Supporting Information for

High throughput transition of tobacco mosaic virus rods to spheres using a mesofluidic device

Michael A. Bruckman[1], Allen VanMeter[1] and Nicole F. Steinmetz[1,2,3,4]*

[1]Department of Biomedical Engineering, [2]Department of Radiology, [3]Department of Materials Science and Engineering, and [4]Department of Macromolecular Engineering, Case Western Reserve University Schools of Medicine and Engineering, 10900 Euclid Ave., Cleveland, OH 44106, USA

* nicole.steinmetz@case.edu

Experimental:

The materials were used as received unless otherwise noted.

Isolation of TMV

Wild-type TMV and lysine mutants (S158K, TMVlys) of TMV were isolated from *Nicotiana benthamiana* plants using a previously established protocol[1]. The TMV concentration was determined based on UV-visible absorbance at 260 nm with an extinction coefficient of 3.0 mL mg^{-1} cm^{-1}. TMVlys was provided by Prof. Christina Wege (University of Stuttgart); its construction is described in detail in Ref 2[2].

Design of mesofluidic device

The SNP maker was designed to enhance simplicity. We used a syringe pump (Harvard Apparatus, Standard infusion only pump 11 plus syringe pump) with silicone tubing (Thermo Scientific, Nalgene pharma grade silicone tubing, 1/8" interior diameter and 1/4" outside diameter) through 800-900 mL silicone oil in a 1 L beaker. A digital thermometer monitored the oil bath temperature.

Mesofluidic set up

The conditions tested to optimize the formation of SNPs from TMV particles were the pump speed, oil bath temperature, and heating time. Varying the heating time was accomplished by

immersing more tubing in the hot oil bath. The specific length of tubing was estimated by relating the volume of the tubing to the pump speed in the following equation:

$$L = \frac{(t * S)}{\pi * r^2}$$

where L is the length of tubing in cm, t is the heating time in minutes, S is the pump speed in mL/min, and r is the interior radius of the tubing in cm.

Bioconjugation

TMV rods were modified using the following sequence of established reactions outlined in Figure S5[3-5]. Unless otherwise noted, chemicals were obtained from Sigma Aldrich. The following chemicals were purchased elsewhere: sulfo-Cyanine5 azide (Cy5, Lumiprobe), azido-mono amide-1,4,7,10-tetraazacyclododecane-*N*,*N*',*N*'',*N*'''-tetraacetic acid (DOTA-azide, Macrocyclics), NHS-LC-biotin (Thermo Scientific), Sulfo-azide-PEG_4-biotin (Click Chemistry Tools), and PEG_{2K}-azide (the MW of PEG was 2,000 Da, NanoCS). All reaction were performed at a TMV concentration of 1 mg/mL and purified by sucrose cushion ultracentrifugation at 160,000g for 2.5 hours.

ext-biotin-TMV, ext-Gd-TMV, ext-PEG-TMV: First, native TMV was mixed with an *in situ* generated diazonium salt (25 molar equivalents (eq) per coat protein (CP) (3-ethynylaniline mixed with sodium nitrite in an acidic solution) in 100 mM borate buffer pH=8.8 for 30 minutes on ice. The reaction was purified to yield ext-alkyne-TMV. Ext-alkyne-TMV was mixed with 2

eq per CP of {biotin, Gd(DOTA), or PEG_{2K}}-azide in the presence of 1 mM $CuSO_4$, 2 mM AMG, and 2 mM Na Asc in 10 mM potassium phosphate buffer pH=7.4 for 30 minutes on ice.

int-Gd-TMV, int-Cy5-TMV, int-biotin-TMV, int-biotin-TMVlys: The interior was labeled with terminal alkynes by targeting glutamic acids 97 and 106 with propargyl amine assisted by ethyldimethylaminopropylcarbodiimide (EDC). TMV(lys) was mixed with propargyl amine (100 eq per cp) with EDC (50 eq per CP), and n-hydroxybenzotriazole (HOBt, 150 eq per cp) in 100 mM HEPES buffer pH=7.4 for 24 hours at room temperature. The reaction was purified to yield int-alkyne-TMV(lys). Int-Alkyne-TMV(lys) was mixed with 2 eq per CP of {Gd(DOTA), sulfo-Cy5, or biotin}-azide in the presence of 1 mM $CuSO_4$, 2 mM AMG, and 2 mM Na Asc in 10 mM phosphate buffer pH=7.4 for 30 minutes on ice.

ext-biotin-TMVlys: TMVlys was mixed with 10 eq per CP of NHS-LC-biotin in 10 mM phosphate buffer pH=7.4 for 24 hours at 4°C.

Modified TMV particles were characterized for labeling efficiency, structural integrity, and magnetic T_1 relaxivity. Labeling efficiency was confirmed using matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS, Figure S6, explanation of MALDI-TOF MS results in Table S2), denaturing gel electrophoresis (SDS-PAGE, Figure S7), western blot analysis (Figure S7), induced coupling plasma optical emission spectroscopy (ICP-OES) (for Gd), and UV-Vis absorbance (for Cy5). The particle integrity was confirmed by transmission electron microscopy (TEM, Figure S8). The ionic relaxivity of engineered Gd-loaded TMV particles was tested using a Bruker Minispec mq60 relaxometer.

Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS):

For MALDI-TOF MS analysis, native and modified TMV (24 μL, 1 mg/mL, dialyzed in pure H_2O for best results) were denatured by adding guanidine hydrochloride (6 μL, 6 M) and mixing for 5 min at room temperature. Denatured proteins were spotted on MTP 384 massive target plate using Zip-$Tips_{\mu C18}$ (Millipore). MALDI-TOF MS analysis was performed using a Bruker Ultra-Flex I TOF/TOF mass spectrometer.

Transmission electron microscopy (TEM):

Drops of TMV formulations in DI water were dried on carbon coated copper TEM grids (2 μL, 0.1 mg/mL), washed with DI water, and negatively stained with 2% (w/v) uranyl acetate for 2 minutes. Samples were examined using a Zeiss Libra 200FE transmission electron microscope operated at 200 kV.

Scanning electron microscopy (SEM):

Drops of SNP formulations in DI water were dried on pre-cut silicon wafers (10 μL, 0.1 mg/mL). The samples were then sputter coated with palladium (5 nm, Agar Scientific, Ltd.). Samples were examined using a Hitachi 4500 scanning electron microscope operated at 5 kV.

UV-visible spectroscopy

UV-visible absorbance measurements were obtained using Thermo Scientific NanoDrop 2000 Spectrophotometer.

Gel electrophoresis:

Denaturing gel electrophoresis was used to analyze protein modifications. Specifically, 10 μg of TMV was loaded on denaturing 4-12% NuPAGE gels (Invitrogen). After separation, the gel stained with Coomassie Blue was photographed using an AlphaImager (Biosciences) imaging system. ImageJ software (rsbweb.nih.gov/ij/) was used for band analysis and to estimate the number of PEG molecules attached.

Western blot

Western blot against streptavidin-alkaline phosphatase (Sigma Aldrich) was used to detect biotin modifications on TMV coat proteins. Specifically, 10 μg of TMV was loaded on denaturing 4-12% NuPAGE gels (Invitrogen). After separation, the separated proteins were transferred to a nitrocellulose membrane for 1.5 hrs at 25V. Next the membrane containing the proteins was blocked with 5% (w/v) milk for 1 hr, followed by primary antibody binding with streptavidin alkaline phosphatase (500:1 in 5% (w/v) milk) for 1 hr. The membrane was then washed and exposed with BCIP.

Induced coupling plasma optical emission spectroscopy (ICP-OES)

The Gd per TMV ratio was determined using an ICP-OES (Agilent 730 Axial ICP-OES).

Relaxivity measurements:

The ionic relaxivity of the Gd(DOTA)-modified TMV was determined using a Bruker Minispec mq60 relaxometer (60 MHz). The Gd concentration was determined using an ICP-OES. Multiple concentrations of TMV (from 0.5 to 1.75 mg/mL) were used with a standard inversion recovery sequence protocol to determine the T_1 values.

Supporting Data:

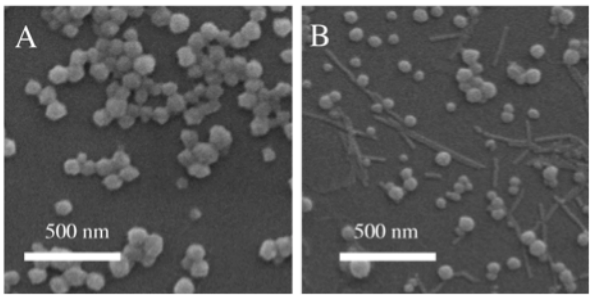

Figure S1 Production of SNPs from TMV rods using a PCR thermocycler is laborious, time consuming, and wasteful. One major reason is due to the inability to obtain 100% conversion of TMV rods to SNPs with volumes greater than 50 μL (in 250 μL PCR tubes). Here, we show the scanning electron microscope (SEM) images of SNPs generated using a volume of (A) 50 μL and (B) 100 μL. A mix of rods and SNPs is obtained in reactions with volumes of 100 μL (B).

Table 1 Here, we determined the Reynold's number and the length of tubing required to heat the TMV solution for 125s at various flow rates. This is important to confirm continuous flow and reduce flow related variability to our device. The Reynold's number (R_e) was calculated using the following equation:

$$R_e = \frac{u * D}{v}.$$

where u is the solution speed in m/sec, D is the diameter of the tubing, and v is the kinematic velocity (0.00000029).

Flow Rate (mL/min)	u (m/s)	R_e	Tube length in oil bath for 125s (m)
0.5	0.001052548	12	0.13
1	0.002105095	23	0.26
2	0.004210191	46	0.53
4	0.008420381	92	1.05
10	0.021050954	230	2.63

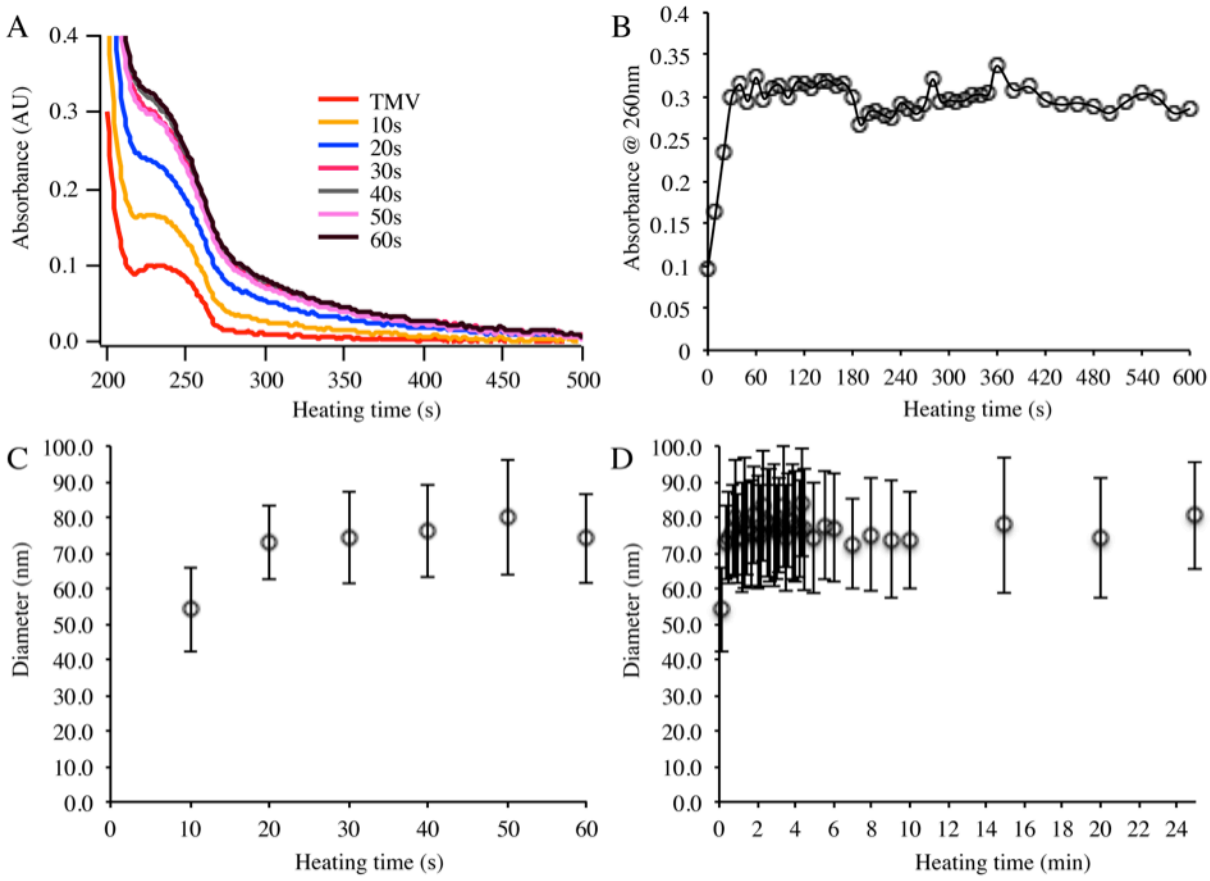

Figure S2 Based on experimental findings from the mesofluidic device, we determined there is a broad heating time window at which the SNPs can be formed, remain stable and intact. One method of determining whether SNPs are formed in solution is to monitor the UV-Vis absorbance properties of the TMV/SNP solution. Additional, more controlled investigation into this phenomenon was performed using the PCR thermocycler. The results are presented in Figure S2 and Figure S3. Figure S4 discusses the thermal stability of modified TMV particles transitioned to SNPs. Specifically, upon transition from TMV rods to SNPs, the absorbance at 260 nm increases, as shown in the UV-Vis absorbance spectra of TMV exposed to 96°C for varying incubation times, see panel (A). Panel (B) shows a plot of the absorbance at 260 nm for incubation times from 10s to 600s. Further, SNPs formed from heating times of 10s to 25 min were measured by SEM (Figure S3) and showed consistent diameter (C) and (D) regardless of heating time. TMV heated for only 10s and 20s have lower absorbance at 260nm; and SEM revealed incomplete conversion of TMV rods to SNPs at these heating times. Therefore, we conclude that UV-Vis absorbance is a useful measure for monitoring of SNP formation.

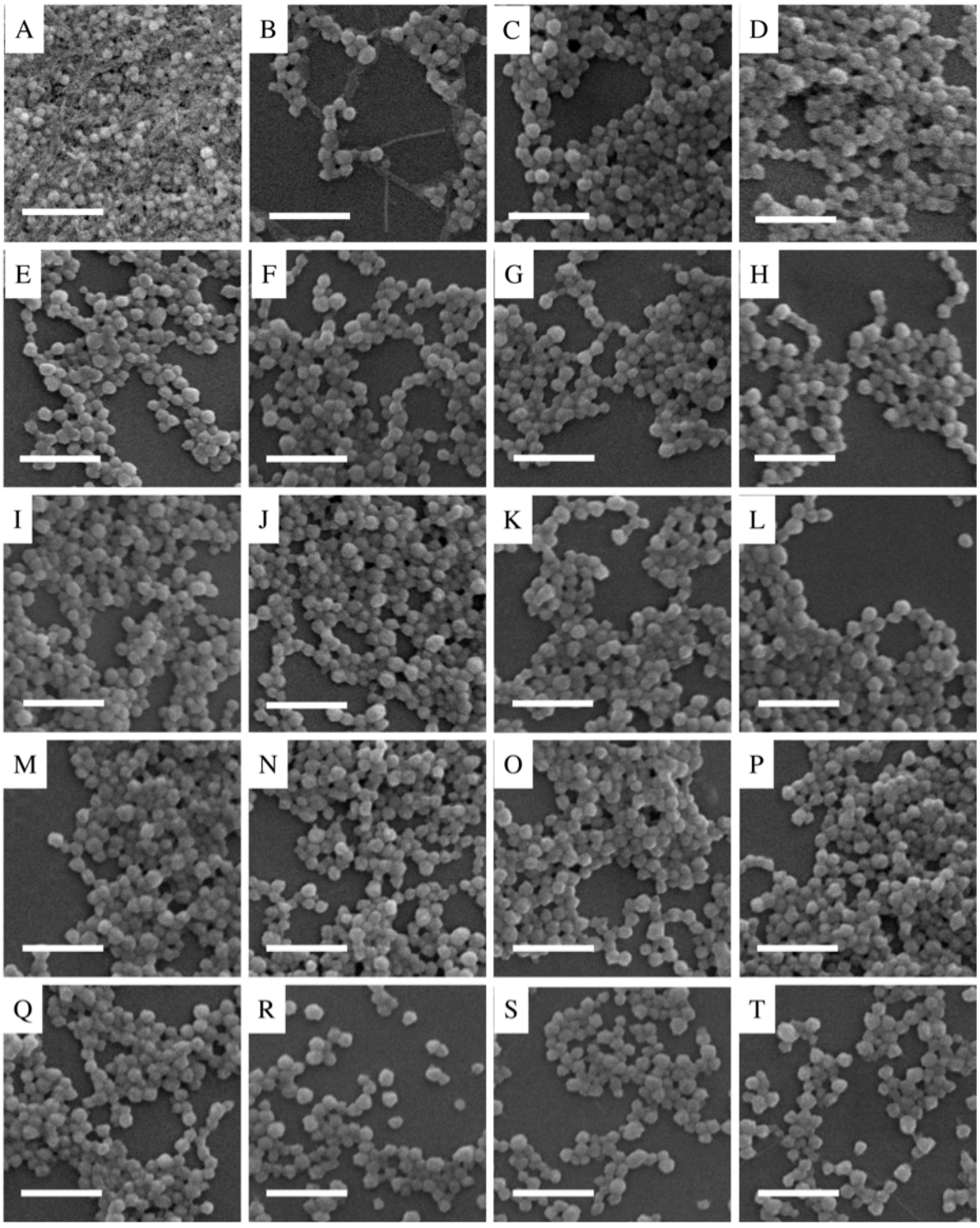

Figure S3 TMV was transitioned to SNPs using a PCR heat cycler set to the following heating times: (A) 10s, (B) 20s, (C) 30s, (D) 60s, (E) 90s, (F) 120s, (G) 150s, (H) 180s, (I) 210s, (J) 240s, (K) 270s, (L) 300s, (M) 360s, (N) 420s, (O) 480s, (P) 540s, (Q) 600s, (R) 900s, (S) 1200s, and (T) 1500s. Scale bar is 500 nm.

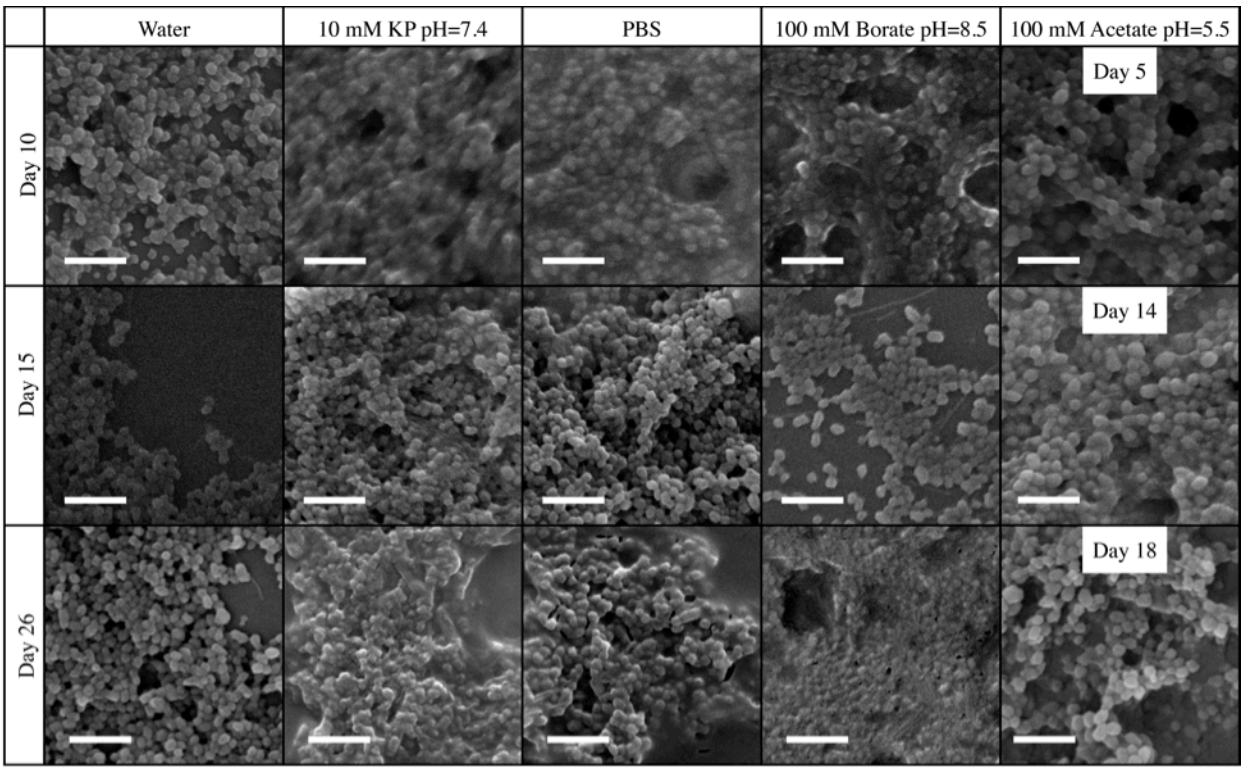

Figure S4 The stability of SNPs in various buffer pH values, salts composition, and salt concentrations was tested. SEM images were used to characterize SNP size and morphology after 10, 15, and 26 days. Data indicate that SNPs remained stable under each condition tested.

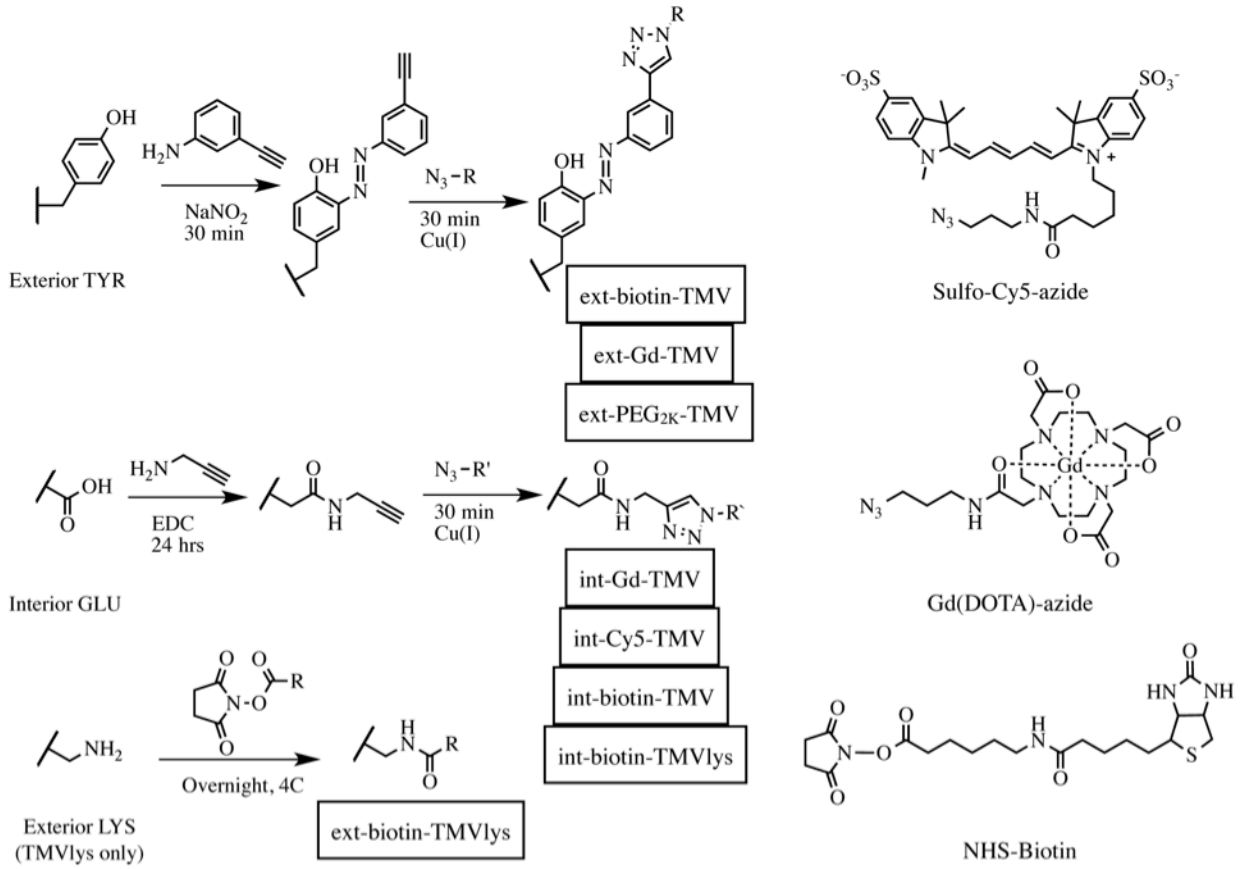

Figure S5 We evaluated the ability of the mesofluidic device to transition chemically modified TMV rods into functionalized SNPs. Here, we show the bioconjugation reaction schemes to incorporate the following molecules onto the interior or exterior of TMV rods; biotin, Cy5 dyes, Gd(DOTA) magnetic resonance contrast agents, and PEG molecules. The characterization of the outlined reactions are shown below; MALDI-TOF MS in Figure S6 and its interpretation in Table S2, SDS-PAGE and western blot against biotin in Figure S7, and TEM images to confirm morphology in Figure S8.

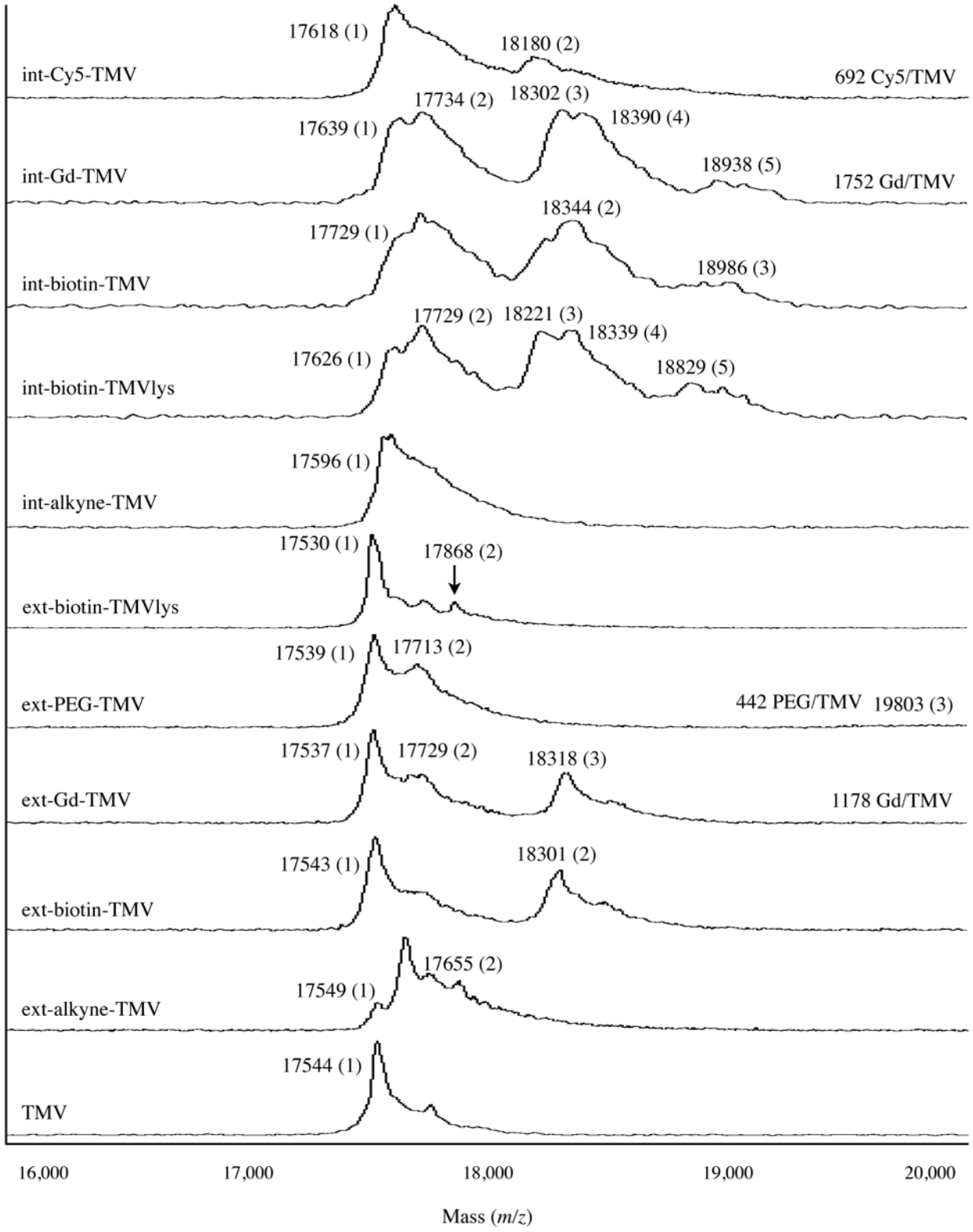
17618 (1)
18180 (2)
int-Cy5-TMV
692 Cy5/TMV
17734 (2)
18302 (3)
17639 (1)
18390 (4)
18938 (5)
int-Gd-TMV
1752 Gd/TMV
18344 (2)
17729 (1)
18986 (3)
int-biotin-TMV
17729 (2)
18221 (3)
18339 (4)
17626 (1)
18829 (5)
int-biotin-TMVlys
17596 (1)
int-alkyne-TMV
17530 (1)
17868 (2)
ext-biotin-TMVlys
17539 (1)
17713 (2)
ext-PEG-TMV
442 PEG/TMV
19803 (3)
17537 (1)
17729 (2)
18318 (3)
ext-Gd-TMV
1178 Gd/TMV
17543 (1)
18301 (2)
ext-biotin-TMV
17655 (2)
17549 (1)
ext-alkyne-TMV
17544 (1)
TMV
16,000
17,000
18,000
19,000
20,000
Mass (m/z)

Figure S6 MALDI-TOF MS was used to determine the molecular weight (as mass per charge (m/z)) of individual coat proteins of denatured TMV rods. The mass of a native TMV coat protein is 17,534 Da. The following changes in molecular weight are expected for the various chemical modifiers: ext-alkyne – 128 Da, biotin-azide – 697 Da, Gd(DOTA)-azide – 641 Da, PEG_{2K}-azide – 2,000 Da, int-alkyne – 37 Da, NHS-LC-biotin – 340 Da, sulfo-Cy5-azide – 723 Da. For peak assignments, see Table S2. Due to the nature of MALDI-TOF MS, some peaks are broad and difficult to define; therefore multiple modified coat proteins are assigned. See previous reports for a more detailed explanation[3,5,6].

Table S2 MALDI-TOF MS peak assignments for modified TMV.

Sample	Mass (peak number)	Peak assignments (predicted m/z)
TMV	17,544 (1)	wt (17,534)
ext-alkyne-TMV	17,549 (1)	wt (17,534)
	17,655 (2)	ext-alkyne (17,662)
ext-biotin-TMV	17,543 (1)	wt (17,534)
	18,301 (2)	ext-biotin (18,359)
ext-Gd-TMV	17,537 (1)	wt (17,534)
	17,729 (2)	ext-alkyne (17,662)
	18,318 (3)	ext-Gd (18,303)
ext-PEG-TMV	17,539 (1)	wt (17,534)
	17,713 (2)	ext-alkyne (17,662)
	19,803 (3)	ext-PEG (19,662)
ext-biotin-TMVlys	17,530 (1)	wt (17,534)
	17,868 (2)	ext-biotin (17,874)
int-alkyne-TMV	19,596 (1)	wt (17,534)
		int-alkyne (17,571)
int-biotin-TMVlys	17,626 (1)	wt (17,534)
		int-alkyne (17,571)
	17,729 (2)	int-alkyne + ?
	18,221 (3)	int-biotin (18,268)
	18,339 (4)	int-biotin + ?
	18,829 (5)	int-biotin + int-biotin (19,002)
int-biotin-TMV	17,729 (1)	int-alkyne (17,571)
	18,344 (2)	int-biotin (18,268)
	18,986 (3)	int-biotin + int-biotin (19,002)
int-Gd-TMV	17,639 (1)	int-alkyne (17,571)
	17,734 (2)	int-alkyne + ?
	18,302 (3)	int-Gd (18,212)
	18,390 (4)	int-Gd + ?
	18,938 (5)	int-Gd + int-Gd (18,853)
int-Cy5-TMV	17,618 (1)	int-alkyne (17,571)
	18,180 (2)	int-Cy5 (18,294)

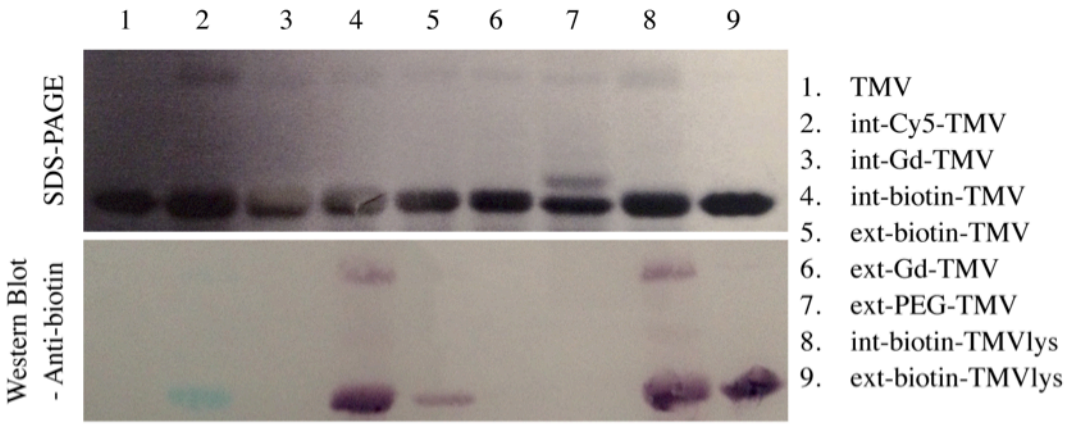

Figure S7 SDS polyacrylamide gel electrophoresis (PAGE) (top) and western blot probed with AP-conjugated streptavidin (bottom) analysis against of modified TMV particles; biotin-modified samples produce a positive signal. The level of PEG modification was determined by using lane analysis function in ImageJ (Lane 7) indicating ~21% (or ~447 PEG per TMV) of coat proteins contained PEG molecules. Quantitative analysis of other TMV modifications is not possible by this method because the ligands are too small (small change in molecular weight); alternative methods such as UV-vis absorbance and ICP-OES were used.

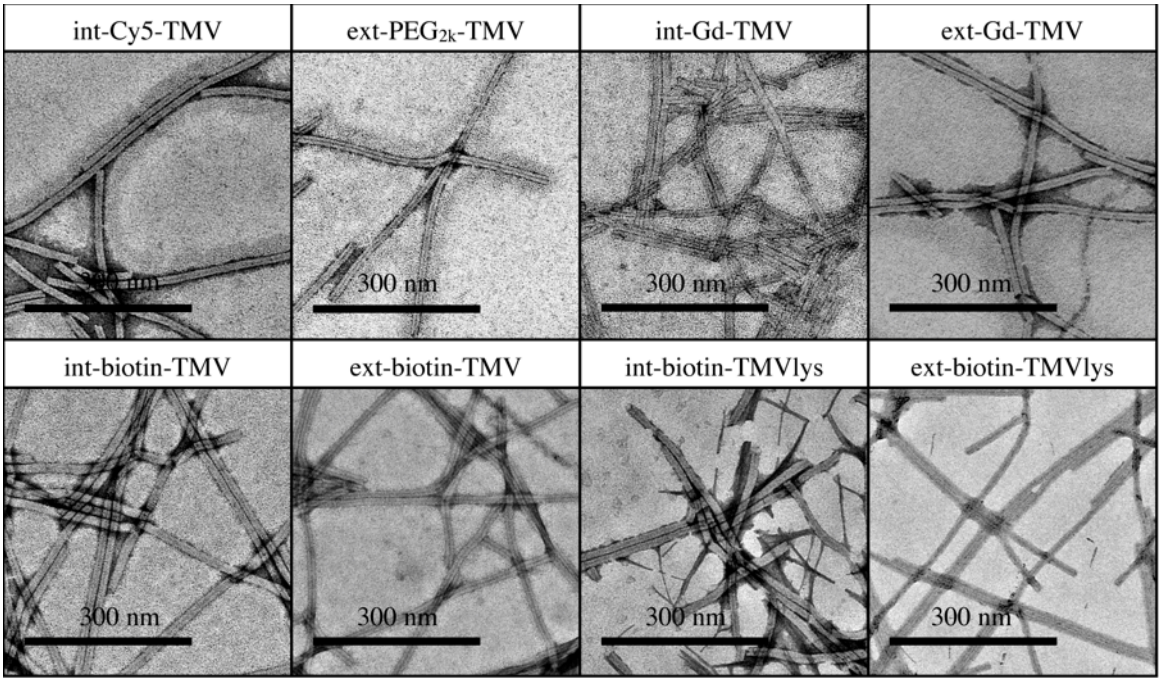

Figure S8 TEM images of modified TMV particles. This confirms that the modifications to TMV did not affect their overall morphology.

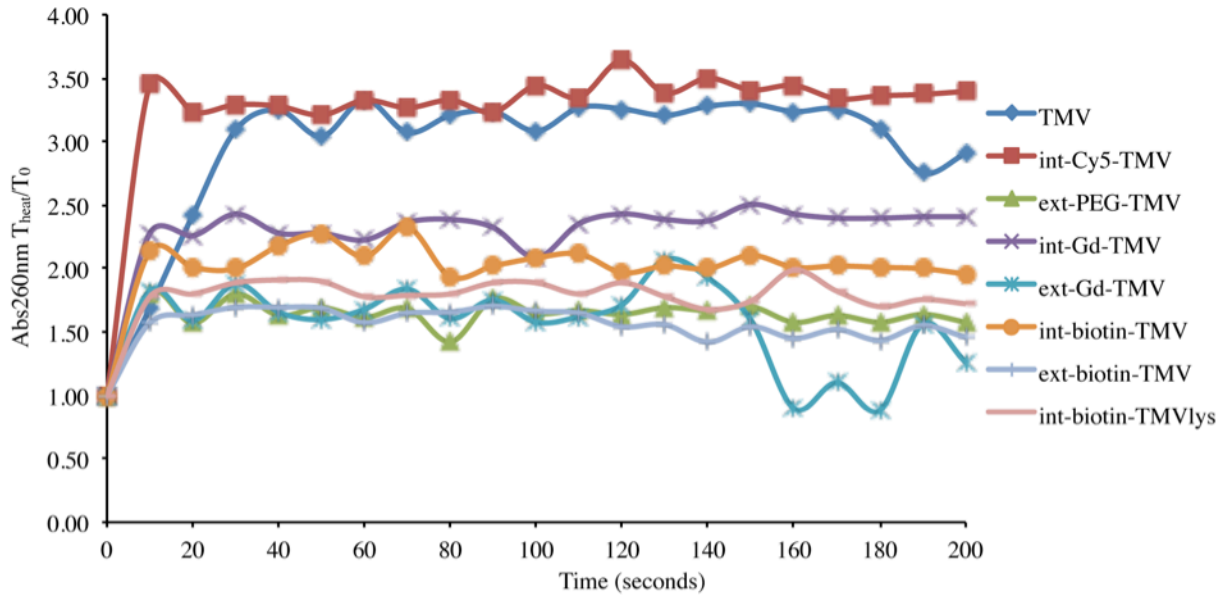

Figure S9 The absorbance at 260 nm was monitored for modified particles before and after transition to SNPs at different heating times using a PCR heat cycler. The data are reported as a ratio of the final absorbance at 260 nm (T_{heat}) over the initial absorbance of the modified TMV (T_0). In summary, the absorbance increases for all modified TMV particles upon transition to SNPs due to increased protein aggregation. Additionally, it can be seen that the SNPs remain stable after heating for up to 200 seconds.

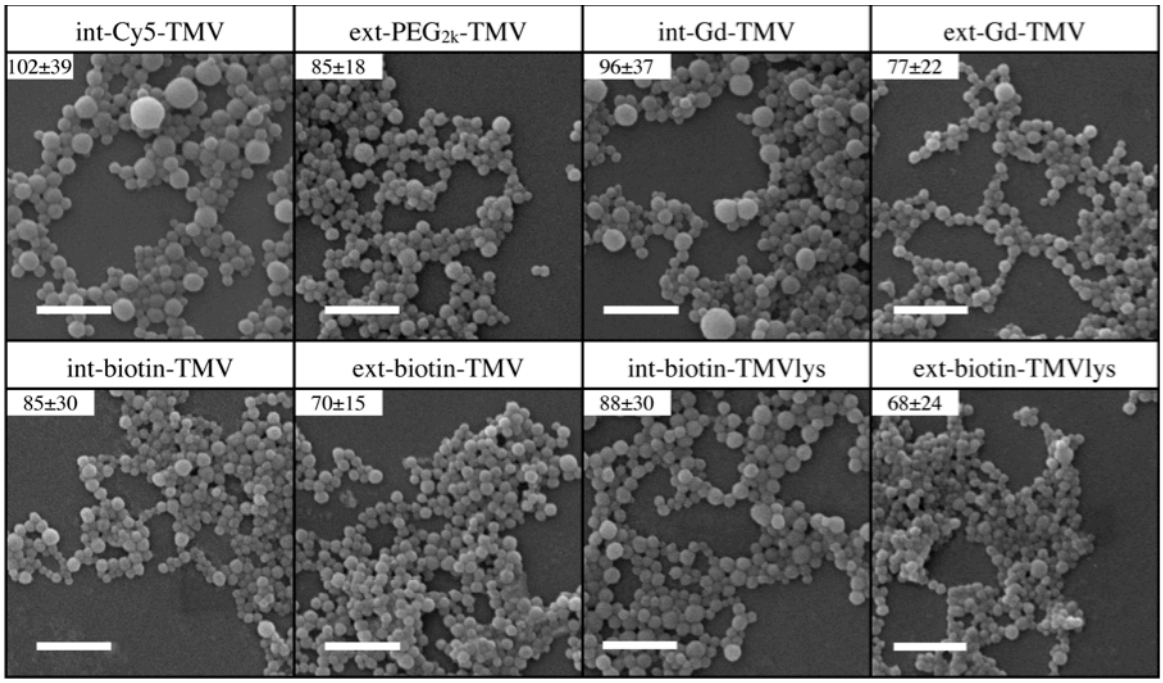

Figure 10 SEM images of SNPs formed from modified TMV particles three weeks after thermal transition. This shows that modified SNPs are quite stable over time. These particles are stored in water. The sizes and distributions are similar to their initial values.

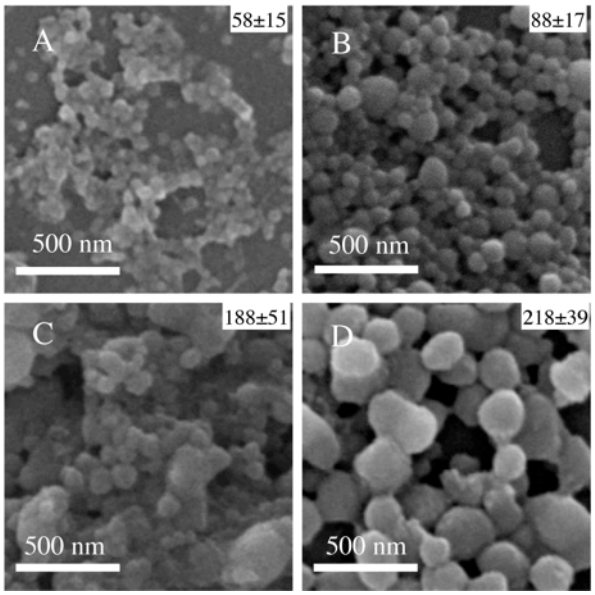

Figure S11 SEM images of SNPs formed from varying concentrations of int-Gd-TMV rods. Here, the followed starting concentrations of TMV formed the corresponding diameter SNPs: (A) 0.1 mg/mL – 58 nm, 1.0 mg/mL – 88 nm, 2.5 mg/mL – 188 nm, and 5.0 mg/mL – 218 nm. The relaxivity values and Gd loading per nanoparticles are reported in Table 1 in the main text.

References:

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(2) Geiger, F. C.; Eber, F. J.; Eiben, S.; Mueller, A.; Jeske, H.; Spatz, J. P.; Wege, C. *Nanoscale* **2013**, *5*, 3808.

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(6) Bruckman, M. A.; Kaur, G.; Lee, L. A.; Xie, F.; Sepulveda, J.; Breitenkamp, R.; Zhang, X.; Joralemon, M.; Russell, T. P.; Emrick, T.; Wang, Q. *Chembiochem* **2008**, *9*, 519-523.