Nanoparticle formation of chitosan induced by 4-sulfonatocalixarenes: Utilization for alkaloid encapsulation

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Abstract

Spontaneous formation of positively charged nanoparticles was observed upon mixing more than stoichiometric amount of chitosan with 4-sulfonatocalix[8]arene (SCX8) in acidic solution. The particle size did not change with SCX8 concentration, polymer chain length and the degree of deacetylation at $0.002 \leq$ SCX8:chitosan ≤ 0.043 molar ratios in 0.01 M HCl. However, larger aggregates were produced when chitosan concentration was increased. The most stable nanoparticles with 160 nm diameter and narrow size distribution were obtained at pH 4 using low molecular weight chitosan. These particles encapsulated coralyne with more than 90% entrapment efficiency and 15% loading capacity. A loading ratio of [coralyne]/[SCX8] = 1.7 was achieved without any stability loss. 4-Sulfonatocalix[4]arene induced the formation of slightly smaller nanoparticles than its homologues comprising 6 or 8 phenol units.

Key words: nanoparticles; self-assembly; host-guest complex; alkaloid; polyelectrolyte

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1. Introduction

Chitosan is a biocompatible and biodegradable polysaccharide produced by partial deacetylation of chitin, which is the main component of the exoskeletons of crustaceans and the cell walls of fungi. The protonation of the D-glucosamine units renders chitosan readily soluble and positively charged in acidic aqueous solution.[1] It is widely used in diverse biomedical applications,[2,3] gene therapy,[4] drug delivery,[5,6] and in removal of water pollutants.[7] Chitosan nanoparticles (NP) can be prepared by ionotropic gelation with tripolyphosphate anions.[8,9] They showed antibacterial activity[10] and were used as efficient carriers for proteins, peptides[11] and various types of drugs.[4] Commercially available chitosan provided NPs with wide size distribution, but the diminution of the molecular weight, degree of acetylation, acetic acid concentration, and the optimization of the temperature of the synthesis resulted in particles of low polydispersity.[12,13] To enhance the bioavailability of the drugs and the loading capacity of the carrier, hybrid NPs comprising cyclodextrin derivatives (CD) were developed.[14-18] The incorporation of CD-bound triclosan and furosemide barely modified the size and zeta potential of chitosan NPs, but driving force of the inclusion of the drugs was significantly reduced.[19]

The main objective of the present study was to reveal the potential of 4-sulfonatocalix[n]arenes (SCXn) to serve simultaneously as a crosslinking agent and a macrocyclic receptor in a self-assembled system with chitosan. Due to their multianionic character, SCXn cavitands are expected to establish electrostatic interactions with the protonated amino groups of chitosan, which may lead to connections between the polymer chains promoting NP formation. SCXn are biocompatible compounds exhibiting antiviral, antibacterial, antithrombic, and ion channel blocking activities.[20,21] Its π-electron rich, flexible cavity is capable of encapsulating a wide variety of organic molecules.[22,23] The

complexation with compounds of biological and pharmaceutical importance received a particular attention.[21,24] We have previously studied the confinement of alkaloids of anticancer properties in SCXn and its effect on the fluorescent behavior. Berberine had a remarkably strong 1:1 binding to SCX8, but the driving force of the association considerably diminished with the macrocycle size, whereas the fluorescence quantum yield vanished with the increase of pH.[25] A structurally related alkaloid, coralyne, showed high affinity and multiple binding to SCXn. As many coralyne were able to associate with these hosts as the number of the 4-hydroxybenzene- sulfonate moieties in the macrocycle.[26] As an extension of these studies, now, we unravel whether the polymer chains of protonated chitosan can be crosslinked by SCXn to produce NPs, and how this process is influenced by alkaloids. The structures of the investigated compounds are presented in Scheme 1.

2. Materials and methods

2.1. Materials

4-Sulfonatocalix[4]arene (SCX4) (Fluka), 4-sulfonatocalix[6]arene (SCX6) and 4-sulfonatocalix[8]arene (SCX8) (TCI) held 1:9, 1:13 and 1:21 stoichiometric amount of water in their crystal structure.[24] Coralyne chloride (Acros Organics) and berberine chloride (Sigma) were purified as described.[27,28] Water was freshly distilled twice from dilute $KMnO_4$ solution. Three types of chitosan were used. Low molecular weight (Mw) compound (ChA) was purchased from Fluka, whereas high purity chitosans from white mushroom $M_w = 60 - 120$ kDa (ChB), and $M_w = 140 - 200$ kDa (ChC) were delivered by Aldrich. Their degree of deacetylation (%) was determined using the ^{1}H NMR method developed by Hirai and coworkers.[29] The polymer amount is always given as the concentration of the saccharide unit. Measurements were performed at 298 K.

2.2. Sample preparation

Chitosan and SCXn were solved in 0.01 M HCl solution. After the adjustment of the pH by NaOH solution to the desired value, the necessary amounts of the two stock solutions were mixed under magnetic stirring at 200 rpm. The counterions of SCXn were H_3O^+ and/or Na^+ depending on pH. Alkaloid chloride loaded NPs were prepared using the solution of alkaloid−SCX8 complex instead of SCX8.

2.3. Instrumentation

The absorption spectra were recorded on an Agilent Technologies Cary 60 spectrophotometer. The turbidity (T) of the samples were calculated from the absorbances (A) at 400 nm by the relationship $T = 1-10^{-A}$. The pH of the solutions was measured with a Consort C832 equipment. To separate nanoparticles from the liquid phase, the solutions were ultracentrifuged in a Beckman Coulter apparatus (Optima Max-XP, type TLA 110 rotor) for 2 hours at 100000 rpm. The size and zeta potential of particles were determined on a Zetasizer Nano-ZS (Malvern Instrument) equipped with a He−Ne laser (λ = 633 nm, scattering angle 173°). Each result was the average of 12 runs of 10 s at 298 K. Data were analyzed with the software developed by the manufacturer, and the mean particle diameters were calculated on the basis of intensity distribution. ^{1}H NMR spectra were recorded on a Bruker Avance 300 NMR spectrometer. Transmission electron microscopy (TEM) were done with a FEI Tecnai F20 microscope operating at an acceleration voltage of 200 kV. The samples were prepared as follows. A drop of the suspension was deposited on a Formvar (poly(vinylformal))/carbon-supported 400 grid mesh previously treated by air plasma for 40 s. After 1 min, excess of liquid was removed with an absorbent paper and the grid was dried in air at room temperature.

3. Results and Discussion

3.1. Turbidity measurements in 0.01 M HCl solution

Despite the lack of absorption of the components above 300 nm, the apparent absorbance increased in this spectral range when chitosan was added to 0.017 mM SCX8 solution in 0.01 M HCl. Fig. 1 displays the SCX8-induced turbidity at 400 nm at various r=[SCX8]/[chitosan] molar ratios for ChA and ChC. The different molecular weight and degree of acetylation of the two types of chitosans (vide infra in Table 1) caused insignificant change in the experimental data. Three well-defined ranges appear, as shown by dashed lines in Fig. 1. At $0.004 \leq r \leq 0.08$, where chitosan was present in substantial excess, the turbidity barely varied. Coalescence was observed in the $0.08 < r < 0.27$ domain, whereas the turbidity gradually vanished at larger r values. In 0.01 M HCl solution, SCX8 macrocycles had 8 negative charges because the sulfonato moieties were deprotonated, but the phenolic OH substituents did not dissociate.[30] Since only the free NH_2 substituents of chitosan were protonated, the mean charge of the polymer repeating units corresponded to the degree of deacetylation. The charge neutralization, which took place at $r = 0.105$ or 0.086 using ChA or ChC, respectively, brought about coalescence.

3.2. Particle size at various SCX8:chitosan mixing ratios in 0.01M HCl

To determine the size of particles in turbid solutions dynamic light scattering (DLS) experiments were performed. Protonated chitosan did not associate under our experimental conditions because of the electrostatic repulsion among the macromolecules. The scattered light intensity of protonated chitosan was low and increased by more than two orders of magnitude after SCX8 addition. Due to its polyanionic character, SCX8 could connect chitosan chains by Coulomb interaction with the NH_3^+ substituents (Scheme 2). This led to

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nanoparticle (NP) formation in the $0.004 \leq r \leq 0.08$ mixing ratios. Fig. 2 displays a typical particle size distribution and the dependence of the mean NP diameter (d) on the molar ratio of the components. At constant 0.59 mM ChA concentration, $d = 268 \pm 25$ nm was found irrespective of the amount of SCX8 ($0.0047 - 0.040$ mM). When 0.018 mM SCX8 concentration was fixed, $d = 190 \pm 20$ nm was obtained in the $0.042 \leq r \leq 0.08$ range, whereas larger d with broader size distribution appeared at lower r values.

Our results demonstrate that the traditionally applied tripolyphosphate crosslinking anion can be replaced by SCX8, which does not lose its polyanionic character even in strongly acidic conditions. Tripolyphosphate cannot induce NP formation in strongly acidic solution because protonation eliminates most of its negative charges. The pK_a values of its conjugated acids[31] are 1.0, 2.2, 2.3, 5.7 and 8.5. Since SCX8 is a stronger acid ($pKa < 0.4$), its sulfonate moieties are not protonated[30] even at pH 0.4. Therefore, SCX8 is able to act as a crosslinking agent in a broad pH range.

3.3. Alteration of molecular weight and deacetylation degree of chitosan

To reveal whether the degree of polymerization and deacetylation influence the self-assembly with SCX8, experiments were performed in 0.01 M HCl with three types of chitosan, whose characteristics are given in Table 1. Insignificant difference was observed in the mean diameter of NPs when various chitosans were used in 0.59 mM concentration and SCX8 amount was altered to reach $0.004 \leq r \leq 0.08$ range (Table 1). The width of the scattered light intensity distribution and the polydispersity index also varied less than the limit of experimental errors indicating that neither the polymer chain length nor the number of the ammonium substituents affected the association. Despite the insubstantial change in particle diameter, the scattered light intensity increased with gradual addition of SCX8 implying that r change influenced only the number of NPs, but did not modify their size. The

NPs were stable. The mean diameter of NPs and the intensity of scattered light altered insignificantly in one day at 0.59 mM chitosan concentration in $0.01 \leq r \leq 0.06$ range irrespective of molecular weight and degree of deacetylation. As a representative example, Figure S1 in Supplementary Information displays that even the size distribution of NPs calculated on the basis of the scattered light intensity barely changed in one day.

Table 1 Characteristics of chitosan and NPs[a] in 0.01 M HCl

	Molecular weight kDa	Degree of deacetylation	Mean diameter nm	PDI[b]
ChA	low[c]	84 %	268 ± 25	0.19
ChB	60 - 120	82 %	279 ± 33	0.19
ChC	140 - 200	69 %	262 ± 31	0.17

[a] The parameters listed in the last two columns are the averages of the results obtained by addition of SCX8 to 0.59 mM chitosan at $0.004 \leq r \leq 0.085$.
[b] polydispersity index; [c] molecular weight range is not specified by the manufacturer.

3.4. Effect of pH and temperature on the particle size and zeta potential

The charge of the polymer chain of chitosan arises from the protonation of its amino groups, which is influenced by the pH. The negative logarithm of the proton dissociation constant (pK_a) was reported to be between 6.17 and 6.51 depending on the molecular weight and degree of deacetylation.[32] The pH enhancement also affects the charge of SCX8 because the phenolic OH moieties[30] have a pK_a of 3.44, 4.26, 7.78 and 10.3. At pH 4, NPs could be prepared only at $0.004 \leq r \leq 0.065$ ratios in 0.59 mM chitosan solutions. At higher r values coalescence was observed. Coalescence took place already at $r \geq 0.02$ when the experiments were performed at pH 6. Due to the smaller degree of protonation of the polymer chain at higher pH, incorporation of less SCX8 ensured charge neutrality, where the lack of

electrostatic repulsion among particles led to coalescence. Zeta potential (ζ) measurements demonstrated the positive charge of NPs. The ζ of ChB−SCX8 NPs gradually diminished with the increase of pH, but did not change with r at a fixed pH in

Table 2 Variation of the parameters of chitosan-SCX8 NPs with pH, molecular weight, and time at 0.59 mM chitosan concentration

pH	Chitosan	Time after mixing / h	Mean diameter[a] nm	PDI[b]
2	ChA $0.004 \leq r \leq 0.080$	0	268 ± 25	0.19
		1	258 ± 20	0.16
		24	283 ± 15	0.17
2	ChB $0.004 \leq r \leq 0.080$	0	279 ± 35	0.19 ± 0.04
		1	298 ± 30	0.19 ± 0.04
		24	320 ± 45	0.21 ± 0.07
4	ChB $0.015 \leq r \leq 0.065$	0	149 ± 18	0.19 ± 0.04
		1	155 ± 16	0.18 ± 0.04
		24	177 ± 17	0.15 ± 0.03
4	ChC $0.015 \leq r \leq 0.065$	0	181 ± 13	0.18 ± 0.04
		1	193 ± 10	0.15 ± 0.03
		24	226 ± 10	0.13 ± 0.03
6	ChB $0.008 \leq r \leq 0.024$	0	149 ± 7	0.28 ± 0.08
		1	159 ± 6	0.23 ± 0.03
		24	153 ± 7	0.24 ± 0.05

[a] Diameter is the average of the values measured in the r range (given in the second column) where no coalescence occurs. [b] polydispersity index

the range where stable NPs were produced. The mean zeta potentials of ChB−SCX8 NPs were 32.5, 29.1, and 21.2 mV at pH 2, 4, and 6, respectively. The zeta potentials were almost insensitive to the variation of the molecular weight and the degree of deacetylation of chitosan. At 0.59 mM chitosan concentration, the diameter and size distribution of NPs did not show any systematic change with SCX8 amount in the r range given in Table 2. Therefore, the parameters in Table 2 represent the average of the values measured at r mixing

ratios where stable NPs is produced. Considerably smaller NPs were prepared from ChB when pH was raised from 2 to 4, but further pH increase to 6 did not lead to any further size decrease. At pH 4, the interaction of SCX8 with ChC of high molecular weight promoted the assembly to somewhat larger NPs compared to ChB, which slightly grew in one day. Contrarily, NPs composed of ChB altered less than the limit of experimental errors during this time indicating the larger stability of NPs comprising shorter chitosan chains. At a given concentration (below 3 mM), the NP size was always insensitive to r within the $0.015 \leq r \leq 0.065$ range at pH 4. However, the NP diameter and polydispersity index (PDI) grew with ChB concentration (Fig. 3). About twice larger particles formed when ChB amount was raised from 0.59 to 3 mM, whereas PDI only slightly increased.

Figure S2 in Supplementary Information presents the diameter and PDI of NPs produced at $r = 0.054$ mixing ratios in 0.01 M HCl as a function of preparation temperature. Only minor effect is observed. Slightly larger NPs were formed with somewhat broader size distribution when the temperature was raised from 298 to 323 K.

3.5. Determination of SCX8 content and morphology *of NPs*

To reveal the extent of SCX8 binding in NPs, the dispersion was ultracentrifuged for 2 hours, and the SCX8 content of the supernatant was determined by spectrophotometric method. The characteristic absorption band of SCX8 always vanished indicating that negligible amount of SCX8 remained in the solvent under all experimental conditions after NP formation. The electrostatic attraction between the oppositely charged SCX8 and protonated chitosan led to crosslinking among the polymer chains inducing thereby NP formation. The multiple charge of the cavitand facilitated the interaction with several macromolecules, and screened the electrostatic repulsion among the ammonium substituents. In stable NPs, less than stoichiometric amount of SCX8 is embedded.

The morphology of the NPs was characterized by TEM and as a representative example, we studied the NPs at r = 0.057 and pH 2 and 4 (Fig. 4). The observed nano-objects are relatively polydisperse, in agreement with the PDI around 0.2. Their size is around 150-200 nm, as determined by DLS. The NPs exhibit a loose structure, which may facilitate the release of the included alkaloid.

3.6. Effect of macrocycle size alteration on NP size

Not only SCX8 but also its smaller homologues SCX6 and SCX4 could induce ChA association to NPs. The charge of these macrocycles corresponds to the number of their 4-sulfonatophenol units[30] at pH 2. Coalescence was always observed around charge neutralization. As seen in Fig. 5, SCX4 promoted the assembly to smaller NPs (d = 150 ± 20 nm), but the NP diameter did not differ for SCX6 and SCX8. The particle size was also independent of the relative amount of the components. The SCX4 has smaller conformational mobility than the other homologues due to the stabilization of the truncated cone shaped macrocycle by hydrogen bonding among the phenolic OH-groups and steric hindrance of the conformation change. The smaller size and the relatively rigid molecular structure lead to smaller NPs compared to those developed with SCX6 and SCX8.

3.7. Alkaloid confinement in NP

We have previously demonstrated that SCXn cavitands are capable of encapsulating alkaloids of pharmaceutical importance.[25,26] Berberine (B^+) was found to bind very strongly to SCX8 with equilibrium constant[23] of 2.4×10^5 M^{-1} at pH 2. In contrast, the lack of change in the B^+ absorption and fluorescence spectra indicated that B^+ did not produce complex with 1.2 mM ChA because the electrostatic repulsion between the two positively charged compounds prevented the binding. The question arose whether SCX8 in NPs could

include this natural isoquinoline alkaloid. ChA in 0.01 M HCl was mixed with solutions of B$^+$–SCX8 complex. The size and stability of NPs produced thereby matched those comprising no B$^+$. Table 3 reports the mean diameter and polydispersity index of NPs. NP diameters increase with ChA concentration, but remain almost constant when B$^+$ and SCX8 concentrations are varied keeping chitosan concentration unchanged. Negligible particle diameter alteration was observed in three days even in the presence of alkaloid and the lack of change in the absorption spectra indicated that no B$^+$ decomposition occurred. To determine the fraction of B$^+$ embedded in NP, the dispersed phase was separated by ultracentrifugation and B$^+$ concentration in the supernatant ([B$^+$]$_S$) was measured by spectrophotometric method. The association efficiency (AE) and loading capacity (LC) was calculated as follows:

$$AE = \frac{A-A_S}{A} \times 100 \qquad (1)$$

$$LC = \frac{A-A_S}{Ch+SCX8} \times 100 \qquad (2)$$

where A, Ch, and SCX8 denotes the total amounts of alkaloid chloride, chitosan, and SCX8, respectively. A$_S$ stands for the amount of alkaloid chloride in the supernatant. The absorption spectrum suggests that the supernatant did not contain B$^+$–SCX8 complex or SCX8 indicating that SCX8 is fully incorporated in NPs. However, B$^+$ appears in the supernatant because competitive binding of the ammonium moieties of protonated chitosan to SCX8 partly expels B$^+$ from the macrocycle cavity. Therefore, AE varies between 17 and 37 %, but LC remains below 1%.

In contrast to the moderate binding capability of B$^+$, its synthetic analogue, coralyne (Cor$^+$) was readily embedded in chitosan−SCX8 NPs. This is in accordance with previous findings that unlike 1:1 complex formation with berberine, SCX8 can associate with 8 Cor$^+$ molecules.[26] Table 4 summarizes the association efficiencies and loading capacities

Table 3 Effect of total concentration of the component on the association efficiency and loading capacity of berberine in 0.01 M HCl (pH 2)

Total concentration			[SCX8]/[ChA]	[B$^+$]/[SCX8]	AE %	LC w%	Mean diameter nm	PDI
[ChA] / mM	[SCX8] / mM	[B$^+$] / mM						
2.37	0.10	0.020	0.043	0.2	37	0.52	550	0.42
1.77	0.10	0.020	0.057	0.2	31	0.54	590	0.32
1.18	0.10	0.020	0.084	0.2	30	0.65	650	0.30
0.59	0.050	0.020	0.084	0.4	22	0.95	270	0.19
0.59	0.030	0.020	0.05	0.66	17	0.88	260	0.19
0.59	0.050	0.010	0.084	0.2	22	0.48	300	0.22

Table 4 Efficiency of Cor$^+$ association to ChB−SCX8 NPs under various experimental conditions at 0.59 mM ChB concentration

pH	Total concentration		[SCX8]/[ChB]	[Cor$^+$]/[SCX8]	AE %	LC%	Mean diameter nm	PDI
	[SCX8]/mM	[Cor$^+$]/mM						
2	0.014	0.008	0.024	0.56	97	2.6	230	0.26
	0.014	0.016	0.024	1.1	98	5.2	170	0.34
	0.014	0.022	0.024	1.6	98	7.1	150	0.37
	0.047	0.022	0.08	0.48	99	5.1	300	0.31
4	0.015	0.008	0.025	0.53	80	2.1	140	0.22
	0.015	0.016	0.025	1.1	81	4.2	160	0.24
	0.015	0.022	0.025	1.5	82	5.9	170	0.27
	0.015	0.045	0.025	3	81	12	200	0.37
	0.015	0.067	0.025	4.5	86	19	180	0.51
	0.039	0.003	0.066	0.083	94	0.7	190	0.17
	0.039	0.016	0.066	0.4	91	3.7	230	0.18
	0.039	0.022	0.066	0.56	93	5.2	220	0.22
	0.039	0.045	0.066	1.1	91	10	230	0.25
	0.039	0.067	0.066	1.7	93	16	240	0.17

calculated on the basis of equations 1 and 2. AE reaches 99%. At pH 2, practically complete binding occurred under our experimental conditions irrespectively of Cor^+ concentration and NP composition. Confinement of even more than 1 Cor^+ per SCX8 molecules was reached. The mean diameter and polydispersity index of NPs are also included in Table 4. At pH 2, the NP size barely changed at constant [SCX8]/[ChB]. However, NP diameter grew from ca. 180 nm to 300 nm when [SCX8]/[ChB] was varied from 0.024 to 0.080. Moreover, Cor^+ incorporation increased the polydispersity, and diminished the temporal stability of NPs. In less acidic solution (pH 4), stable, smaller and more monodisperse Cor^+–NP aggregates were found, although the association efficiency became slightly lower than at pH 2 (Table 4). Nevertheless, the fraction of bound Cor^+ was always above 80 and 90% at [SCX8]/[ChB] = 0.025 and 0.067, respectively. The NP diameter slightly grew upon addition of 22 μM Cor^+ from 140 to 170 nm at [SCX8]/[ChB] = 0.025 and from 190 to 220 nm at [SCX8]/[ChB] = 0.066. At the latter [SCX8]/[ChB] ratio, NPs had larger stability, narrower size distribution, and Cor^+ concentration could be raised to 70 μM without any stability loss and 15% loading capacity was reached. Above $[Cor^+]$/[SCX8] molar ratio of 1.7, reduced temporal stability and larger polydispersity of NPs were observed. Confinement in NP did not alter the stability of Cor^+. The temporal stability of the absorption spectra indicated that neither of the applied experimental conditions induced decomposition.

4. Conclusions

SCX8-induced supramolecular crosslinking of protonated chitosan is a straightforward, mild method for the preparation of NPs capable of carrying pharmaceutically important alkaloids. In these self-assembled systems, SCX8 not only promotes the association to NPs, but also provides binding sites for inclusion complex formation with alkaloid molecules. The substantial binding affinity to SCX8 ensured B^+ or Cor^+ confinement in NPs despite the

positive charge of NPs. SCXn macrocycles could replace the often employed tripolyphosphate crosslinking agent with the additional benefit of forming host-guest inclusion complexes. The NP size can be fine-tuned by the variation of pH, SCXn size and chitosan concentration, whereas other experimental conditions slightly affect NP formation. A significant advantage of SCXn utilization for the initiation of self-organization into NPs is that these biocompatible compounds keep polyanionic character and as a consequence, the crosslinking ability even in strongly acidic conditions where conventional polyanions such as for example tripolyphosphate cannot be applied.

Acknowledgement

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/

Conflict of Interest: The authors declare that they have no conflict of interest.

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Captions

Scheme 1 Chemical structure of the studied compounds

Scheme 2 Illustration of the association between protonated chitosan molecules and SCX8

Fig. 1 Alteration of the turbidity at 400 nm as a function of the [SCX8]/[chitosan] molar ratio in 0.01 M HCl. SCX8 concentration was kept constant (0.017 mM) and ChA (▲) or ChC (○) amount were varied. Measurements were carried out 2 min after mixing of the components. Dashed lines indicates the range of coalescence ($0.08 < r < 0.27$).

Fig. 2 (A) Typical size distributions of NPs after mixing 0.036 mM SCX8 and 0.59 mM ChA in 0.01 M HCl. (B) Variation of the mean diameter of NPs as a function of the molar ratio of the components at [ChA] = 0.59 mM.

Fig. 3 (A) Alteration of the particle diameter (d) and (B) polydispersity index (PDI) with ChB concentration at pH 4 solution immediately after mixing (■), in an hour (○), and in a day (▲). Each point represents the average d and PDI values in the $0.015 \leq r \leq 0.065$ domain at constant ChB concentration.

Fig. 4 TEM images of SCX8–ChB NPs formed at r = 0.057 and (A) pH 2 and (B) pH 4. [ChB] = 0.59 mM

Fig. 5 Particle diameter as a function of the mixing ratio of the components in the case of SCX4 (■), SCX6 (▼), and SCX8 (○) in 0.01 M HCl. [ChA] = 0.30 mM