Figure S6 The SEM micrographs (A-C) and size distributions of three batches HBsAg-DDAB/PLA-alginate microcapsules

Table S7 The characteristics of three batches HBsAg-DDAB/PLA-alginate microcapsules

Batches.	**Size/μm**	**Span value**	**Encapsulation efficiency/%**
1	27.060	1.774	70.52
2	20.473	2.465	64.64
3	26.780	1.947	63.70

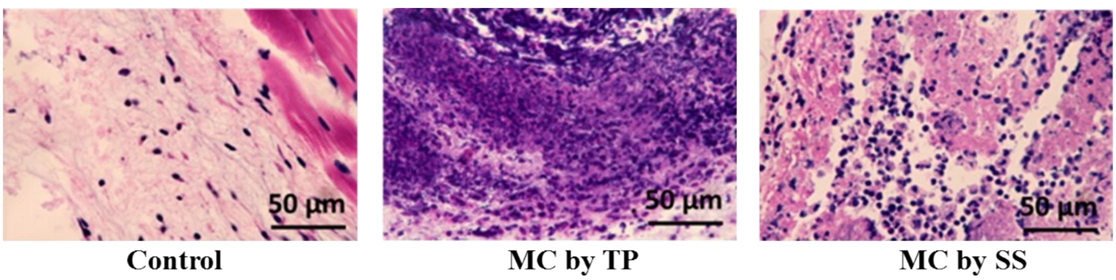


Figure S7 The local inflammation of HBsAg-DDAB/PLA-alginate microcapsules by traditional preparation (MC by TP) and spray-solidification method (MC by SS) employing in this study