Ethosomal Nanocarriers for Hydrophilic Peptide Encapsulation: Formulation Optimization, Stability, and In Vitro Release Performance

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SUPPLEMENTARY INFORMATION

S1. Detailed Lowry Assay Procedure and Method Validation Data

The Lowry assay was performed using freshly prepared reagents. Solution A was prepared by dissolving sodium hydroxide (2.8598 g) and sodium carbonate (14.3030 g) in distilled water and diluting the mixture to a total volume of 500 mL. Solution B consisted of copper(II) sulfate pentahydrate (1.4232 g) dissolved in 100 mL of distilled water, while Solution C was prepared by dissolving sodium–potassium tartrate (2.85299 g) in 100 mL of distilled water. Immediately before use, the Lowry reagent was prepared by mixing Solutions A, B, and C in a 100:1:1 (v/v/v) ratio. The Folin–Ciocalteu reagent (1 N) was also prepared fresh on the day of analysis and protected from light.

Hydrolyzed collagen working standards were prepared in distilled water at concentrations of 20, 40, 60, 80, and 100 µg/mL. All standards were prepared immediately prior to analysis to minimize degradation and ensure consistency.

For each determination, 0.5 mL of sample or standard solution was transferred into a 10 mL glass test tube. Subsequently, 0.7 mL of the freshly prepared Lowry reagent was added, and the mixture was vortexed to ensure complete mixing. The tubes were then incubated for 20 minutes in the dark at 25 ± 2 °C. Following this incubation period, 0.1 mL of 1 N Folin–Ciocalteu reagent was added to each tube, and the contents were mixed immediately. The reaction mixtures were left to stand at room temperature for 30 minutes to allow for full color development. Absorbance values were recorded at 750 nm using a UV–Visible spectrophotometer. A reagent blank containing all assay components except hydrolyzed collagen was included in each analytical run

and used to correct all absorbance values. Collagen concentrations in the unknown samples were calculated by interpolating absorbance values onto the standard calibration curve.

S.1.1. Calibration Curve and Linearity

The calibration curve was constructed by plotting absorbance at 750 nm against standard hydrolyzed collagen concentrations ranging from 20 to 100 µg/mL. Linear regression analysis yielded a strong linear relationship, with a correlation coefficient (r^2) greater than 0.99. The calibration plot is provided in Figure S1. Method linearity was assessed in accordance with ICH guidelines (ICH Q2(R1), 1995).

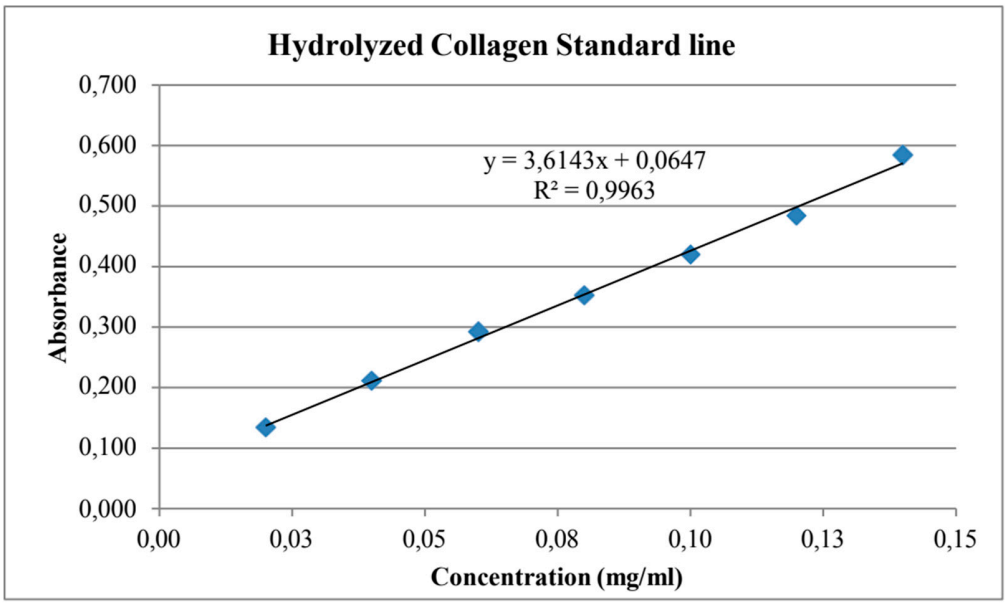


Figure S1.The standard line of hydrolyzed collagen obtained by Lowry method

S.1.2. Accuracy (Recovery Studies)

Accuracy was assessed by recovery experiments at three concentration levels corresponding to 20, 60, and 80 µg/mL prepared from the hydrolyzed collagen stock solution. Each solution was analyzed using the Lowry method, and concentrations were calculated from the calibration curve. Known quantities of hydrolyzed collagen were added to pre-analyzed matrices, and each level was analyzed in triplicate. Percent recovery was calculated by comparing the measured concentrations to the theoretical spiked amounts. Percent recovery (%R) was calculated using Formula where C_{real} is the concentration determined from the calibration curve and C_{spiked} is the nominal concentration.

$$R\% = \frac{Creal}{Cspiked} x100$$

Recovery values ranged from 98 to 102 percent, indicating excellent accuracy of the analytical method. Detailed recovery data, including individual replicate values and calculated means, are provided in Table S1.

Table S1. Accuracy (Recovery) Data for Hydrolyzed Collagen at Three Concentration Levels (20, 60, and 80 μg/mL)

Sample	**Measured value (μg/ml)**	**Recovery (%)**	**Measured value (μg/ml)**	**Recovery (%)**	**Measured value (μg/ml)**	**Recovery (%)**
	20 μg/ml level		**60 μg/ml level**		**80 μg/ml level**	
1	18.62	93.10	62.06	103.43	78.38	97.98
2	18.34	91.72	62.06	103.43	78.94	98.67
3	18.07	90.34	61.51	102.51	77.83	97.29
4	17.51	87.57	61.51	102.51	77.83	97.29
5	17.51	87.57	62.34	103.89	77.55	96.94
Statistics	**20 μg/ml level**		**60 μg/ml level**		**80 μg/ml level**	
Average recovery (%)	90.06		103.16		97.63	
Reliability level (p)	0.05		0.05		0.05	
Standard deviation	2.47		0.62		0.69	
Coefficient of Variation	0.0275		0.0060		0.0071	
n (sample size)	5		5		5	
Confidence interval	2.17		0.54		0.61	

S.1.3. Precision

Precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability was assessed by analyzing six replicates of a single concentration within the same day, while intermediate precision was determined by conducting the analysis over three separate days under identical conditions. Precision was assessed at three levels: repeatability, intermediate precision, and reproducibility. For both studies, relative

standard deviation (RSD) values were below 2%, confirming that the method demonstrates both high repeatability and reproducibility. Comprehensive precision data are summarized in Table S2a and S2b.

Table S2a. Pecision (Repeatability) of Hydrolyzed Collagen in PBS at Three Concentration Levels (20, 60, and 90 μg/mL)

Sample	Measured value (μg/mL)	Measured value (μg/mL)	Measured value (μg/mL)
	20 μg/ml level	60 μg/ml level	90 μg/ml level
1	18.62	62.06	93.05
2	18.34	61.51	92.77
3	18.07	61.51	93.32
Average	18.34	61.69	93.05
Reliability level	0.05	0.05	0.05
Standard deviation	0.28	0.32	0.28
Coefficient of Variation	0.0151	0.0052	0.0030
n (sample size)	3	3	3
Confidence interval	0.31	0.36	0.31

Table S2b. Repeatability of Hydrolyzed Collagen in PBS at 100 μg/mL

Sample	Concentration
	100μg/ml
1	0.428
2	0.428
3	0.428
4	0.435
5	0.484
6	0.476

Average	0.447
Reliability level	0.05
Standard deviation	0.026
Coefficient of Variation	0.0589
n (sample size)	6
Confidence interval	0.021

S.1.4. Limit of detection (LOD) and Limit of Quantification (LOQ)

The lowest concentration at which hydrolyzed collagen can be detected with acceptable accuracy and repeatability was calculated as 20μg/ml and the lowest concentration at which hydrolyzed collagen can be determined with acceptable accuracy and repeatability was calculated as 60μg/ml.

S.1.5. Range

The range of the analytical procedure was defined as the interval between the lowest and highest concentrations of hydrolyzed collagen in which the method demonstrated acceptable precision, accuracy, and linearity.

S.1.6. Robustness

Robustness was evaluated by introducing small, intentional variations in critical assay parameters, including slight changes in incubation temperature (25 ± 3 °C), incubation time following Lowry reagent addition (20 ± 2 minutes), and the volume of Folin–Ciocalteu reagent (0.10 ± 0.01 mL). These variations did not result in significant differences in absorbance values, with deviations remaining below 2 %. The results confirmed that the method is robust and reliable under minor operational fluctuations. Detailed robustness results are presented in Table S3.

Table S3.Hydrolyzed collagen stability test results in PBS (Robustness Assessment Data)

Concentration	1st Measurement (A)	2nd Measurement (A)	% Difference
100μg/ml	0.456	0.420	7.89

S.1.7. Specificity

Specificity was assessed by analyzing reagent blanks and blank matrices containing formulation excipients without hydrolyzed collagen. No measurable absorbance at 750 nm was observed in these samples, confirming that the excipients did not contribute to any interfering background signal.

S.1.8. Summary of Validation

All evaluated validation parameters, including linearity, accuracy, precision, LOD, LOQ, robustness, specificity, and analytical range, complied with the acceptance criteria outlined in ICH Q2(R2) (2023). The Lowry method was therefore validated as a reliable and sensitive analytical approach for the quantitative determination of hydrolyzed collagen in the investigated formulations.

S2. Cryo-SEM Analysis

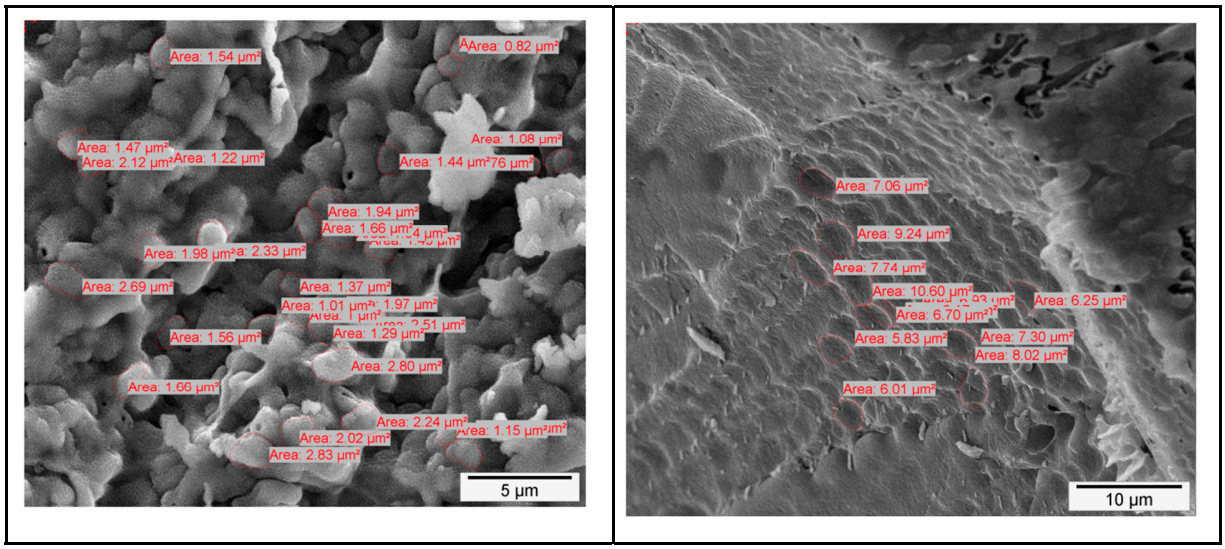


Figure S2. Annotated Cryo-SEM images of blank ethosomes (T24, left) and hydrolyzed collagen-loaded ethosomes (T11, right).

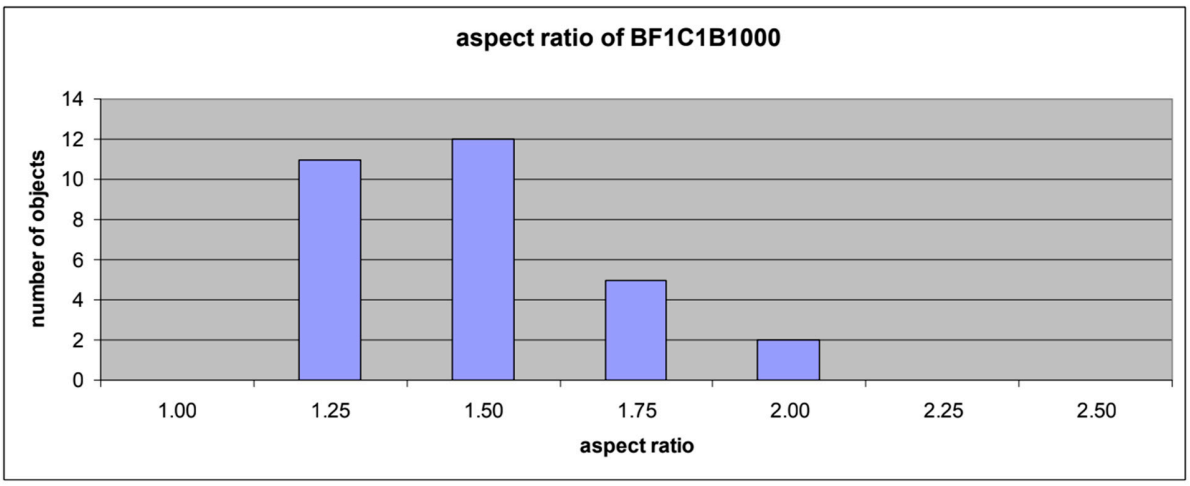


Figure S3. Distribution of mean aspect ratio versus number of analyzed vesicles for blank (T24) ethosomal formulation. Data were obtained from cryo-SEM image-based morphometric analysis, where individual vesicle contours were annotated and projected area measurements were used to calculate aspect ratios. The figure illustrates the slight increase in mean aspect ratio upon peptide incorporation while maintaining overall vesicle homogeneity.

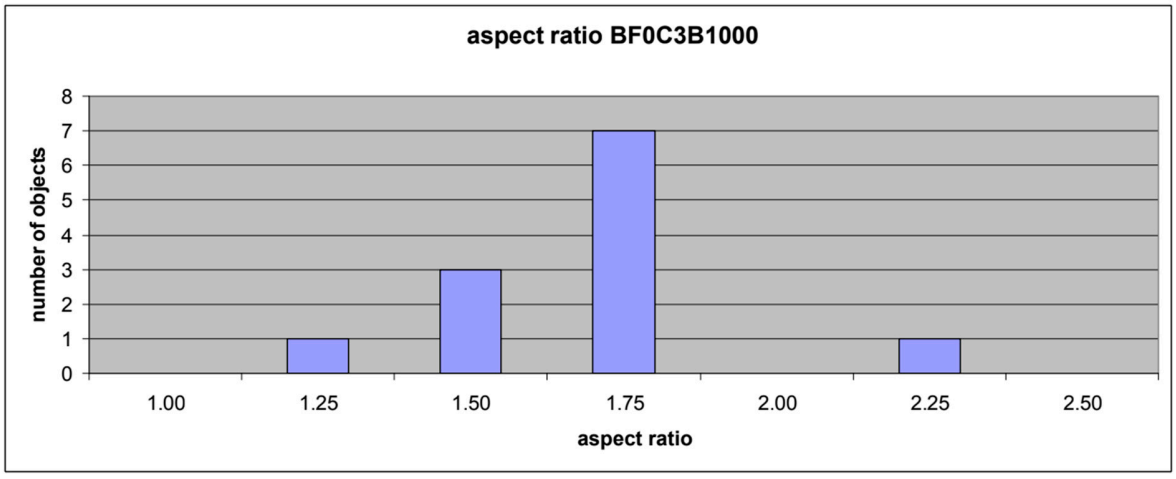


Figure S4. Distribution of mean aspect ratio versus number of analyzed vesicles for hydrolyzed collagen peptide-loaded (T11) ethosomal formulation. Data were obtained from cryo-SEM image-based morphometric analysis, where individual vesicle contours were annotated and projected area measurements were used to calculate aspect ratios. The figure illustrates the slight increase in mean aspect ratio upon peptide incorporation while maintaining overall vesicle homogeneity.

S3. Particle Size Distribution and Stability Over Time

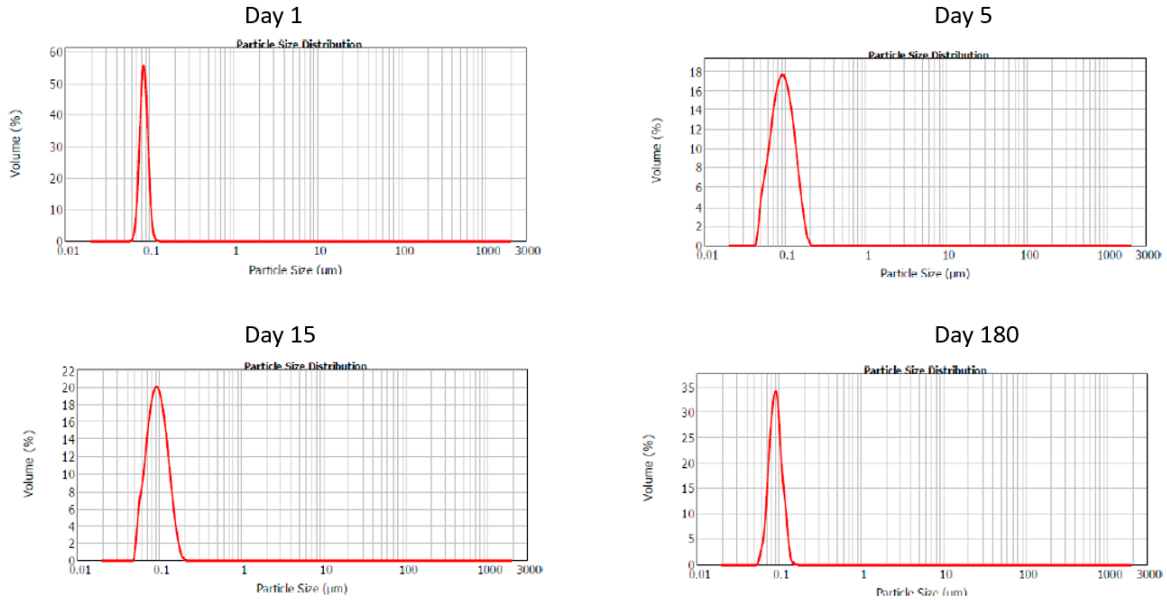


Figure S5. Particle size distribution of blank ethosomes (T24) measured on day 1, 5, 15, and 180, showing long-term physical stability.

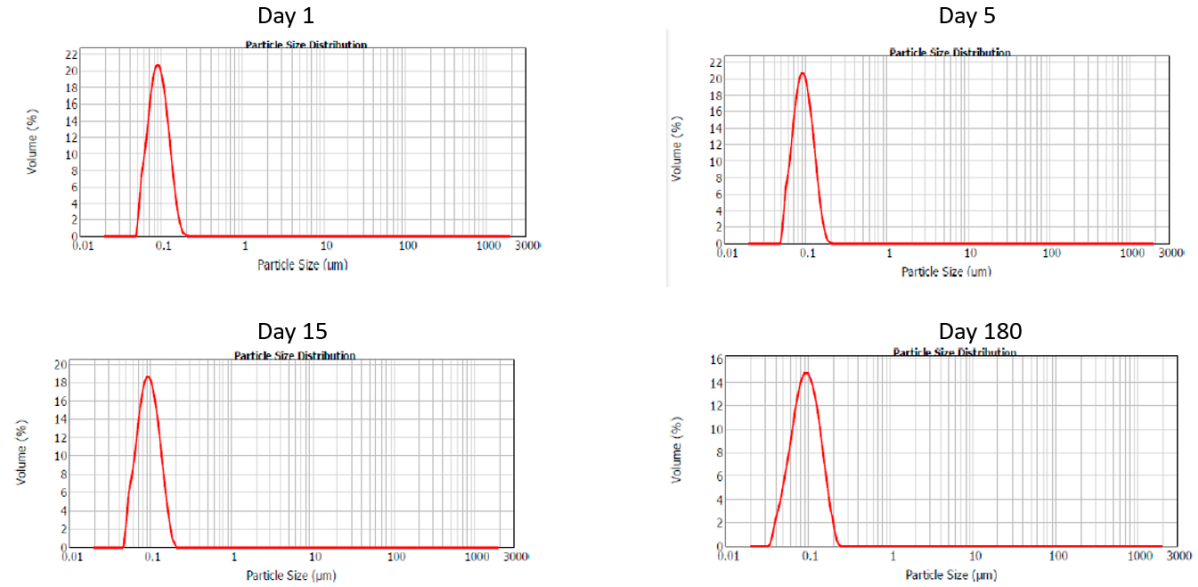


Figure S6. Particle size distribution of hydrolyzed collagen peptide-loaded ethosomes (T11) measured on day 1, 5, 15, and 180, showing long-term physical stability.

Measurements

Name	Temperature [°C]	Processed runs	Mean zeta potential [mV]	Distribution peak [mV]	Conductivity [mS/cm]	Electrophoreti c Mobility [µm*cm/Vs]	Color
T21	25,0	100	-69,4	-70,8	0,039	-5,4121	
T22	25,0	100	-65,5	-64,6	0,014	-5,1066	
T23	25,0	100	-70,3	-68,0	0,016	-5,4754	
T24	25,0	100	-64,8	-68,3	0,015	-5,0519	

Zeta potential distribution

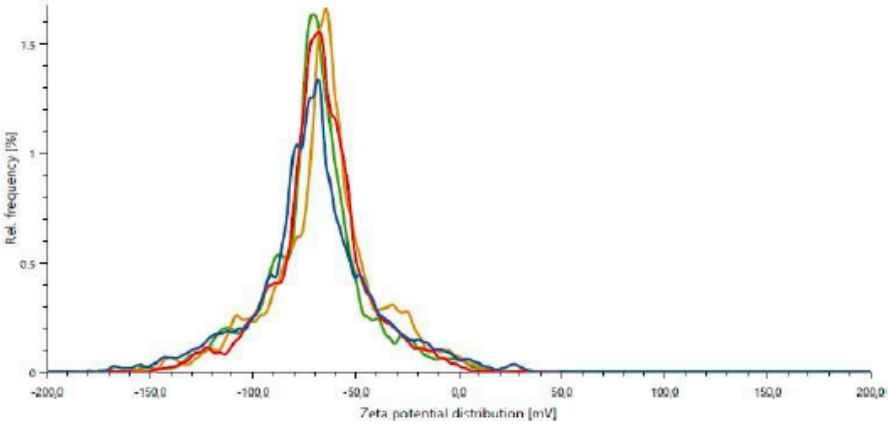


Figure S7. The data from zeta potential measurements of blank ethosomes (T21-T22-T23-T24) on Day 1.

Measurements

Name	Temperature [°C]	Processed runs	Mean zeta potential [mV]	Distribution peak [mV]	Conductivity [mS/cm]	Electrophoreti c Mobility [µm*cm/Vs]	Color
T9	25,0	100	-46,6	-43,5	0,040	-3,6334	
T10	25,0	100	-44,9	-44,6	0,028	-3,4985	
T11	25,0	100	-43,2	-41,9	0,025	-3,3647	
T12	25,0	100	-42,9	-44,1	0,025	-3,3454	

Zeta potential distribution

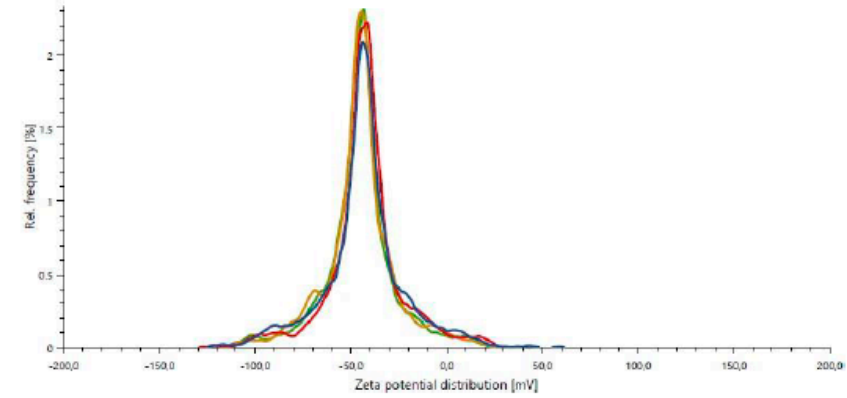


Figure S8. The data from zeta potential measurements of HC peptide-loaded ethosomes (T9-T10-T11-T12) on Day1.

Measurements

Name	Temperature [°C]	Processed runs	Mean zeta potential [mV]	Distribution peak [mV]	Conductivity [mS/cm]	Electrophoreti c Mobility [µm*cm/Vs]
T21	25,0	100	-43,4	-45,2	0,025	-3,3863
T22	25,0	100	-52,4	-52,7	0,030	-4,0819
T23	25,0	100	-51,0	-55,3	0,030	-3,9768
T24	25,0	100	-40,7	-41,4	0,026	-3,1723

Zeta potential distribution

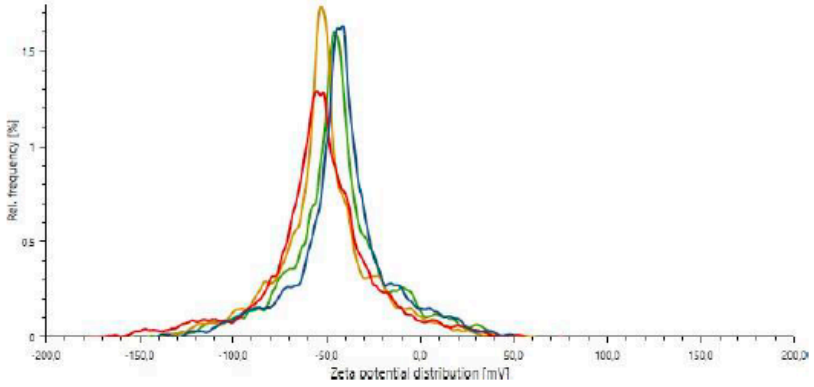


Figure S9. The data from zeta potential measurements of blank ethosomes (T21-T22-T23-T24) on Day 15.

Measurements

Name	Temperature [°C]	Processed runs	Mean zeta potential [mV]	Distribution peak [mV]	Conductivity [mS/cm]	Electrophoreti c Mobility [µm*cm/Vs]
T9	25,0	100	-38,8	-35,3	0,043	-3,0274
T10	25,0	100	-41,4	-37,6	0,039	-3,2231
T11	25,0	100	-43,3	-43,7	0,042	-3,3783
T12	25,0	100	-41,1	-42,7	0,041	-3,2039

Zeta potential distribution

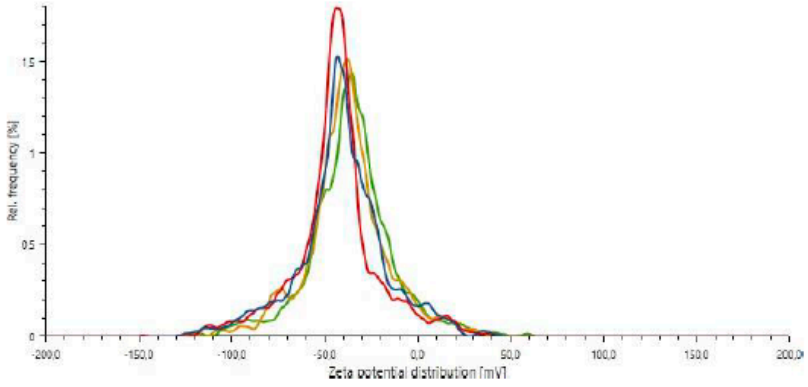


Figure S10. The data from zeta potential measurements of HC peptide-loaded ethosomes (T9-T10-T11-T12) on Day15.

Measurements

Name	Temperature [°C]	Processed runs	Mean zeta potential [mV]	Distribution peak [mV]	Conductivity [mS/cm]	Electrophoreti c Mobility [µm*cm/Vs]
T21	25,0	100	-43,4	-45,2	0,025	-3,3863
T22	25,0	100	-52,4	-52,7	0,030	-4,0819
T23	25,0	100	-51,0	-55,3	0,030	-3,9768
T24	25,0	100	-40,7	-41,4	0,026	-3,1723

Zeta potential distribution

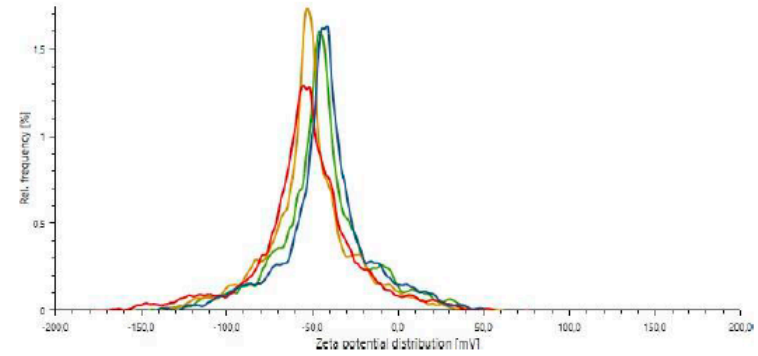


Figure S11. The data from zeta potential measurements of blank ethosomes (T21-T22-T23-T24) on Day 30.

Measurements

Name	Temperature [°C]	Processed runs	Mean zeta potential [mV]	Distribution peak [mV]	Conductivity [mS/cm]	Electrophoreti c Mobility [µm*cm/Vs]
T9	25,0	100	-38,8	-35,3	0,043	-3,0274
T10	25,0	100	-41,4	-37,6	0,039	-3,2231
T11	25,0	100	-43,3	-43,7	0,042	-3,3783
T12	25,0	100	-41,1	-42,7	0,041	-3,2039

Zeta potential distribution

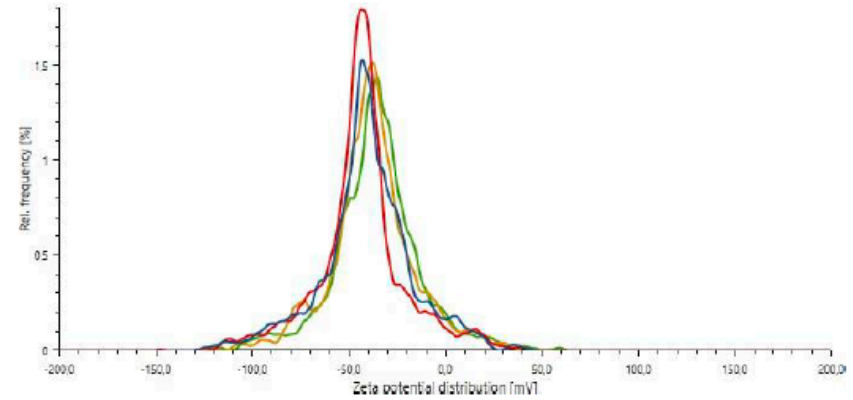


Figure S12. The data from zeta potential measurements of HC peptide-loaded ethosomes (T9-T10-T11-T12) on Day30.