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Enzymatically hydrolyzed sodium caseinate nanoparticles efficiently enhancing solubility, stability, antioxidant and anti-biofilm activity of hydrophobic Tanshinone IIA

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Method S1. Details of the HPLC method

0.8 mL of ethanol (95%) was added to 0.2 mL of TA-NPs solution and vortexed for 5 min to completely extract encapsulated TA. The absorption peak of the extract was measured by HPLC and the TA content was calculated by referring to the standard curve. TA detection was performed at a wavelength of 270 nm, using a Venusil XBP C18 reversed-phase column (5 μm, 4.6 mm × 250 mm, Agela Tech, CA, USA) at 25 °C. The flow rate was maintained at 1.0 mL/min. The elution solvent was phase A (methanol) and phase B (Ultra-pure water), the ratio was 90% A and 10% B.

Method S2. MIC and MBC assay

The minimum inhibitory concentration (MIC) of TA-NPs and free TA was determined by the 96-well microdilution method of resazurin [1] and the minimum bactericidal concentration (MBC) was tested by solid media diffusion assays [2].Briefly, 200 μL of free TA aqueous solution and TA-NPs solution (same TA concentration) were transferred to the first row of a sterile 96-well plate and 100 μL of BHI was added to all other wells in the same column. 100 μL of liquid was pipetted at a time into the next wells in the same column for serial dilution, discarding the tips after each dilution so that each well in the column had 100 μL of the test solution in successive decreasing concentration. After that, 100μL of *S. mutans* bacterial dilution solution (the following bacterial dilutions, if not specified otherwise, all refer to the bacterial solution diluted with fresh BHI to OD_{600}=0.5) was added to each well. After 24h of anaerobic incubation at 37°C, 40μL of resazurin (0.015%) indicator solution was added to each well and left at room temperature for 20-40min to observe the color change. The concentration of the drug corresponding to the row that turned pink for the first time was the MIC.

The solid medium (2% w/v) was divided into 6 zones and 10 μL of the test solution

mixed with the bacterial solution was added to each zone. The number of colonies on the solid medium was observed after incubation at 37°C for 24 h. The lowest concentration that results in a bacterial colony count of less than 10 is the MBC.

Method S3. Details of washing

The bacterial solution was centrifuged at 5000×g for 5 min at 4°C. The supernatant was discarded and the precipitate was resuspended in 1 mL of sterile saline, referred to as a single wash. The purpose was to completely remove the medium components.

Method S4. Crystal violet (CV) staining assay of *S. mutans* biofilms

Bacteria used for biofilm experiments were cultured in BHIS (sucrose, 1% w/v) medium and diluted with BHIS, as the involvement of sucrose contributes to biofilm formation [3]. The crystal violet (CV) analysis method was used to quantitatively evaluate the biomass of biofilms in 96-well plates [4]. 100 μL of bacterial dilution (OD_{600}=0.5) was mixed with 100 μL of TA-NPs or free TA solution (same concentration as above). Blank control contained bacterial diluent and deionized water. 200 μL of methanol was added for 15 min and removed by rinsing with sterile PBS. Then 200 μL of 0.25% CV solution was added to each well for 30 min, followed by 4-5 times with sterile distilled water. Finally, 200 μL of 95% ethanol was added to dissolve the CV. OD_{550} was measured using an enzyme-labeled instrument. three sets of experiments were performed independently for each experimental group at each time point.

Method S5. Bacterial surface hydrophobicity assay

The surface hydrophobicity of microorganisms was measured based on the adhesion of microorganisms to hydrocarbons[5]. Briefly, *S. mutans* dilution were incubated with TA-NPs (the final concentration of encapsulated TA was 1/8MIC, 1/4MIC) or free TA (the final concentration of TA was 100μg/mL) under aerobic conditions at 37°C for 30 min. After that, the mixed solution was washed twice and resuspended with 1 mL sterile PBS (pH=7.4) solution. The OD_{550} was measured and recorded as OD_1. Then we added 20% (v/v) xylene dropwise to the resuspension solution, and vigorously vibrated the mixture for 2 min. The mixture was then allowed to stand until the water phase and the organic phase were completely separated. The OD_{550} of the water phase was measured (recorded as OD_2). The hydrophobicity calculation formula is: $H = (OD_1-OD_2)/OD_1\times100\%$. In this experiment, each group of solutions was independently measured for three times.

Method S6. Bacterial aggregation assay

The 96-well plate method was used to determine the aggregation of bacteria [2]. In brief, 2 mL of the bacterial dilution was washed twice and resuspended in 1 mL of sterile PBS. 200μL of resuspension was transferred to a well of 96-well plate. The OD_{600} of the initial solution was recorded as $OD_{Initial}$, and then TA-NPs (the final concentration of TA was 1/8MIC, 1/4MIC) were added to the resuspension solution, and incubated at 37°C for 2 h without stirring. The OD_{600} after 2h incubation was recorded as OD_{2h}. The percentage of bacterial aggregation was obtained by the following equation: Aggregation rate= $(OD_{Initial}-OD_{2h})$ / $(OD_{Initial}-OD_{Blank})\times100\%$. Where OD_{Blank} was the blank absorbance of the container. Each experiment was carried out independently in three groups in parallel.

Method S7. Bacterial glycolytic activity assay

The inhibitory effect of TA-NPs on the glycolytic activity of *S. mutans* was measured, and the method was modeled on other experiments [6]. Simply, the *S. mutans* bacterial dilution (OD_{600}=0.5) was washed twice with sterile saline to remove the medium components (BHI). The bacteria were resuspended in a saline solution containing TA-NPs (the final concentration of encapsulated TA was 1/8MIC,1/4MIC) or free TA (the final concentration of TA was 50μg/mL), and then glucose (1% w/v) was added. Then the pH of resuspension was adjusted to 7.2-7.4 with 0.2M NaOH solution. The blank control group was added with the same volume of salt solution without nanoparticles. The positive control group was added with an equal volume of saline solution containing 0.2mg/mL erythromycin. Within 120 minutes, pH tester was used (PB-10, Sartorius, Beijing, China) to check the pH of the solution every 20 min. The difference in pH values indicated the effect of nanoparticles on the glycolytic activity of *S. mutans*. Three parallel experiments were performed independently for each group of solutions.

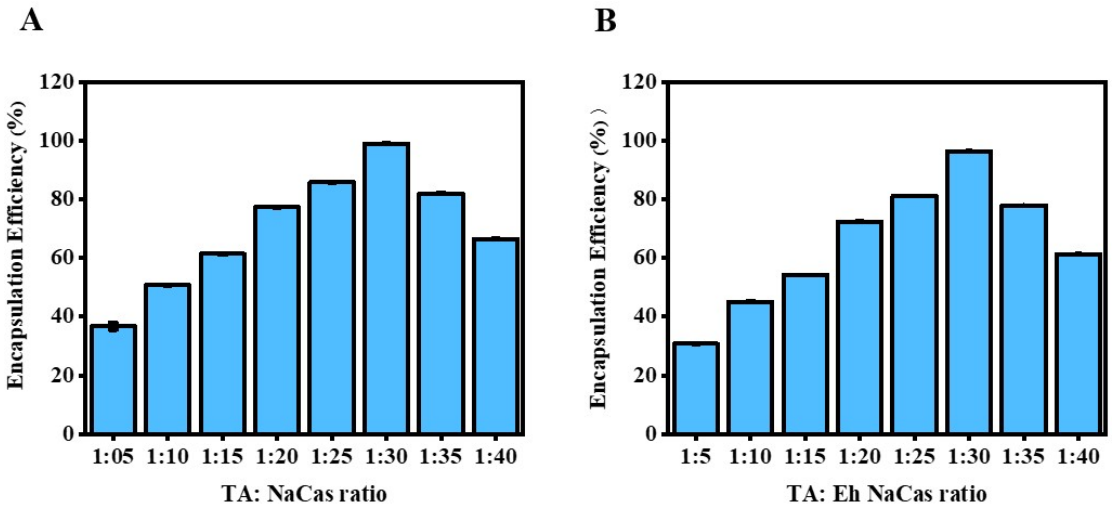

Fig. S1 Encapsulation efficiency (EE) of nanoparticles with different drug loading mass ratios. (A)TA: NaCas and (B)TA: Eh NaCas

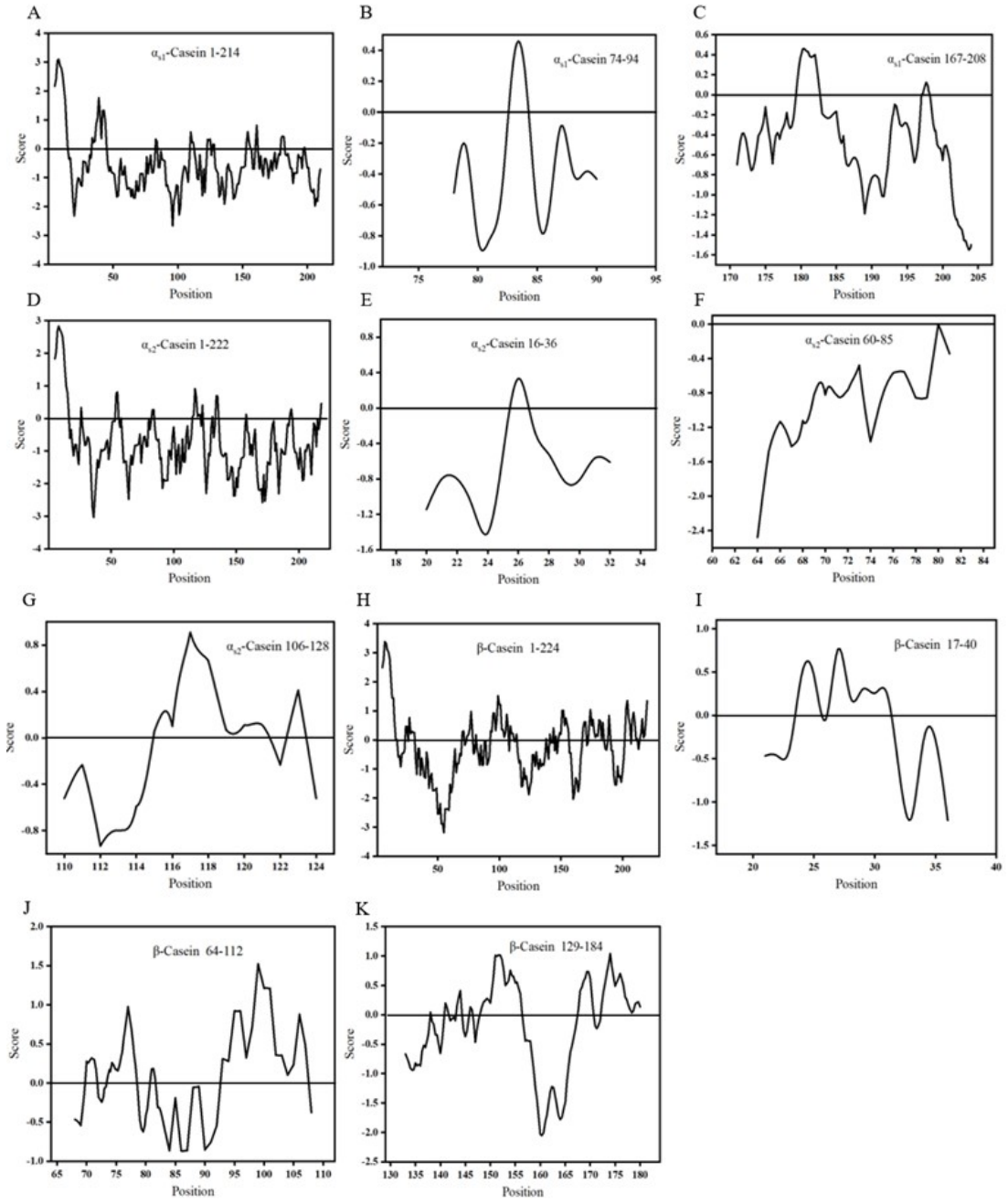

Fig. S2 Hydropath of peptide output by ProtScale (web.expasy.org/protscale/). a_{s1}-Casein sequence position: (A) 1-214, (B) 74-94, (C) 167-208. a_{s2}-Casein sequence position: (D) 1-222, (E) 16-36, (F) 60-85, (G) 106-128. β-Casein sequence position: (H) 1-224, (I) 17-40, (J) 64-112, (K) 129-184. The curve with lower values indicates better hydrophilicity [7]. Detailed information about the peptides was shown in Table S1.

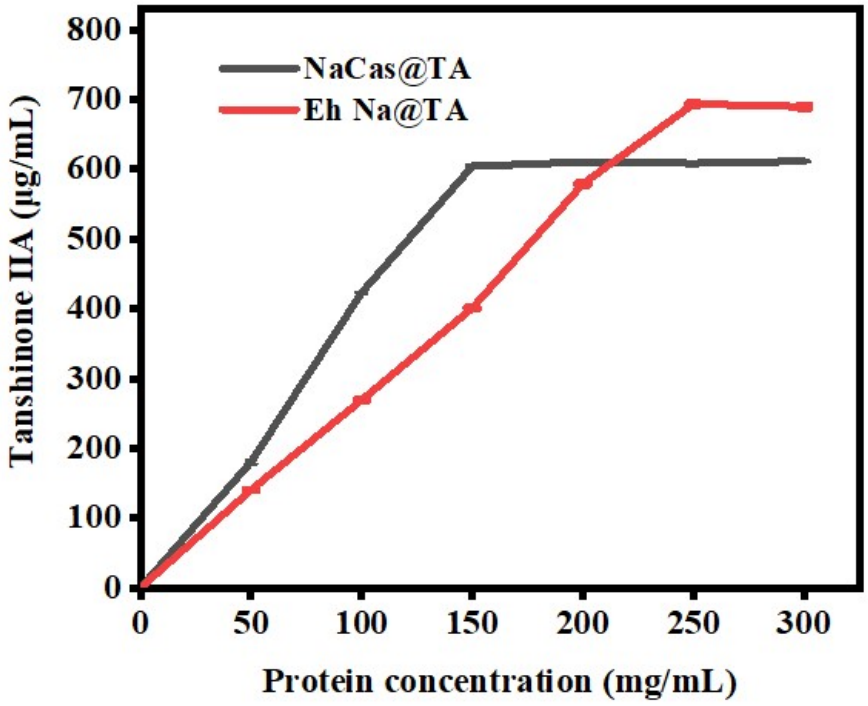

Fig. S3 Linear relationship between maximum encapsulated TA solubility and protein concentration

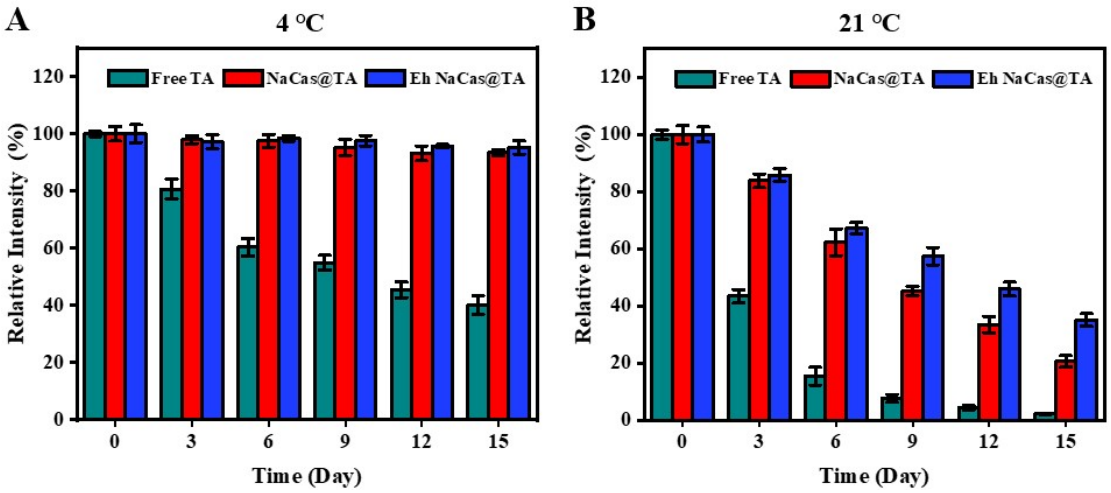

Fig. S4 Free TA, NaCas@TA and Eh NaCas@TA's storage stability at (A) 4°C and (B) 21°C.

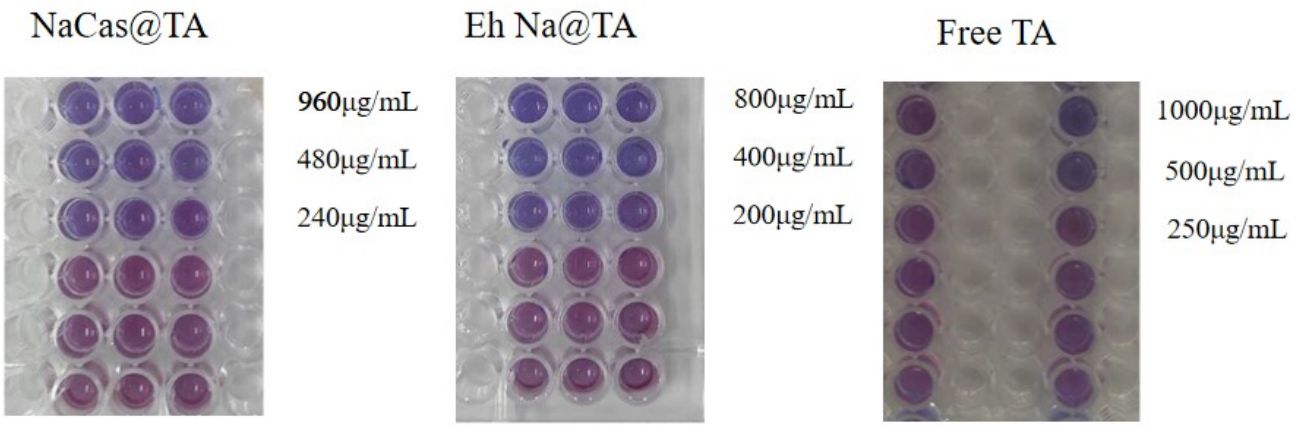

Fig. S5 96-well plate experiment based on resazurin

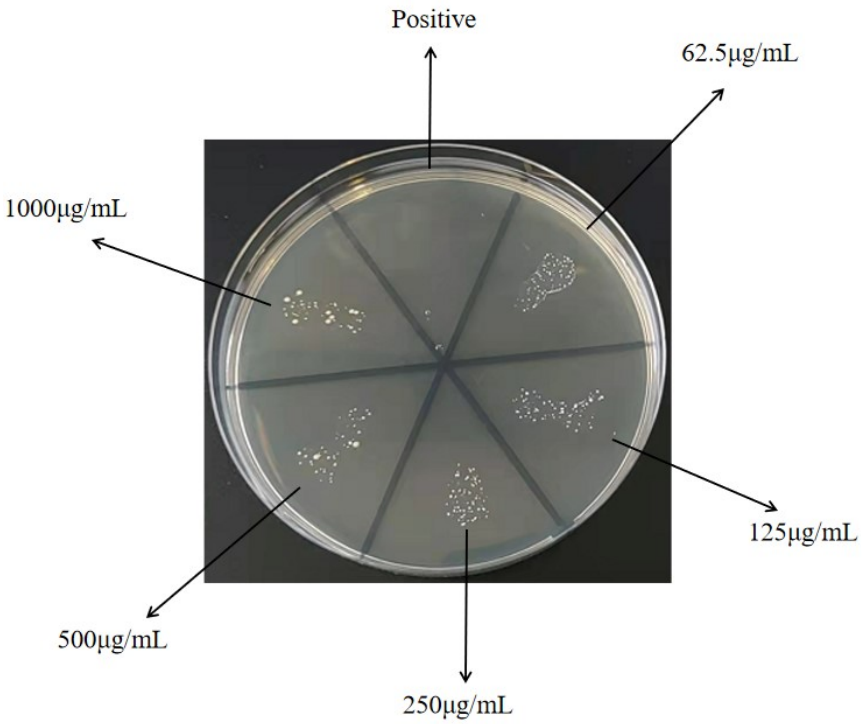

Fig. S6 Free TA's solid medium experiment

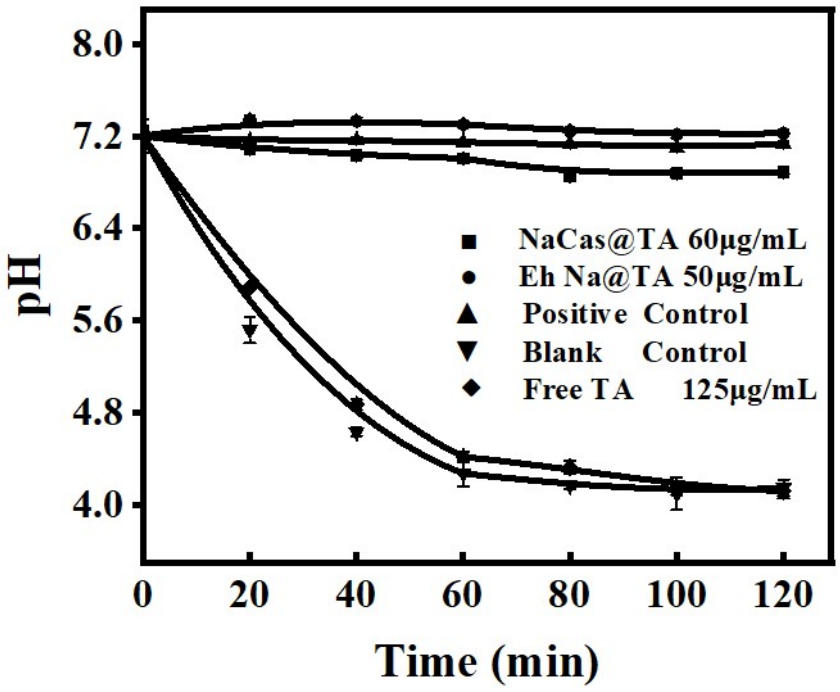

Fig. S7 Effects of different nanoparticles on the acid production ability of *S. mutans*

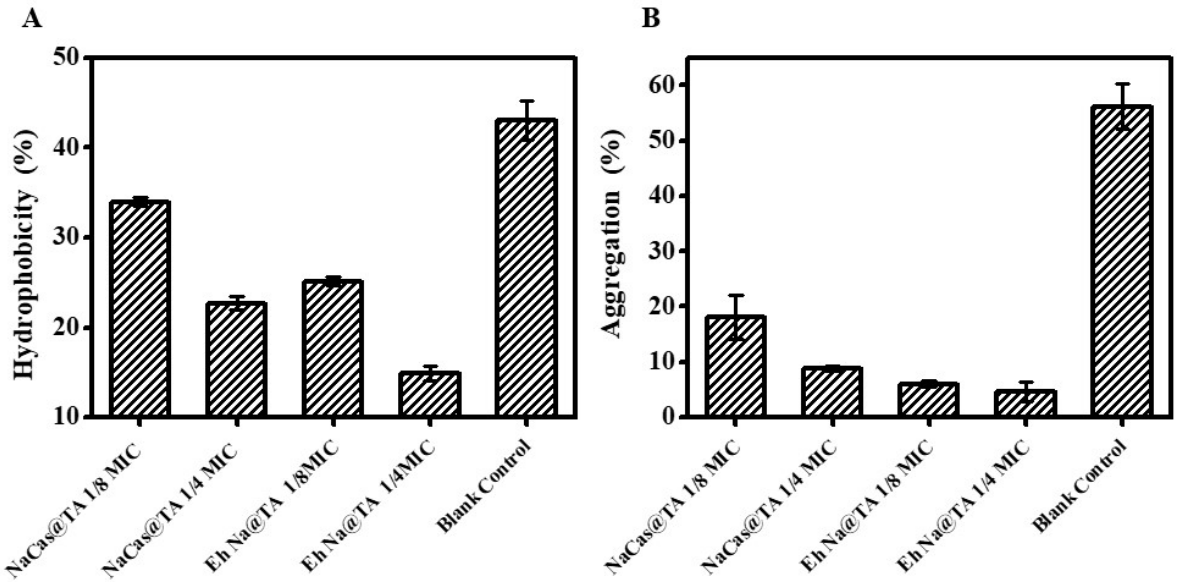

Fig. S8 The effect of TA-NPs on the (A) hydrophobicity and (B) aggregation of *S. mutans*

Table S1 Properties of different fragments of the main components of NaCas hydrolyzed by trypsin.

Peptide	Number of amino acids	Mol. weight, Da. [d]	GRAVY [e]	Hydrophobicity/Kyte & Doolittle [f]
α_{s1}–Casein (P02662) [a]				
1-214 [b]	214	24,528	-0.481	
74-94 [c]	21	2,321	-0.781	
167-208 [c]	42	4,718	-0.693	
α_{s2} –Casein (P02663) [a]				
1-222 [b]	222	26,018	-0.704	
16-36 [c]	21	2,427	-1.124	Fig. S2
60-85 [c]	26	2,844	-1.212	
106-128 [c]	23	2,838	-0.509	
β-Casein (P02666) [a]				
1-224 [b]	224	25,107	-0.154	
17-40 [c]	24	2,646	-0.433	
64-112 [c]	49	5,319	0.104	
129-184 [c]	56	6,362	-0.257	

[a] Peptide identifier in Uniport (www.uniprot.org/) denoted in parentheses. Peptides from different sources may vary slightly.

[b] Whole peptide, not cleaved by trypsin.

[c] Numbers indicated the possible cleavage sites of trypsin analyzed by Peptide Cutter (web.expasy.org/peptide_cutter/). Only peptides longer than 20bp are shown here.

[f] Graph of hydrophobicity index of peptides (ProtScale, web.expasy.org/protscale/). Individual values of 20 amino acids are given by the scale Hydropath. / Kyte & Doolittle [7], the smaller the value, the lower the hydrophobicity.

Abbreviations: [d] Mol. weight, Da. Molecular weight, Dalton. [e] GRAVY, Grand average of hydropathicity. Both from Proparam software (web.expasy.org/protparam/).

Table S2 Size, Zeta-potential and PDI of protein nanoparticles and TA-NPs

	NaCas	Eh NaCas	NaCas@TA	Eh NaCas@ TA	Free TA (PBS)
Size (d. nm)	108.22 ± 7.83	85.39 ± 5.58	362.53 ± 9.54	122.53 ± 4.87	2766.33 ± 153.94
Zeta-potential (mV)	-23.66 ± 2.33	-27.77 ± 1.91	-27.50 ± 1.87	-32.13 ± 1.58	-3.86 ± 0.22
PDI	0.422 ± 0.016	0.313 ± 0.015	0.357 ± 0.022	0.254 ± 0.016	0.617 ± 0.039

Table S3 The maximum solubility of TA and protein peptides in a transparent solution, and the increase rate of the solubility of TA by nanoparticles

	NaCas@TA	Eh NaCas@TA	Free TA
Maximum concentration of TA (μg/mL)	603.10 ± 1.60	693.29 ± 0.81	0.0028
Maximum concentration of protein peptide (μg/mL)	149.58 ± 0.01	249.52 ± 0.05	
TA solubility increase (times)	215,391	247,603	

Table S4 The DLS information (Size, Zeta-potential and PDI) of free TA and TA-NPs during 15-day storage at 4℃

		DLS information (4℃)		
	day	Size (d. nm)	ζ-potential (mV)	PDI
Free TA				
	0	1421.68 ± 305.39	-3.51 ± 0.43	0.609 ± 0.34
	3	2766.12 ± 153.91	-3.07 ± 0.26	0.831 ± 0.16
	6	3486.84 ± 201.59	-2.25 ± 0.45	1[a]
	9	5015.36 ± 219.91	-1.53 ± 0.38	1
	12	5972.17 ± 158.99	-1.01 ± 0.27	1
	15	6589.12 ± 302.36	-0.76 ± 0.29	1
NaCas@TA				
	0	327.92 ± 9.54	-25.14 ± 1.91	0.324 ± 0.018
	3	346.91 ± 8.36	-23.62 ± 2.08	0.393 ± 0.013
	6	359.35 ± 14.91	-21.23 ± 1.25	0.405 ± 0.015
	9	362.53 ± 8.36	-20.85 ± 0.98	0.415 ± 0.024
	12	396.61 ± 11.28	-19.26 ± 1.01	0.429 ± 0.017
	15	474.55 ± 9.48	-18.36 ± 0.45	0.448 ± 0.021
Eh NaCas@TA				
	0	129.73 ± 5.31	-34.12 ± 1.39	0.23 ± 0.042
	3	149.24 ± 6.24	-33.64 ± 1.21	0.254 ± 0.123
	6	153.43 ± 8.64	-33.19 ± 0.88	0.256 ± 0.031
	9	188.45 ± 3.60	-31.97 ± 0.69	0.309 ± 0.041
	12	207.91 ± 5.93	-31.45 ± 0.51	0.315 ± 0.016
	15	220.75 ± 11.01	-30.28 ± 1.07	0.335 ± 0.043

[a] indicates that the reading exceeds the maximum range of the system to measure PDI and displays a value of 1

Table S5 The DLS information (Size, Zeta-potential and PDI) of free TA and TA-NPs during 15-day storage at 21℃

		DLS information (21℃)		
	day	Size (d. nm)	ζ-potential (mV)	PDI
Free TA				
	0	1421.68 ± 305.39	-3.51 ± 0.43	0.609 ± 0.34
	3	3124.23 ± 420.15	-2.41 ± 0.72	1 [a]
	6	5214.91 ± 280.63	-2.04 ± 0.29	1
	9	7539.28 ± 368.17	-1.23 ± 0.54	1
	12	9045.39 ± 202.61	-0.81 ± 0.16	1
	15	9723.58 ± 200.34	-0.24 ± 0.19	1
NaCas@TA				
	0	327.92 ± 9.54	-25.14 ± 1.91	0.324 ± 0.018
	3	395.36± 10.98	-21.12 ± 1.74	0.394 ± 0.014
	6	481.51 ± 12.24	-15.16 ± 1.27	0.586 ± 0.092
	9	667.25 ± 20.01	-10.87 ± 1.39	0.782 ± 0.013
	12	741.11 ± 4.66	-8.36 ± 0.91	0.939 ± 0.031
	15	935.84 ± 39.73	-7.36 ± 1.28	0.954 ± 0.065
Eh NaCas@TA				
	0	129.70 ± 5.31	-34.12 ± 1.39	0.23 ± 0.042
	3	146.41 ± 6.86	-30.74 ± 1.58	0.257 ± 0.080
	6	184.29 ± 6.21	-28.83 ± 0.93	0.382 ± 0.015
	9	246.94 ± 19.00	-26.16 ± 1.13	0.483 ± 0.021
	12	315.45 ± 3.75	-24.26 ± 0.88	0.504 ± 0.023
	15	413.55 ± 19.45	-23.28 ± 1.24	0.660 ± 0.039

[a] indicates that the reading exceeds the maximum range of the system to measure PDI and displays a value of 1

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