Electronic Supplementary Material (ESI) for Green Chemistry.

Aqueous nanoprecipitation for programmable fabrication of versatile biopolymer nanoparticles

Dongming Ding, Li Gong, Miao Li, Xie Cheng, Huahong Peng, Zesheng Zhang, Shuai Wang, Xibo Yan

Table of content

Materials

Pectin from citrus fruits (galacturonic acid=85%, methoxy content=9.5%) was purchased from Sigma Chemical Co. Chitosan from shrimp shells (≥95% deacetylated, viscosity=100-200 mPa·s), curcumin (natural extract), catalase from fungi (activity≥200000 units/g) were purchased from Aladdin Shanghai Biochemical Technology Co., Ltd. Citric acid (AR grade), sodium hydroxide (AR grade), hydrogen peroxide (30 wt% in H_2O, AR grade), titanium dioxide (99%), ammonium sulfate (99%) and all the solvents (AR grade) were purchased from Tianjin Jiangtian Chemical Technology Co., Ltd. Unless otherwise stating, other reactants were purchased from Aladdin Shanghai Biochemical Technology Co., Ltd. Dialysis membranes (MW cut-off 1000 Da) were purchased from Spectrum Laboratories, Inc.

Methods

Particle size measurements were carried out by dynamic light scattering (DLS) using a Malvern Instruments Zetasizer nano series instrument and using the cumulant method. The aqueous solutions were prepared at certain concentrations, and at least five measurements were made for each sample. ζ-potential was calculated from the electrophoretic mobility measurements using the Smoluchowski model.

TEM images were obtained on a JEOL JEM-1400Flash electronic microscope, by observations in transmission at an accelerating voltage of 80 kV. Samples were made by placing a drop of sample onto a carbon grid. The excess solution was carefully blotted off using filter paper and samples were dried for a few minutes. The

nanoparticles were stained by RuO_4 through deposition of the sample grid in the sealed culture dish containing 50 μL of 0.5 % RuO_4 aqueous solution for overnight[1, 2].

Fluorescence spectroscopy analyses were performed using a Varian Cary Eclipse Fluorescence Spectrophotometer. All the fluorescent spectra were measured in aqueous solution at room temperature.

Absorption spectra (UV-Vis) were recorded on a SHIMADZU UV-1800 spectrophotometer.

Sulfuric Acid–UV method: 1 mL of pectin solution (or supernatant solution after centrifugation) is rapidly mixed with 3 mL of concentrated sulfuric acid and then vortexed for 1 min. After cooling down to room temperature, the solution is then characterized by using a UV spectrophotometer at 315 nm. Reference solutions are prepared following the same procedure as above by replacing the pectin solution with the aqueous solution without pectin.

Cell culture: Human Hepatocellular carcinoma (HepG2) was cultured in RPMI 1640 cell culture medium supplemented with 10% FBS and 1% penicillin-streptomycin in the cell incubator (ThermoFisher Scientific) at 37 ^oC with an atmosphere of 5% CO_2 and 70% humidity.

The degradation of hydrogen peroxide with different concentrations (2, 5 and 10 mM) treated by catalase-loaded pectin nanoparticles was characterized by a classic titanium salt spectrophotometry with slight modification[3]. The titanium salt solution was prepared by dissolving 1 g of titanium dioxide and 4 g of ammonium sulfate in 100 mL of concentrated sulfuric acid (heating at 150 °C for 16 h). The catalase-loaded

nanoparticles were added to a certain concentration of H_2O_2. 100 μL of titanium salt solution and 200 μL of distilled water were added into 200 μL of the reaction solution (taken at each time point). The mixture was then co-incubated at the room temperature for 10 min. The H_2O_2 concentration was measured by absorbance at 430 nm using a Thermo Scientific Multiskan FC microplate photometer. The kinetic assays of free and loaded catalase were carried out at H_2O_2 concentrations of 0, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50 and 25.00 mM. The Michaelis-Menten plots[4] were determined by non-linear regression analysis with software GraphPad Prism for obtaining the Michaelis constant (K_m).

The cytotoxicity evaluation of the nanoparticles was performed by using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a microplate reader (Multiskan FC, ThermoFisher Scientific)[5].

Multi-inlet vortex mixer (MIVM, 4 streams): The internal configurations and dimensions of the mixer are basically the same as in the literature[6, 7]. While stainless steel is the main material for fabricating the MIVM. The mixing chamber height was 1.5 mm. The inlet channel width was 1.1 mm. The mixing chamber diameter was 6.0 mm, and the outlet diameter was 1.3 mm. Four-channel syringe pump (WH-SP-04, Suzhou Wenhao Microfluidic Technology Co., Ltd.) was integrated with the MIVM to manipulate the flow rates of the inlet streams.

Particle preparation

Preparation of pectin nanoparticles through dialysis induced nanoprecipitation. Citric acid solution of pectin was prepared by dissolution of pectin in 2 M citric acid

solution at 65 °C (stirring for 5 min). Then, the solution was transferred into dialysis bag (cut-off 1000 Da) for dialysis against ionized water for 48 hours at room temperature.

Preparation of pectin nanoparticles through batch nanoprecipitation. Citric acid solution of pectin was prepared by dissolution of pectin in 2 M citric acid solution at 65 °C (stirring for 5 min). Here, the nanoparticle dispersion with a final pH of 6 was used as an example. To perform a rapid solvent-shifting, 4 M NaOH solution (1.5 mL) was added into 1 mL of citric acid solution containing pectin (1 mg/mL) at room temperature, resulting in cloudy pectin colloidal dispersion immediately.

Preparation of pectin nanoparticles through continuous nanoprecipitation. Citric acid solution of pectin was prepared by dissolution of pectin in 2 M citric acid solution at 65 °C (stirring for 5 min). Here, the nanoparticle dispersion with a final pH of 6 was used as an example. Citric acid solution of pectin (channel 1), water (channel 2), 5.9 M NaOH solution (channel 3) and water (channel 4) was continuously injected into MIVM mixer chamber at the same time (pump rate of 10-40 mL/min) at room temperature. The pectin nanoparticles can be obtained in the outstream.

Preparation of curcumin-loaded pectin nanoparticles through continuous nanoprecipitation. Citric acid solution of pectin was prepared by dissolution of pectin in 2 M citric acid solution at 65 °C (stirring for 5 min). Aqueous solution of curcumin was prepared by dissolution of curcumin in 5.9 M NaOH solution at room temperature. Citric acid solution of pectin (channel 1 and 3) and NaOH solution of curcumin (channel 2 and 4) was continuously injected into MIVM mixer chamber at the same

time (pump rate of 40 mL/min) at room temperature. The nanocarriers can be obtained in the outstream.

Preparation of catalase-loaded pectin nanoparticles through stepwise nanoprecipitation

*Step 1. Preparation of catalase nanoparticles.*10 mg of catalase was dissolved into 1 mL of deionized water. 0.4 mL of 0.002 M citric acid was gradually added into aqueous solution of catalase in 30 mins (the final pH reached around 4), resulting in enzyme nanoparticles.

Step 2. Preparation of catalase loaded nanoparticles. 0.5 mL of 2 M citric acid solution of pectin (1 mg/mL) and 0.65 mL of 4 M NaOH solution was simultaneously added into catalase nanoparticle dispersion (the final pH reached around 6) resulting in nanoparticles immediately.

Preparation of pectin/chitosan hybrid nanoparticles through continuous nanoprecipitation. Citric acid solution of pectin was prepared by dissolution of pectin in 2 M citric acid solution at 65 °C (stirring for 5 min). Citric acid solution of chitosan was prepared by dissolution of chitosan in 2 M citric acid solution at room temperature. 2 M citric acid solution of pectin (channel 1 and 3) and 5.9 M NaOH solution (channel 2 and 4) was continuously injected into MIVM mixer chamber at the same time (pump rate of 40 mL/min) at room temperature. The hybrid nanoparticles can be obtained in the outstream.

Supplementary figures

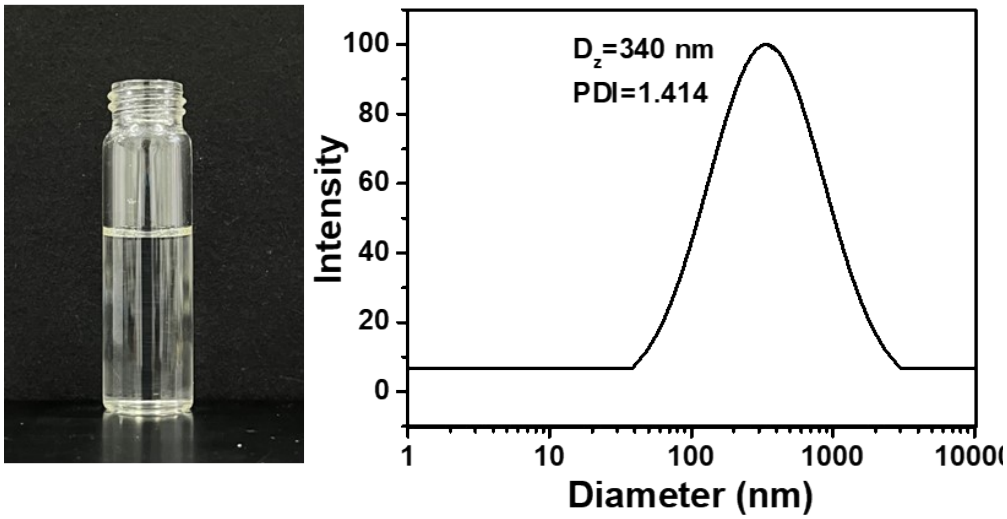

Figure S1. (Left) Digital image of pectin in hot deionized water (65 °C, 1 mg/mL) and **(Right)** DLS measurement of the solution.

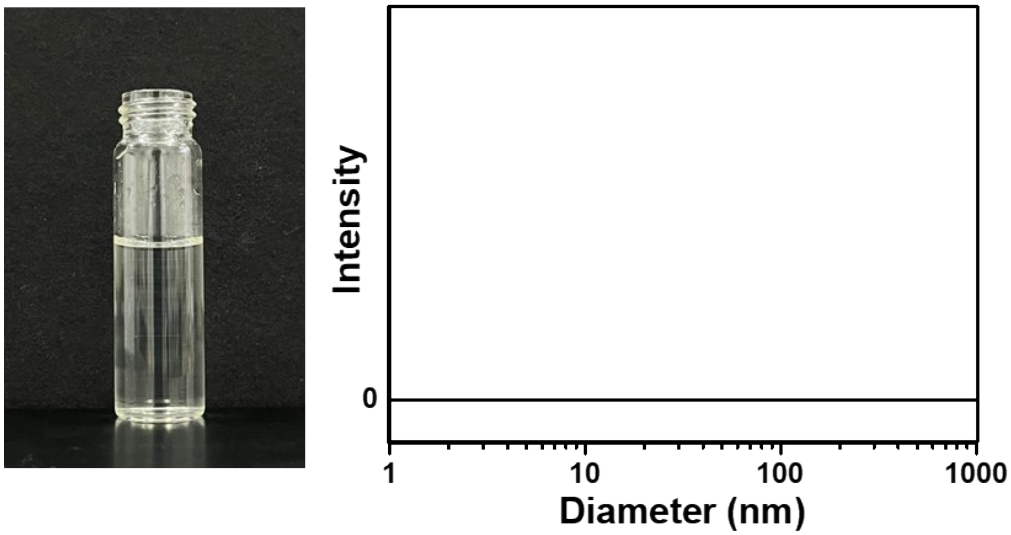

Figure S2. (Left) Digital image of pectin in 2M citric acid solution (1 mg/mL) and **(Right)** DLS measurement of the solution.

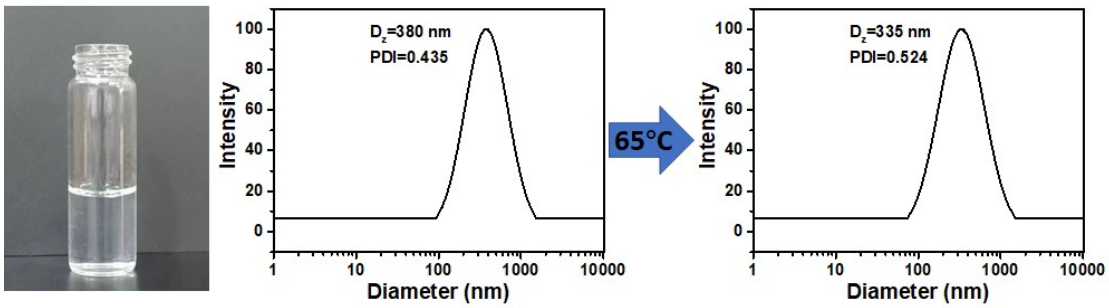

Figure S3. (Left) Digital image of pectin in pH HCl solution (1 mg/mL) and **(Right)** DLS measurement of the solution before and after heating at 65°C.

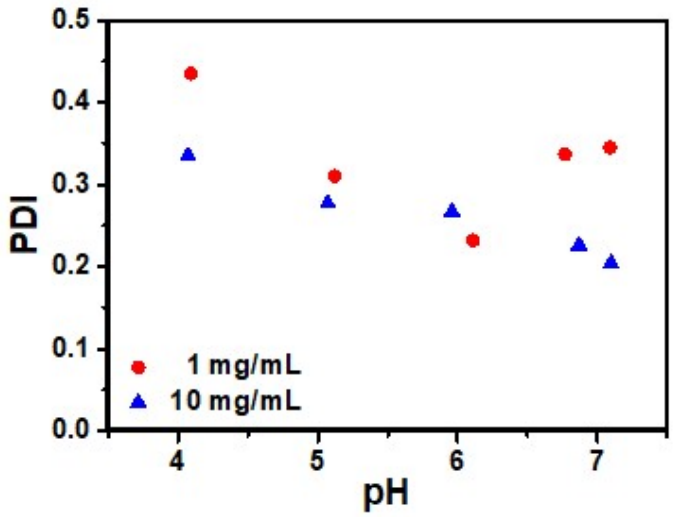

Figure S4. DLS results of pectin nanoparticles prepared by continuous nanoprecipitation using different feed concentration

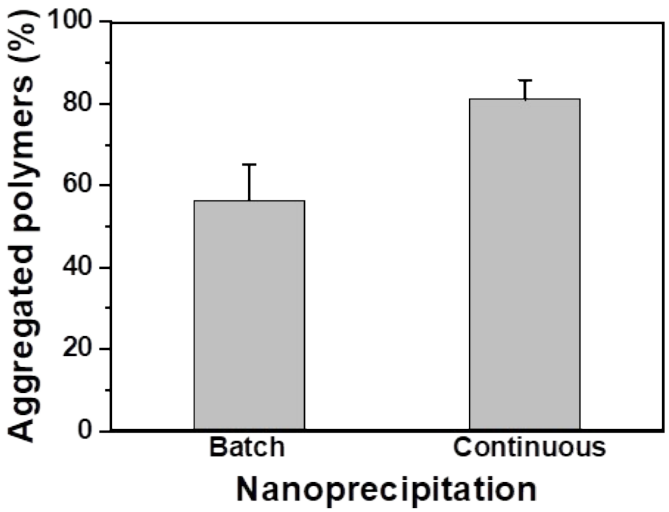

Figure S5. Aggregated percentages of pectin in the solution after batch or continuous nanoprecipitation (final pH around 6), the amount was determined at 10 min after solvent-shifting.

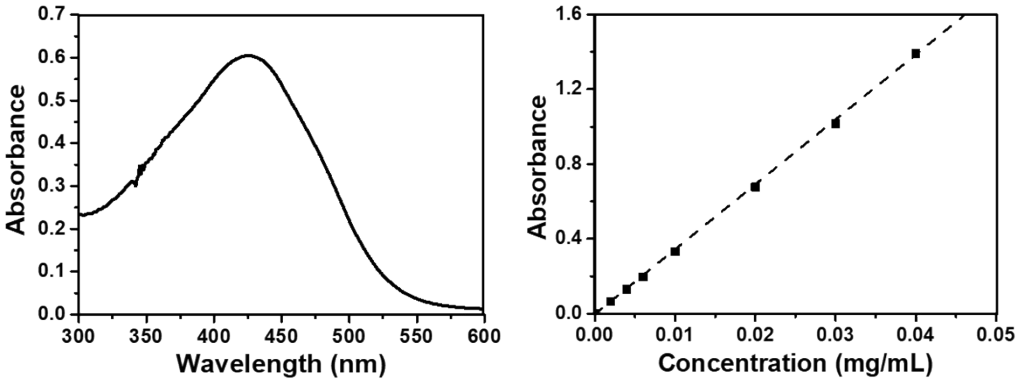

Figure S6. (Left) Absorbance of curcumin-loaded pectin nanoparticles; **(Right)** Calibration curve of curcumin in 5.9 M NaOH solution (λ_{abs}=450 nm).

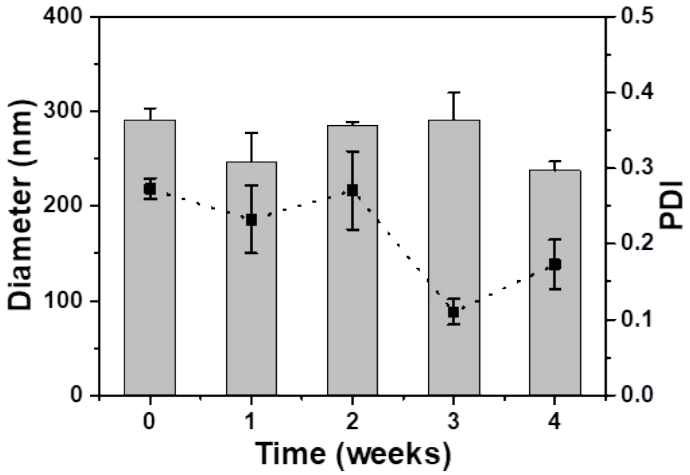

Figure S7. Colloidal stability of curcumin-loaded pectin nanoparticles for weeks at room temperature.

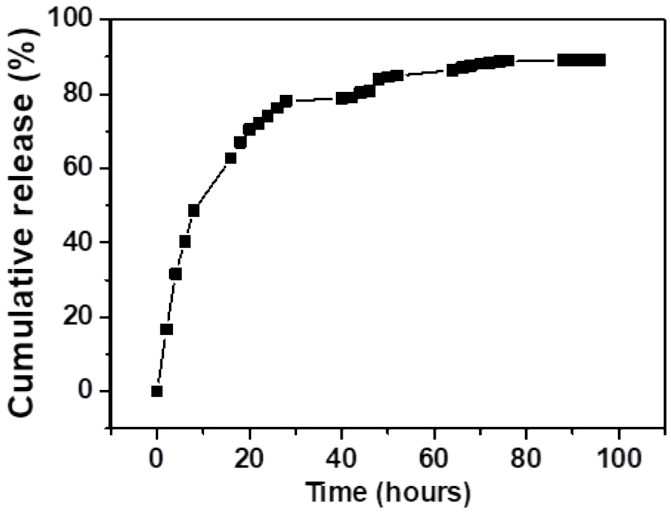

Figure S8. Cumulative release of curcumin from pectin nanoparticles at room temperature (pH 7.4 PBS buffer).

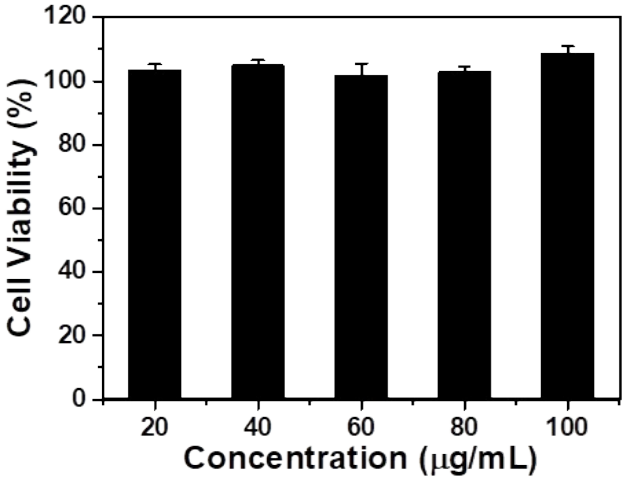

Figure S9. Cytotoxicity of pectin nanoparticles with different concentrations (HepG2 cells, MTT assay).

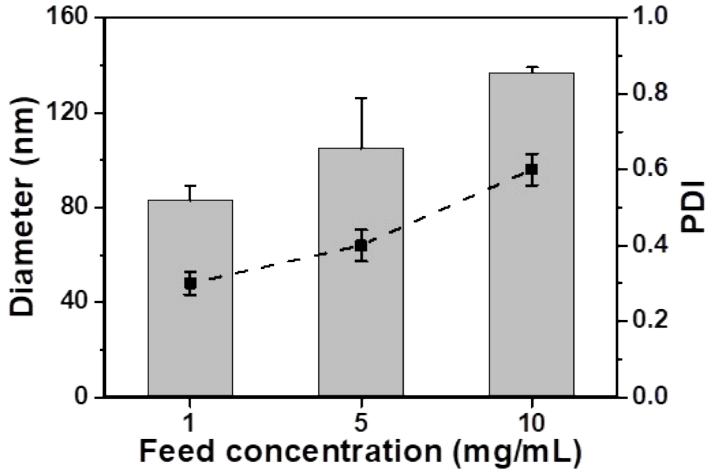

Figure S10. Particle size and PDI of catalase nanoparticles with different feed concentrations through pH shifting process.

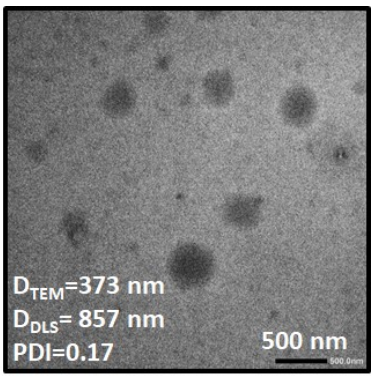

Figure S11. TEM image of catalase-loaded pectin nanoparticles.

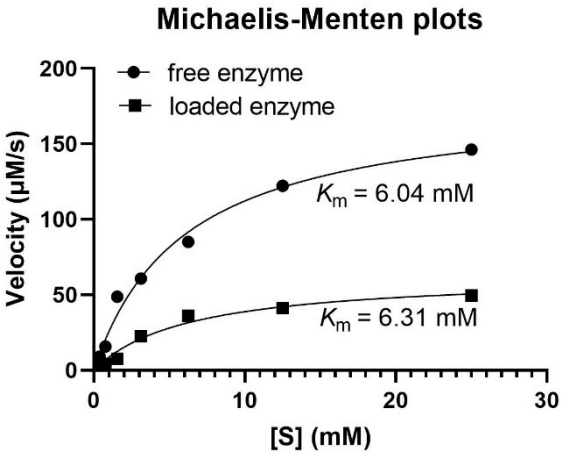

Figure S12. Michaelis-Menten plots of free and loaded catalase. The kinetics assays were carried out at various concentrations of H_2O_2 of 0, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50 and 25.00 mM. Non-linear regression analysis with software GraphPad Prism was used to obtain K_m values.

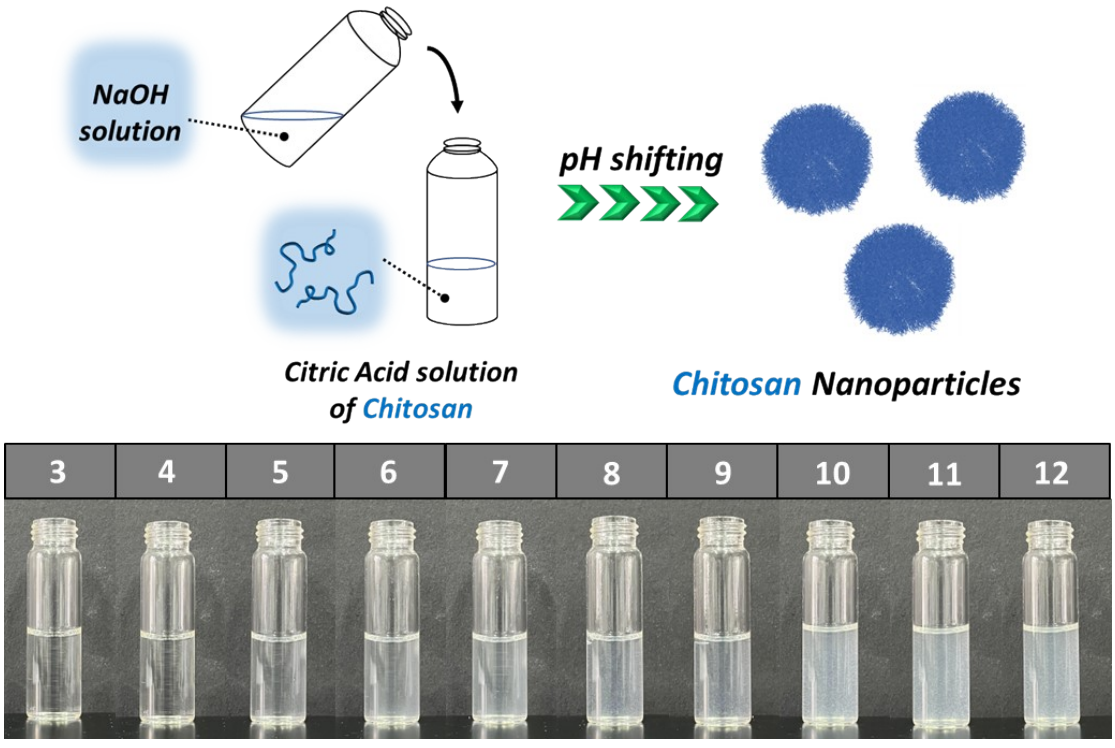

Figure S13. (Top) Schematic illustration of preparation of chitosan nanoparticles through aqueous nanoprecipitation; **(Bottom)** Digital image of the chitosan solution with different pH after pH-shifting process.

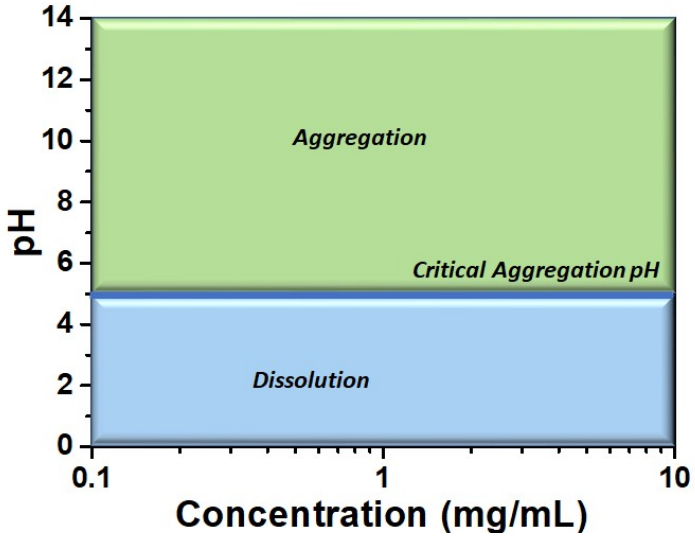

Figure S14. Phase diagram of chitosan at different pH after pH-shifting.

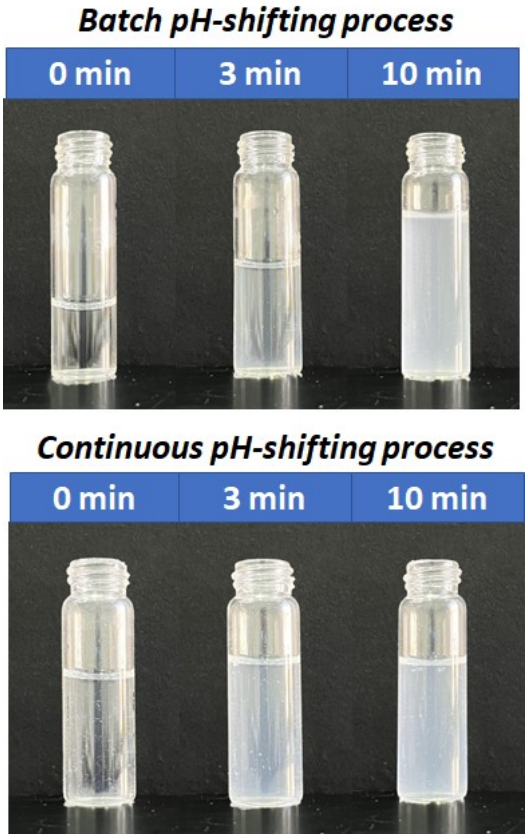

Figure S15. Digital images of solution colours with time prepared by batch or continuous pH shifting process.

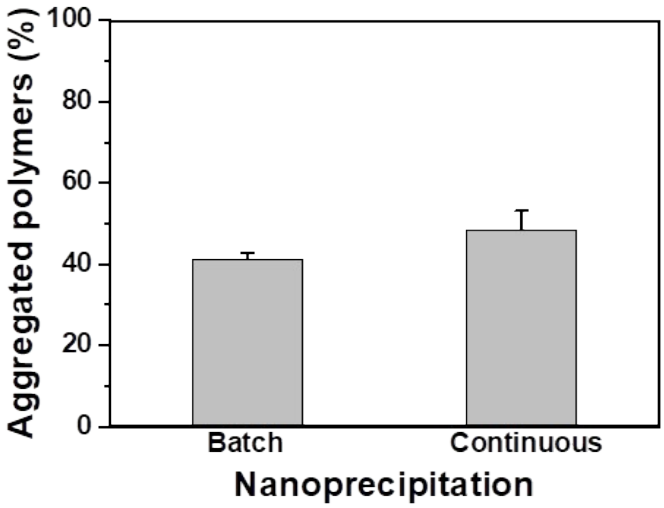

Figure S16. Aggregated percentages of chitosan in the solution after batch or continuous pH shifting process (final pH around 6), the amount was determined at 10 min after pH-shifting.

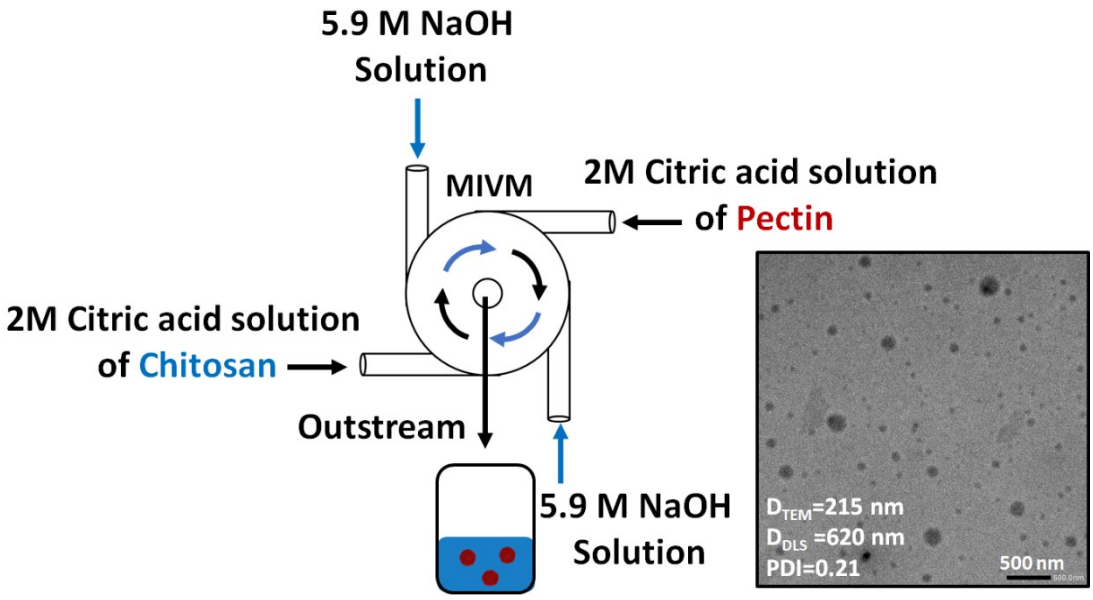

Figure S17. **(Left)** Schematic illustration of preparation of pectin/chitosan hybrid nanoparticles through continuous aqueous nanoprecipitation (pump rate: 40 mL/min); **(Right)** TEM of pectin/chitosan hybrid nanoparticles (concentration of pectin: 0.4 mg/mL, $wt_{pectin}/wt_{chitosan}$=4/0.3).

Table S1. Preparation of biopolymer nanoparticles through organic solvent-involved nanoprecipitation.

	Biopolymers[a]	S[b]	NS[c]	V_{NS} %	Feed Con. mg/mL	D_{DLS}[d] (nm)	PDI	Ref.
1	Polygalacturonate (pectin backbone)	Water	EtOH[e]	94	45	300-600		8
2	Glycogen	Water	EtOH[e]	91	10	300-500		9
3	Maltodextrin	Water	Acetone	90	5	170-450	0.02-0.15	10
4	Delonix	Water	EtOH[e]	91-95	10-20	215-360	0.14-0.22	11
5	Ganoderma lucidum polysaccharides	Water	EtOH[e]	74-95	20	100-150	0.2-0.4	12
6	Momordica charantia polysaccharides	Water	EtOH[e]	74-95	20	100-250	0.3-0.4	12
7	Ginseng polysaccharide	Water	Acetone	95	1	65		13
8	Chitosan	Water	MeOH[f]	75-98	0.5-2	980-1900		14
9	Gelatin	Water	EtOH[e]	91	20	250	0.12	15
10	Dextran	Water	Acetone	41-66	1.4-5.7	150-300		16
11	Pullulan	Water	Acetone	51-66	2.2-3.1	250		16
12	Hyaluronic acid	Water	Acetone	41-66	1.6-3.1	150		16
13	Glycogen	Water	Acetone	41-66	1.6-3.1	120-180		16
14	BSA	Water	EtOH[e]	50-75	10-30	50-180		17
15	Soy protein isolate	Water	THF	50	0.8	160-250	0.3-0.5	18
16	Silk Sericin	Water	Acetone		1-10	130-350		19

[a]The biopolymers were directly used for particle formation without structure modification; [b]Solvent; [c]Nonsolvent; [d]The particle diameters were determined by DLS measurement of monocomponent polymeric nanoparticles (without encapsulation); [e]Ethanol; [f]Methanol.

Table S2. Pectin nanoparticles prepared by traditional or aqueous nanoprecipitation (batch process)

Entry	Polymer	Solvent 1 mL	Nonsolvent 1.5 mL	Feed Con. (mg/mL)	D_{DLS} (nm)	PDI
Organic solvent-involved nanoprecipitation						
1	Pectin	Citric acid solution	Ethanol	1	Gels	
2	Pectin	Citric acid solution	Acetone	1	Gels	
3	Pectin	Citric acid solution	Tetrahydrofuran	1	Gels	
4	Pectin	Citric acid solution	Dimethylformamide	1	647	0.287
5	Pectin	Citric acid solution	Dimethylsulfoxide	1	1041	0.875
Aqueous nanoprecipitation (final pH=6)						
6	Pectin	Citric acid solution	NaOH solution	1	733	0.084

Table S3. Pectin nanoparticles prepared by aqueous nanoprecipitation using various organic acid solutions as solvent (batch process, final pH: ~6)

Entry	Polymer	Solvent 1 mL	Nonsolvent 1.5 mL	Feed Con. (mg/mL)	D_{DLS} (nm)	PDI
1	Pectin	2 M Citric acid	4 M NaOH	1	733	0.084
2	Pectin	1 M Acetic acid	0.43 M NaOH	1	673	0.355
3	Pectin	1 M Malic acid	1.25 M NaOH	1	714	0.291

Reference

1. J. S. Trent, J. I. Scheinbeim and P. R. Couchman, *Macromolecules*, 1983, **16**, 589-598.
2. B. K. Wilson and R. K. Prud'homme, *Journal of Colloid and Interface Science*, 2021, **604**, 208-220.
3. C. N. Satterfield and A. H. Bonnell, *Analytical Chemistry*, 1955, **27**, 1174-1175.
4. R. A. Copeland, *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, Second Edition*, Wiley-VCH, New York, 2000.
5. R. Hamid, Y. Rotshteyn, L. Rabadi, R. Parikh and P. Bullock, *Toxicology in Vitro*, 2004, **18**, 703-710.
6. C. E. Markwalter and R. K. Prud'homme, *Journal of Pharmaceutical Sciences*, 2018, **107**, 2465-2471.
7. S. F. Chow, C. C. Sun and A. H. L. Chow, *European Journal of Pharmaceutics and*

Biopharmaceutics, 2014, **88**, 462-471.

8. E. R. Gasilova, G. P. Aleksandrova and I. V. Tyshkunova, *Carbohydrate Polymers*, 2022, **291**, 119521.
9. M. Pais, S. D. George and P. Rao, *International Journal of Biological Macromolecules*, 2021, **182**, 2117-2129.
10. S. Barthold, M. Hittinger, D. Primavessy, A. Zapp, H. Groß and M. Schneider, *European Journal of Pharmaceutics and Biopharmaceutics*, 2019, **142**, 405-410.
11. A. T. Ogunjimi, S. M. G. Melo, C. G. Vargas-Rechia, F. S. Emery and R. F. V. Lopez, *Carbohydrate Polymers*, 2017, **157**, 1065-1075.
12. Y. Qin, L. Xiong, M. Li, J. Liu, H. Wu, H. Qiu, H. Mu, X. Xu and Q. Sun, *Journal of Agricultural and Food Chemistry*, 2018, **66**, 4373-4383.
13. K. F. Akhter, M. A. Mumin, E. K. Lui and P. A. Charpentier, *ACS Biomaterials Science & Engineering*, 2016, **2**, 96-103.
14. A. G. Luque-Alcaraz, J. Lizardi-Mendoza, F. M. Goycoolea, I. Higuera-Ciapara and W. Argüelles-Monal, *RSC Advances*, 2016, **6**, 59250-59256.
15. S. A. Khan and M. Schneider, *Macromolecular Bioscience*, 2013, **13**, 455-463.
16. X. Yan, R. A. N. S. Ramos, P. Alcouffe, L. E. Munoz, R. O. Bilyy, F. Ganachaud and J. Bernard, *Biomacromolecules*, 2019, **20**, 3915-3923.
17. M. Tarhini, I. Benlyamani, S. Hamdani, G. Agusti, H. Fessi, H. Greige-Gerges, A. Bentaher and A. Elaissari, *Materials*, 2018, **11**, 394.
18. X. Wang, M. Wang, H. Zhao, J. Liu, M. Xing, H. Huang, M. A. Cohen Stuart and J. Wang, *Food Hydrocolloids*, 2022, **131**, 107798.
19. I.-C. Radu, C. Zaharia, A. Hudiță, E. Tanasă, O. Ginghină, M. Marin, B. Gălățeanu and M. Costache, *Polymers*, 2021, **13**, 2047.