Supporting Information for

Nanogel@Fe₃O₄ via UOx/HRP Initiated Surface Polymerization for

pH Sensitive Drug Delivery

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1. Materials

Ferric chloride hexahydrate (FeCl₃ • 6H₂O), ethylene glycol (EG), diethylene glycol (DEG),
Urate oxidase (UOx, MW = 34 kDa, EC 1.7.3.3), Horseradish Peroxidase (HRP, MW = 44 kDa,
EC 1.11.1.7), Poly (ethylene glycol) dimethacrylate (PEGMA, mw=360) and Poly (ethylene
glycol) diacrylate (PEGDA, mw=250) and Methacrylic acid were purchased from Sigma-Aldrich.
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were
purchased from Energy Chemical, sodium acetate (CH₃COONa, NaOAc), poly (vinylpyrrolidone)
(PVP, K30), 3-aminopropyltriethoxysilane (APTES) and succinic anhydride was purchased from
Aladdin. Glucose, o-phenylenediamine (OPD), uric acid and acetylacetone (ACAC) were obtained
from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Doxorubicin hydrochloride (DOX)
was purchased from Shanghai Baoman Biotechnology Co., Ltd. (Shanghai, China). All materials
were used without further purification.

2. Instruments

UV-vis spectra were obtained by UV-2700 (Shimadzu Corporation). Dynamic light scattering
(DLS) studies of the microgels were conducted on Zetasizer Nano instrument (Malvern
Instruments Ltd., United Kingdom). Scanning electron microscope (SEM) images were obtained
on Hitachi S-4800 with 3 kV accelerating voltages. TEM images were taken with a JEOL 2100
microscope (Japan) operated at 200 kV. Absorbance of the MTT assay was measured at 490 nm
using the ELx800 reader (BioTek Instruments, Inc, Winooski, VT). FACS was analyzed using the
Becton–Dickinson spectrophotometer (Becton, Dickinson and Company). Confocal microscopic
studies were performed on a confocal laser scanning microscope (FV1000 Fluoview; Olympus
Corporation) equipped with 405 and 488 nm laser lines using a 60× oil immersion objective.
3. The detailed preparation of the nanogels

The magnetic Fe$_3$O$_4$ nanoparticles (MNPs) were synthesized according to the literature. In detail, FeCl$_3$·6H$_2$O (0.54 g) was dissolved in a mixture of EG and DEG (V$_{\text{EG}}$/V$_{\text{DEG}}$=3/17, total volume is 20 mL) in a flask under magnetic stirring. After 30 min, 2 g of PVP (K30) was added into the above solution and was heated at 120°C to form a transparent solution under a continuous flow of N$_2$ with stirring. After 1 h, 1.5 g NaOAc was added into the above solution and stopped heating. After vigorous stirring for 30 min, the obtained solution was then transferred to a Teflon-lined stainless-steel autoclave (50 mL capacity) and sealed. The autoclave was heated at 200 °C and maintained for 12 h reaction period, and then cooled to room temperature. The obtained ferrite nanospheres were washed three times with ethanol and water, and then resuspended in deionized water for further use.

2 mL the above MNPs was resuspended in a mixture of 80 mL ethanol and 80 mL deionized water. After sonication for 30 min, 6 mL APTES was added and then stirred at 70°C for 24 h under continuous N$_2$ flow. The amino-functionalized MNPs were washed three times with ethanol and deionized water. Subsequently, the MNPs-APTES was further treated with 10% succinic anhydride in dimethylformamide (DMF) and stirred for 18 h to achieve carboxylic functionalized nanoparticles MNPs-COOH. The resultant mixture was purified by magnetic separation and washed with ethanol and deionized water for further use.

The above carboxyl-functionalized MNPs were activated by EDC (200 mg) and NHS (200 mg) in 40 mL phosphate buffer solution at pH 5.8 for 2 h. After 3 times wash, the nanoparticles were then resuspended in 20 mL enzyme UOx and HRP solution (molar ratio of UOx:HRP=1:1) to covalently attach the UOx and HRP simultaneously on the surface of the carboxyl-modified magnetic nanoparticles. The residual unreacted UOx and HRP were separated from the magnetite nanoparticles by magnetic decantation and then washed with deionized water for three times. The supernatant and the washed solution were kept for determination of enzyme concentration. The obtained MNPs@UOx/HRP nanoparticles were dispersed in 10 mL deionized water at approx. 4 °C for further use.

The nanogel shell synthesis was fabricated around the interface of UOx/HRP-MNPs with the addition of coating precursor solution, which was composed of PEGMA$_{360}$ (4%, v/v), methacrylic acid (MAA, 2%, v/v), PEGDA$_{250}$ (1%, v/v), ACAC (1%, v/v) and uric acid (0.02 M). The following interfacial polymerization was initiated by the bienzyme (UOx and HRP) for the appropriate stirring time. The core-shell nanogels were acquired by magnetic separation from the coating solution and re-dispersed in aqueous solution for further characterization.
4. Test of magnetization saturation value (Ms)

The magnetic properties of the obtained magnetic nanoparticles and core-shell nanogels were investigated with a vibrating sample magnetometer (VSM). The samples of Fe₃O₄ and magnetic core-shell nanogels were freeze-dried before this test. The Fe₃O₄ and nanogels have the magnetization saturation values (Ms) of 36.31 emu/g and 32.39 emu/g, respectively (Fig. S3), indicating that the core-shell nanogels retained strong magnetization of the magnetic nanoparticles, thus providing an extremely efficient way for separating these enzymes.

5. Test of catalytic activity

We used uric acid and o-phenylenediamine (OPD) as the substrates to investigate the catalytic activity of the immobilized and free bienzyme by UV-vis spectroscopy. Kinetic measurements were performed as follows: The mixture of the OPD (20 mM) and uric acid (0.2mg/ml) in 2 mL buffer was catalyzed by 0.5mM UOx and HRP in free or co-immobilized state. The reaction course was continued for 3 min at 25°C. During the incubation, absorbance at 450 nm was collected continuously with a UV-vis spectrometer. The concentrations of the product in buffer were corrected according to the molar extinction coefficients in aqueous buffer. (Fig. S4)

6. In vitro drug release

A certain amount of DOX-loaded nanogels including 600 µg DOX was first placed into dialysis bag (MWCO 8000), and then the dialysis bag was put into a 100 mL-beaker against 50 mL of PBS at different pH values of 4.5, 5.8, and 7.4. Subsequently, this device was kept in water bath maintained at 37 °C, 150 rpm. The absorbance of PBS in beaker was measured by UV-vis (absorbance at 480 nm) at different time intervals, which was used to assess the pH-triggered cumulative drug release from the nanogels. The release experiments were carried out for three independent times.

7. Cytotoxicity assay

The cell viability was estimated by MTT assay. SH-SY5Y cells were cultured in 96-well plates with 5×10⁴ cells in 100 µL of medium per well. After 24 hours of incubation, triplicate wells were treated with nanogels, DOX and DOX-loaded nanogels at the DOX concentration of 1, 5, 10 and 20 µg/mL. The plates were incubated at 37 °C in 5% CO₂ for 24 h and 48 h respectively. The control group was conducted under the same conditions with 100 µL of medium. After the cells were incubated with 20 µL of MTT dye solution (5 mg/ml) for 4 h at 37 °C under a light-blocking condition, the medium was removed and 150 µL of DMSO was added into each well. Absorbance was measured at 492 nm by the ELx800 reader (BioTek Instruments, Inc, Winooski, VT).
\[ \text{Cell viability(\%)} = \frac{OD_{492}(\text{test}) - OD_{492}(\text{blank})}{OD_{492}(\text{control}) - OD_{492}(\text{blank})} \times 100\% \]

8. Cellular uptake study

SH-SY5Y cells were seeded in 12-well plates at a density of 1 \times 10^5 cells per dish. After 24 hours of incubation, cells were treated with free DOX and DOX-loaded nanogels (5 \mu g/mL) for 0.5, 2, 4, 8 and 24 h. Afterwards, the cells were washed three times with PBS (pH 7.4), and then harvested and centrifugated. Subsequently, the cells were washed twice and resuspended in PBS (pH 7.4) for quantitative determination by flow cytometry. (Fig. S5)

As for the laser confocal scanning microscopy (LCSM), the pretreatment was as follows. The SH-SY5Y cells were seeded in confocal dishes at a density of 2 \times 10^5 cells per dish, and then were treated with DOX-loaded nanogels (5 \mu g/mL) for 0.5, 2, 8 and 24 h. After incubation, the cells were washed 3 times with PBS (pH 7.4) and fixed with 4% paraformaldehyde for 15 min and stained with DAPI for another 15 min at room temperature. Finally, the cells were observed under a confocal laser scanning microscope (FV1000 Fluoview; Olympus Corporation) equipped with 405 and 488 nm laser lines using a 60× oil immersion objective.

9. Figures

![TEM image of the single Fe₃O₄ nanoparticles.](image)}
Fig. S2 Zeta potential of the Fe$_3$O$_4$ nanoparticles with the different surface modification.

Fig. S3 Magnetization curve of Fe$_3$O$_4$ and the core-shell nanogels.
Fig. S4 Catalytic reactions in 3 minutes of the co-immobilized and native UOx/HRP (0.5 mM) with uric acid (0.2 mg/mL) and OPD (10 mM) in buffer.

Fig. S5 The FACS analyses of cellular uptake for the DOX-loaded nanogels against SH-SY5Y cells for respective 0.5 h, 2 h, 4 h, 8 h and 24 h.

References