#### **Supplementary Material**

# A progressive approach on zebrafish toward sensitive evaluation of nanoparticles' toxicity

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#### Materials

Ferric chloride (FeCl<sub>3</sub>·6H<sub>2</sub>O) was purchased from Tianjin Guangfu Chemical Engineering Institute. Ammonium hydroxide (25%, aqueous solution) was provided by Tianjin Dongzheng Fine Chemical Reagent Factory, China. Trisodium citrate and Ethylene glycol (EG) were obtained from Tianjin Chemical Reagents I Co. Tetraethylorthosilicate (TEOS) was bought from Aldrich and used without further purification. Cetyltrimethylammonium bromide (CTAB) was purchased from Tianjin Daomao Chemical Reagent II Co. N, N'-Methylenebisacrylamide (MBAAm, chemical grade, Tianjin Bodi Chemical Engineering Co.) was recrystallized from acetone. Methacrylic acid (MAA) was purchased from Tianjin Chemical Reagent II Co and purified by vacuum distillation. 2, 2'-Azobisisobutyronitrile (AIBN) was provided by Chemical Factory of Nankai University and recrystallized from methanol. Acetonitrile (analytical grade, Tianjin Chemical Reagents II Co.) was dried over calcium hydride and purified by distillation before use. All the other reagents were of analytical grade and used without any further treatment.

#### **Preparation of MSRMs**

The synthesis of Fe<sub>3</sub>O<sub>4</sub> microspheres and coating of P(MBAAm-co-MAA) on the Fe<sub>3</sub>O<sub>4</sub> microspheres please refer to our previous work.<sup>1</sup> The MSRMs were then prepared through the sol-gel process to coat the  $Fe_3O_4(a)P(MBAAm-MAA)$ microspheres with a silica shell using CTAB as mesoporous directing agent and the subsequent calcination of the tri-layer microspheres. Briefly, 0.28 g Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA) microspheres were dispersed in 35 mL water and 280 mL ethanol mix solution with the aid of ultrasound radiation and 4.2 mL  $NH_3 H_2O$ was added under the mechanical stirring, then 0.7 mL TEOS was dropped into the solution and 0.14 g CTAB was immediately added. The reaction lasted for 12 h and the product was purified by magnetic collection and washing with ethanol and water 3 times respectively. The microspheres were dried in a vacuum oven until constant weight. The tri-layer microspheres were calcinated at 500 °C for 6 h to obtain MSRMs.

#### **Methods of Materials Characterization**

The morphology of the particles was determined by transmission electron microscopy (TEM) using a Technai G2 20-S-TWIN microscope. The samples for TEM characterization were dispersed in ethanol and a drop of the dispersion was dropped onto the surface of a copper grid coated with a carbon membrane and then dried under vacuum state at room temperature. All the size and size distribution reflect the averages about 100 particles each, which are calculated according to the following formula:

$$U = D_W / D_n \qquad D_n = \sum_{i=1}^k n_i D_i / \sum_{i=1}^k n_i \qquad D_w = \sum_{i=1}^k n_i D_i^4 / \sum_{i=1}^k n_i D_i^3$$

where, U is the polydispersity index,  $D_n$  is the number average diameter,  $D_w$  is the weight-average diameter,  $D_i$  is the diameter of the determined microspheres. The thickness of the shell-layer is calculated as half of the difference between the average diameter of the core-shell particles and that of the cores.

Fourier transform infrared spectra were determined on a Bio-Rad FTS 135 FT-IR spectrometer over potassium bromide pellet and the diffusion reflectance spectra were scanned over the range of 4000-400 cm<sup>-1</sup>.

The magnetic properties of  $Fe_3O_4$  microspheres,  $Fe_3O_4@P(MBAAm-co-MAA)$  microspheres and MSRMs were studied in the dried state with a vibrating sample magnetometer (9600 VSM, BOJ Electronics Inc., Troy, MI) at room temperature.

The crystalline structure of the samples was analyzed on a D/max 2500 V X-ray diffractometer using Cu K $\alpha$  ( $\lambda$ =0.15406 nm) radiation at 40 kV and 100 mA. The crystal size of Fe<sub>3</sub>O<sub>4</sub> and maghemite core in MSRMs were estimated by applying the Scherrer equation ( $\varphi$ =k $\lambda$ / $\beta$ cos $\theta$ ), where  $\varphi$  is the crystal size,  $\lambda$  is the wavelength of the X-ray irradiation, k is usually taken as 0.89 here,  $\beta$  is the peak width at half-maximum height of the (311) peak of magnetite and rattle-type microspheres after subtracting the instrumental line broadening, and  $\theta$  is the diffraction angle.

N<sub>2</sub> sorption-desorption was performed on a TriStar 3000 V6.07 A.

### **Results of Material Characterization**

Scheme S1 illustrated the synthesis of rattle-type magnetic mesoporous microspheres. The Fe<sub>3</sub>O<sub>4</sub>@SiO<sub>2</sub> rattle-type microspheres with strong magnetization cores and mesoporous shells were prepared through a three-stage process: 1, the polymer was directly coated onto the magnetite microspheres to form Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-*co*-MAA) core-shell microspheres, as our previous work demonstrated;<sup>1</sup> 2, a sol-gel process was performed on the core-shell microspheres to obtain Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-*co*-MAA) tri-layer microspheres by using TEOS as the silica source with CTAB as mesoporous structure directing agent; 3, the tri-layer microspheres was calcinated to prepare MSRMs.



Scheme S1. The process for synthesis of rattle-type magnetic mesoporous silica microspheres

The magnetite microspheres were firstly synthesized through a solvothermal method according to the literature with a minor modification.<sup>2</sup> The TEM image of  $Fe_3O_4$  microspheres shown in Figure S1A revealed that the  $Fe_3O_4$  microspheres had a spherical shape with a rough surface and a relatively uniform size of about 180 nm. With the clear observation of the magnetite microspheres through HRTEM (high resolution transmittance electronic microscopy) in inset of Figure S1A, the microspheres comprised of small magnetite particles with the size of about 5-10 nm. That was to say, the magnetite microsphere was a secondary aggregate of small particles which was smaller than the critical size of the single magnetic domain (<15 nm) and may exhibit the superparamagnetic property.<sup>3</sup> Figure S2A-b presented the

XRD spectrum of the Fe<sub>3</sub>O<sub>4</sub> microspheres, the diffraction peaks at  $2\theta$ =30.1°, 35.4°, 43.0°, 53.4°, 56.9° and 62.5° attributed to the (220)(311)(400)(422)(333) and (440) lattice planes of cubic magnetite (Fe<sub>3</sub>O<sub>4</sub>), which fit well with the standard PDF card 74-748 in absence of any other peaks. This result meant that the acquired particles were magnetite with high purity. The grain size of the crystalline was calculated to be 7.4 nm based on the strongest 311 diffraction peak through the Debye-Scherrer equation, which was consistent with the size acquired through statistical analysis of the particles in the HRTEM graphs.



**Figure S1.** TEM graphs of **A**) magnetite microspheres (inset in (**A**) was the HRTEM image, scale bar=20nm); **B**)  $Fe_3O_4@P(MBAAm-co-MAA)$  microspheres; **C**)  $Fe_3O_4@P(MBAAm-co-MAA)@SiO_2$  microspheres; **D**) rattle-type microspheres(inset in (**D**) was the TEM image with higher magnification, scale bar=50nm ).



Figure S2. (A) XRD spectra of a) magnetite microspheres, b) rattle-type microspheres; FT-IR spectra of a) magnetite **(B)** microspheres, b) Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA) microspheres, c) Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA)@SiO<sub>2</sub> microspheres, d) rattle-type microspheres; (C)  $N_2$  absorption-desorption isotherm of rattle-type microspheres(the inset was the pore size distribution graph); (**D**) hysteresis loops of a) magnetite microspheres, b) Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA) microspheres, c) rattle-type microspheres. Inset in (D): Rattle-type microsphere was suspended in water and collected by an external magnetic field.

In our previous work, the functional groups such as hydroxyl or carboxyl groups on the surface of the polymer or inorganic particles have played an important role for coating of a new polymer layer through distillation-precipitation polymerization.<sup>1, 4-7</sup> The hydrogen bonding interaction between the functional monomers and the functional groups on the core particles can effectively capture the oligomer to form the well-defined core-shell particles.<sup>1, 4-7</sup> As the surface of the obtained magnetite microspheres in this paper was abundant with hydroxyl and carboxyl groups,<sup>1, 8, 9</sup>

distillation-precipitation polymerization method was used to encapsulate a polymer shell onto the magnetite microspheres through the hydrogen-bond interaction between the monomer and the hydroxyl groups on the surface of the magnetite microspheres without any surface modification of the templates. As shown in Figure S1B of the TEM images, the Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA) microspheres had a spherical morphology remaining the shape of the core template but a smoother surface compared to the initial magnetite microspheres. The deep contrast core and light contrast shell presented the well-defined core-shell structure. These results meant that the polymer shell with thickness of 18 nm was successfully coated onto the magnetite microsphere core and the shell thickness was calculated by the difference between the radii of core-shell particles and those of the cores. There were not any secondary polymer particles appeared in the TEM graph, indicating that the hydrogen bond was strong enough to capture the oligomer to form the core-shell structure during the polymerization process. The FT-IR spectrum was further applied to characterize the polymer component of the core-shell structure as shown in Figure S2B-b. The absorption peak at 588 cm<sup>-1</sup> corresponded to Fe-O vibration mode.<sup>1,10,11</sup> Further, the new absorption peak at 1708 cm<sup>-1</sup> was attributed to the stretching vibration of the carbonyl groups of the MAA as well as MBAAm segment and the absorption peak at 1550 cm<sup>-1</sup> assigned to the vibration of the second-amide group of the MBAAm segment, suggesting that the Poly(MBAAm-co-MAA) was coated onto the magnetite microspheres through one-pot distillation-precipitation polymerization without any surface modification of the template. This functional P(MBAAm-co-MAA) polymer shell abundant with carboxyl groups would facilitate depositing with an additional silica layer as our previous work demonstrated.<sup>12, 13</sup>

The silica was coated onto the Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA) microspheres via a sol-gel method and the CTAB was used as mesoporous structure directing agent for preparing tri-layer microspheres, which was similar to the case for the synthesis of PMAA/TiO<sub>2</sub>@PMAA@SiO<sub>2</sub> tri-layer hybrid microspheres.<sup>14</sup> The corresponding TEM images of Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA)@silica tri-layer microspheres were shown in Figure S1C. The microspheres had distinct tree layers: the deepest contrast core, the lightest contrast middle layer and the outer shell with a size of 260 nm. The thickness of silica shell was calculated to be about 22 nm. The presence of a new peak at 1090 cm<sup>-1</sup> for the FT-IR spectrum of Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA)@silica in Figure S2B-c (comparing to that of  $Fe_3O_4(a)P(MBAAm-co-MAA)$  in Figure S2B-b) that silica shell successfully suggested the was coated the onto Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA) microspheres.

The mesoporous rattle-type silica containing maghemite cores were afforded by the calcination  $Fe_3O_4(a)P(MBAAm-co-MAA)(a)silica$ of the resultant tri-layer microspheres, during which the sandwiched P(MBAAm-co-MAA) mid-layer were selectively decomposed to give the large cavity and mesoporous silica shell-layers were formed via the selective pyrolysis of the organic surfactant. Figure S1D presented the TEM images of MSRMs: a void between the deep contrast core and light contrast shell. During the calcination, most of the acquired MSRMs retained the spherical shape without fragmentation and kept the initial size of Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA)@silica microspheres of about 260 nm. The FT-IR spectrum of the MSRMs showed the disappearance of the absorption peak of the carboxyl group at 1708 cm<sup>-1</sup> while retaining the strong absorption peak of Si-O at 1090 cm<sup>-1</sup>. This result was consistent with the successful removal of the polymer layer to form the rattle-type microspheres. In this paper, a series of rattle type microspheres

with different void space were prepared by changing the thickness of the polymer layer as shown in Figure S3 of ESI. All of the rattle-type microspheres had similar structure but with different void layer of about 5 nm, 10 nm, 18 nm, 24 nm. In the literature,<sup>15</sup> it was relatively difficult to tune the void space especially to a large content between the core and shell in the rattle type microspheres. While in our rattle type microspheres, the sandwiched voids were varied in the range of 5-24 nm through altering the middle polymer thickness.



Figure S3 TEM images of MSRMs with different void space

The MSRMs characterized by XRD was shown in Figure S2A. After the calcination at high temperature, the diffraction peak of the rattle type microspheres was different from that of the initial magnetite microspheres and had a little shift to the higher angle. The diffraction peaks at  $2\theta$ =30.2°, 35.6°, 43.3°, 53.7°, 57.2° and 63.0° were attributed to the (220)(311)(400)(422)(511) and (440) lattice planes of cubic maghemite ( $\gamma$ -Fe<sub>2</sub>O<sub>3</sub>). All of these diffraction peaks fit well with the PDF card 39-1346. It meant that the magnetite microspheres were transferred to maghemite

during the elevated temperature treatment.<sup>10, 16, 17</sup> According to Debye–Scherer formula, calculations based on the strongest 311 diffraction peak revealed that grain size of maghemite was about 8.2 nm for the as-synthesized rattle-type nanoparticles. This grain size was almost the same with the particle in the initial magnetite microspheres, indicating that the particles didn't aggregate during the high temperature treatment. In addition, there was an additional broad peak between 20 and 30 degree compared to initial magnetite microspheres due to the existence of amorphous silica.<sup>18</sup>

Room temperature magnetization curves of the Fe<sub>3</sub>O<sub>4</sub> microspheres, Fe<sub>3</sub>O<sub>4</sub>@P(MBAAm-co-MAA) microspheres, rattle-type microspheres were shown in Figure S2D. The corresponding saturation magnetization and coercive force were summarized in Table S1. The saturation magnetization values of these microspheres measured at 298 K was 53.02, 32.55 and 21.67 emug<sup>-1</sup> respectively, the coercive force of these three microspheres were 18.6, 13.57 and 24.71 Oe for each. Importantly, the rattle-type microsphere with the strong saturation magnetization value of 21.67 emug<sup>-1</sup> was strong enough for magnetic separation. As shown in inset of Figure S2D, the suspension of MSRMs in water could be separated completely in 4 minutes. It also retained the superparamagnetic properties after the high temperature treatment during which time the magnetite transferred to the maghemite and may be a superior candidate for MRI. The mesoporous structure of MSRMs was studied by N2 adsorption-desorption isotherm as shown in Figure S2C. It revealed the type IV loop of the mesoporous structure with a Brunauer-Emmett-Teller (BET) surface area of 251 m<sup>2</sup>/g. The average pore size and pore volume obtained from the analysis of the adsorption branch using the Barrett-Joyner-Halenda (BJH) method was about 3 nm and 3.2  $\text{cm}^3/\text{g}$ .

$Fe_3O_4(w)P(MBA-co-MAA)$ incrospheres, MSRMs							
Material	Saturation magnetization	Coercive force (Oe)					
	(emug <sup>-1</sup> )						
Fe <sub>3</sub> O <sub>4</sub>	53.02	18.6					
Fe <sub>3</sub> O <sub>4</sub> @P(MBAAm-co-MAA)	32.55	13.57					
MSRMs	21.67	24.71					

Table	<b>S1.</b>	Magnetization	properties	of	Fe <sub>3</sub> O <sub>4</sub>	microspheres,
Fe <sub>3</sub> O <sub>4</sub> @P(MBA-co-MAA) microspheres, MSRMs						

## Zebrafish Scoring Spectra:



**Figure S4** Semi-quantitative scoring spectrum for assessing the toxicity of MSRMs by screening the level of 144 hpf zebrafish malformation. In each level, five representative micrographs of zebrafish were demonstrated. Scale bar = 1 mm.





**Figure S5** Semi-quantitative scoring spectrum of zebrafish embryos at 48 and 72 hpf. In each level, five representative micrographs of zebrafish were demonstrated. In level 3 of 48 hpf zebrafish, only three zebrafish with severe malformations could be found. Scale bar = 0.5 mm.

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