

Supporting Information

Biodegradable Nanosyringes for Intracellular Amplification-based Dual-Diagnosis and Gene Therapy in Single Living Cells

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Experimental Sections

1. Chemical reagents.

Hydrofluoric acid (HF, $\geq 40\%$) and hydrogen peroxide (H_2O_2 , $\geq 30\%$) were purchased from Sinopharm Chemical Reagent Co., Ltd (China). Silver nitrate (AgNO_3) was purchased from Nanjing Chemical Reagent Co., Ltd (China). Silicon wafers (boron-doped (p type), 0.01-0.05 Ω sensitivity) were purchased from Hefei Kejing Materials Technology Co., Ltd (China). All chemicals were used without additional purification. All solutions were prepared using Milli-Q water (Millipore) as the solvent.

SplintR ligase and phi29 DNA polymerase were bought from New England Biolabs (Beijing, China). The Oligonucleotide sequences (Supplementary Table S1), Diethyl pyrocarbonate (DEPC)-treated water, Deoxyribonucleotides mixture (dNTPs) and agarose were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). siRNA (Table S1) were purchased from GenePharma Co., Ltd (Shanghai, China). Vascular endothelial growth factor (VEGF) was purchased from Sigma (USA).

Phosphate buffered saline (PBS) (0.01 M, pH 7.2~7.4) was obtained from Solarbio (Beijing Solarbio Science & Technology Co., Ltd.). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640 medium and Penicillin-streptomycin were from Thermo Fisher Scientific (USA). 4', 6- diamidino-2-phenylindole (DAPI) was purchased from Beyotime Institute of Biotechnology (Haimen, China). Chloroform, ethanol, dimethyl sulfoxide (DMSO), and other reagents of analytical grade were obtained from Beijing Chemical Co. (Beijing, China). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). All the solution and deionized water were treated with DEPC and autoclaved to protect from RNase degradation.

2. Apparatus and software.

STEM and TEM were performed on a Tecnai G2 F20 transmission electron microscope (FEI Company, Hillsboro, OR, USA) under accelerating voltage of 200 kV. Fluorescence (FL) spectra were collected by FS-5 fluorescence spectrophotometer (Edinburgh instruments, UK). 50 μ L quartz fluorescence cuvette was purchased from Yixing Purshee Optical Elements Co., Ltd (Zhejiang, China). Dynamic light scattering (DLS) was carried out on a Nano-ZS Zetsozer ZEN3600 (Malvern Instruments Ltd., U.K.). Tissue slides were examined by a Zeiss fluorescence microscope using the AxioVision MTB2004 configuration and images were acquired by Zeiss AxioVision Rel. 4.7 image manager software. The vitro cytotoxicity assay was carried out at 490 nm in a microplate reader (BioTek Instruments Inc, USA). All primers (Table S1) were designed by using Mfold software (<http://unafold.rna.albany.edu/?q=DINAMelt/Quickfold>), NUPACK software (<http://www.nupack.org/>), and Primer 5.0 software (Primer-E Ltd., Plymouth, UK). FT-IR spectra were performed on an IR Affinity-1 spectrometer (Shimadzu, Japan). X-ray photoelectron spectroscopy (XPS) analysis was employed on an ESCALAB 250Xi X-ray photoelectron spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Confocal laser scanning microscopy (CLSM, Nikon A1R, Japan) was used for cell imaging.

3. Synthesis of NSs.

Synthesis of mesoporous silicon nanoneedles:

Free mesoporous silicon nanoneedles (MSNs) arrays were first prepared via a well-established HF-assisted etching method. In brief, MSNs arrays were produced via a selective etching process by immersing a freshly cleaned Si wafer in AgNO_3/HF solution based on electrochemical reaction. In our experiment, silicon wafer was cleaned with acetone and ethyl alcohol by ultrasonic treatment for 10 min respectively, and washed with Milli-Q water (Millipore) for 3 times. After blowing the surface with nitrogen gas, the silicon wafer was

immersed in piranha solution containing H_2SO_4 (98%) and H_2O_2 (30%) with a volume ration of 3:1 for 30 min, and rinsed with Milli-Q water for three times to remove organics. After the blowing of the surface with nitrogen gas, the cleaned silicon wafer was further immersed in hydrogen fluoride (HF, 5%) aqueous solution for 30 min to obtain fresh H-terminated silicon wafer covered by Si-H bonds. The H-terminated silicon wafer was then immediately placed into a freshly prepared reduction solution containing 0.02 mol L^{-1} silver nitrate (AgNO_3) and HF (10%) by slow stirring for 2 min. Then silicon wafer was etched in 4.8 mol L^{-1} HF and 0.1 mol L^{-1} H_2O_2 for 15 min. To shape the thread into a needle, the silicon wafer was immersed in 10% KOH for 2 min. The resultant NSs were detached from the surface of silicon wafer by scrapping, and collected for synthesis of NSs.

Synthesis of NH_2 -Modified MSNs:

According to a previous report,⁸ the MSNs surfaces were amino-functionalized by APTES. Briefly, 1 mL MSNs were dispersed in 4 mL ethanol, followed by adding 10 μL APTES for the reaction for 3 h under stirring at room temperature. The obtained NH_2 -modified MSNs were washed twice with ethanol and three times with water to remove excess reactants. The NH_2 -modified MSNs were then dispersed in water for further experiment.

Obtaining of NSs with biomolecules loaded:

The as-prepared NH_2 -modified MSNs (500 μL) and EDC (4 mg mL^{-1} , 50 μL) were mixed and stirred in an ice water bath. Subsequently, 12.5 μL splintR ligase, 5 μL phi29 DNA polymerase and 5 μL BSA were added into the mixture and stirred for 2 h. The enzymes were then conjugated onto the NH_2 -modified MSNs surfaces with the help of EDC in the ice bath, forming enzyme-functionalized MSNs. Enzyme functionalized MSNs were then collected by centrifugation and washed three times with diethyl pyrocarbonate (DEPC)-treated water to remove any unreacted species. Subsequently, 5 μL $1 \times 10^{-6} \text{ M}$ padlock probes and 5 μL $1 \times 10^{-6} \text{ M}$ VEGF-siRNA were added into the mixture and stirred for 30 min in an ice bath, wherein siRNA was loaded on NSs based on electrostatic self-assembly with nucleic acids.

Finally, the products were purified, re-dispersed in the DEPC-treated water, and stored at 4 °C for further experiments. The total synthesis of NSs was facile and rapid, taking about 3 h.

4. Electrophoresis Analysis—Gel Electrophoresis.

RCA amplicons in 6 μL $1 \times$ gel loading buffer were separated by agarose gel electrophoresis. Agarose was prepared with 40×10^{-3} M TAE buffer (40×10^{-3} M Tris, 20×10^{-3} M acetic acid, and 2×10^{-3} M ethylenediamine tetraacetic acid (EDTA), pH 8.0) to form a 1% agarose-TAE sol-gel with $1 \times$ Sybr Green I. Gel electrophoresis was performed on the prepared gel in TAE at 120 V for 35 min. After electrophoresis, the gel was visualized using ChampGel 5000 (Beijing Sage Creation Science Co., Ltd, China).

5. *In Vitro* Response by NSs -Based RCA Reaction.

Every 50 μL reaction solution contained 5 μL target (let-7a), 12 μL dNTPs solution (10 mM), 2.5 μL splintR ligase reaction buffer, 2.5 μL phi29 DNA polymerase reaction buffer, 2.5 μL FAM- Probe (5×10^{-8} M) and 2.5 μL Cy5- Probe (5×10^{-8} M). For the NSs-based RCA reaction, 23 μL NSs solution was added to the above solution to react at 37 °C for 2 h.

6. Cell Culture.

The CCRF-CEM and HepG2 cell lines were provided by the National Infrastructure of Cell Line Resource (Beijing, China) and were cultured in the cell-specific media. CCRF-CEM cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin. HepG2 cells were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and 1% penicillin. All cells were cultured at 37 °C in a humidified incubator containing 5% CO_2 .

7. Real-Time RT-PCR Analysis of mRNA in Living Cells.

RT-PCR was performed to analyze the expression of VEGF-mRNA. Total RNAs were extracted from NSs or NSs + siRNA treated CCRF-CEM cells, and complementary DNA was generated using the PrimeScript^{RT} reagent kit (Takara). Then the SYBR Select Master Mix was employed according to the manufacturer's instructions on a Bio-Rad IQ5 Thermal Cycler (Bio-Rad, USA). A 20 μ L aliquot of the reaction solution consisted of 2 μ L cDNA, 10 μ L $2 \times$ SYBR Select Master Mix, 2 μ L forward primer (5×10^{-6} M), 2 μ L reverse primer (5×10^{-6} M), and 4 μ L RNase-free water. The PCR conditions were as follows: kept at 95 °C for 5 min to ensure a hot start, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s.

8. *In Vitro* Cytotoxicity Assay.

The standard CCK-8 assay was applied to examine cellular toxicity. In a 96-well plate, HepG2 cells were incubated in 10% FBS-DMEM at 37 °C under a 5% CO₂ atmosphere for 24 h. Varying amounts (from 0.5 to 50 μ g/mL) of NSs were then added into various wells and incubated with HepG2 cells for a further 24 h. Subsequently, 10 μ L of CCK-8 solution was added to each well, followed by 3 h incubation in the CO₂ incubator. Finally, the absorbance at 450 nm was measured using a Bio-Rad 680 microplate reader (USA) to determine the relative cell viability.

9. Cellular Uptake and Confocal Imaging.

CCRF-CEM cells were seeded in RPMI-1640 medium containing 10% FBS overnight at 37 °C and 5% CO₂ in confocal microscopy petri dishes. HepG2 cells were seeded in DMEM containing 10% FBS in confocal microscopy petri dishes overnight at 37 °C and 5% CO₂.

RCA was performed using a 500 μ L of solution containing 120 μ L of dNTPs (10×10^{-3} M), 50 μ L of $10 \times$ mix buffer (splintR ligase reaction buffer: phi29 DNA polymerase reaction buffer at 1:1), 80 μ L of NSs, and 250 μ L of RPMI-1640 medium in a humidified incubator at 37 °C under 5% CO₂ for 4 h. The cells were then washed three times with PBS (pH 7.4) and

incubated with Cy5- Probe and FAM-Probe for 10 min at 37 °C, followed by three washes with PBS (pH 7.4). Finally, the cells were fixed using chilled methanol (1 mL) for 10 min at -20 °C and rinsed three times with PBS. The cells were then incubated with 500 ng mL⁻¹ 4'-6-diamidino-2-phenylindole (DAPI) for cell nuclei counterstaining. Finally, images of the CCRF-CEM and HepG2 cells were recorded by Confocal Laser Scanning Microscope (CLSM).

10. *In Vivo* Imaging and Antitumor Assessment.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Beijing Normal University and Experiments were approved by the Animal Ethics Committee of Beijing Normal University. To establish CCRF-CEM tumor xenograft-bearing models, healthy male BALB/c nude mice aged six weeks (16–20 g) received a subcutaneous injection of 1×10^7 cells/mouse in the right armpit region. Tumor size was monitored by a Vernier caliper, and tumor volume (V) was calculated as $V = \text{Length} \times \text{Width} \times (\text{Length} + \text{Width})/2$. When the tumor volume reached a certain size, the mice were randomly distributed into three groups: PBS, NSs, and NSs + siRNA. Each experimental group contained five mice. The solution of PBS/NSs/NSs + siRNA was subcutaneously injected into the BALB/c nude mice for approximately another 2 weeks. Subsequently, the tumor size and body weight of each mouse were recorded every other day. On the 14th day, some mice were sacrificed, and tumors and vital organs (heart, liver, spleen, lung and kidney) were collected. Next, images of the tumors were taken using a digital camera (Nikon, Japan). Subsequently, the tumors and organs were washed with saline three times and fixed in 4% paraformaldehyde. For the hematoxylin and eosin (H&E) staining and TUNEL assay (Roche, Switzerland), paraffin tumor sections were stained and observed using an inverted fluorescence microscope system (Olympus, Japan). For *in vivo* imaging, NSs with two RCA detection probes were subcutaneously injected into the tumor tissues, and the mice were fed

for approximately another 6 h. Next, fluorescence imaging of the mice was performed with an *in vivo* imaging system under 480 and 640 nm excitation.

Table S1. Oligonucleotide sequences.

Name	Sequences (5'-3')
Padlock Probe	p- <u>CTACTACCTCATTTC</u> CCGGT <u>TACCCACCCAGTCCACCC</u> CACA <u>TTTAACTATAACAAC</u>
SiRNA	GGAGUACCCUGAUGAGAUC-dTdT
FAM-Probe	FAM- AACTATAACAACCTACTACCTCA
Cy5-Probe	Cy5- TACCCACCCAGTCCACCCCACA
VEGF Forward	CGAAACCATGAACTTTCTGC
VEGF Reverse	CCTCAGTGGGCACACACTCC

The underlined portion in sequences of target represented the complementary sequence of miRNA target (Let-7a). The part that draws the wavy line is the aptamer sequence of VEGF.

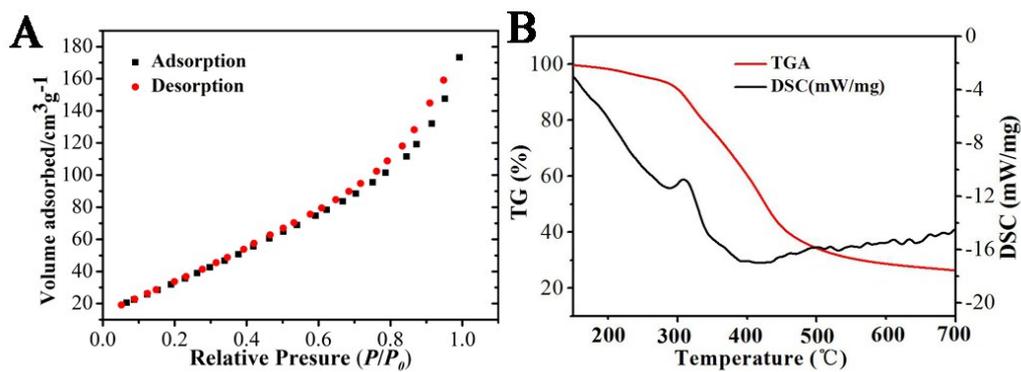


Fig. S1 (A) Nitrogen adsorption-desorption experiment (BET) of mesoporous silicon nanoneedles. (B) Thermal-gravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves of NSs.

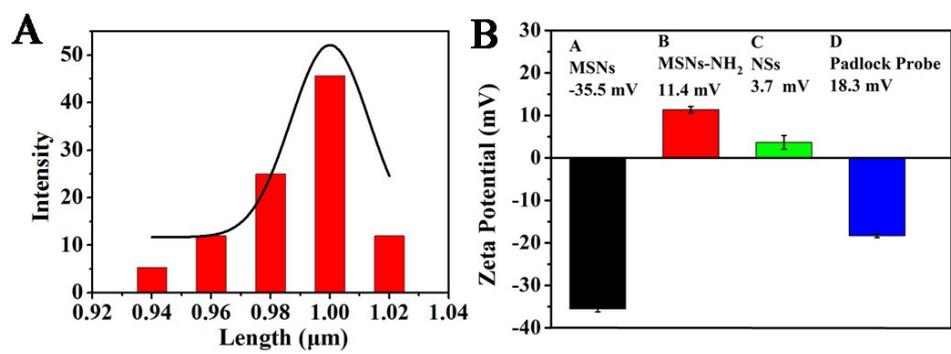


Fig. S2 (A) DLS analysis of the corresponding nanoneedles. (A) Diameter of silicon nanoneedles. (B) Zeta potential of different nanoneedles.

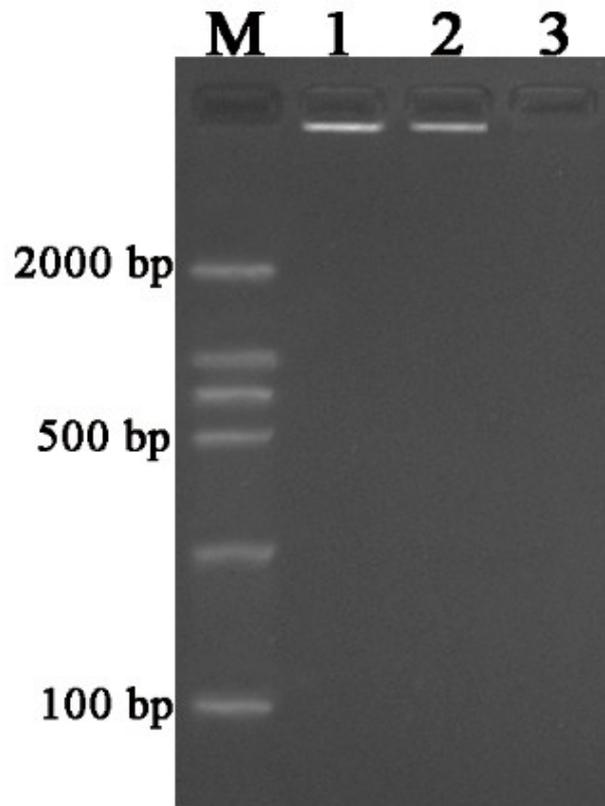


Fig. S3 Comparison between the traditional RCA and our method by agarose gel electrophoresis. M:2000-bp DNA markers; 1: traditional RCA; 2: NSs-RCA; 3: NSs-RCA without Let-7a.

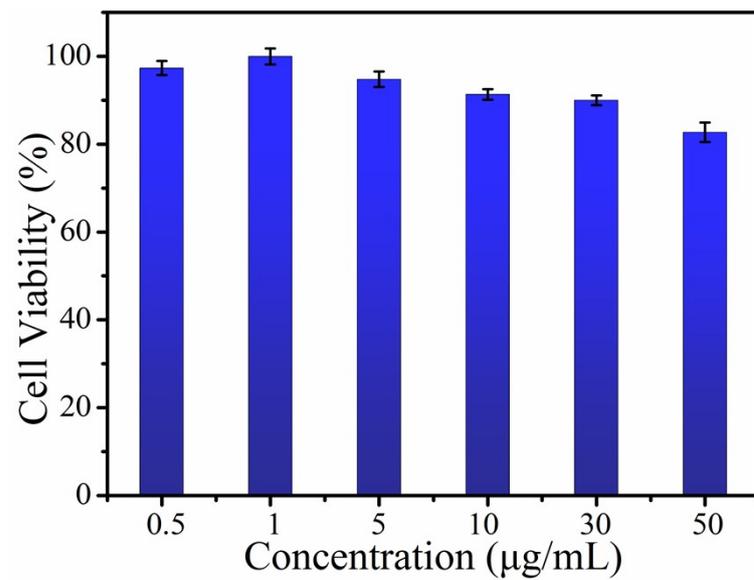


Fig. S4 The CCK-8 assay for *in vitro* cytotoxicity of NSs against HepG2 cells after 24 h incubation.

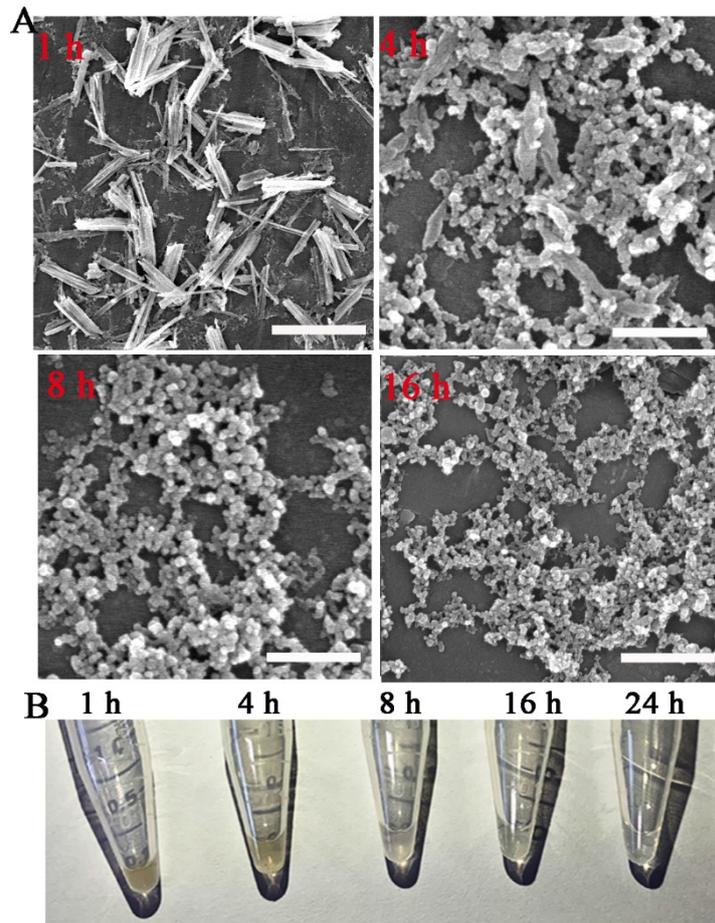


Fig. S5 Time course of NSs incubated in cell-culture medium at 37 °C. Progressive biodegradation of the needles appears, with loss of structural integrity between 4 and 8 h. Complete degradation occurs at 16 h. Scale bars, 1 μm.

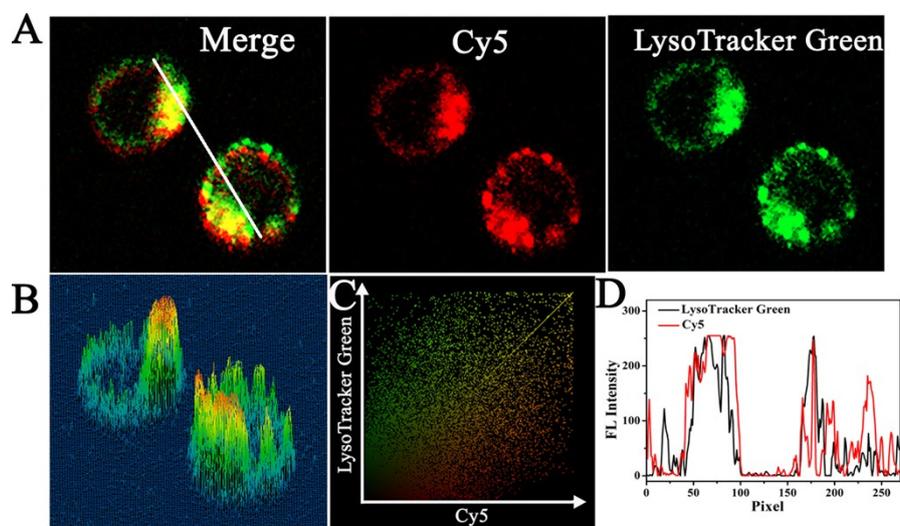


Fig. S6 Colocalization experiments of Cy5 and LysoTracker Green in CCRF-CEM cells. (A) Images for Cy5 and LysoTracker Green. (B) The 3D perspective observation. (C) The correlation between red and green channels. (D) Fluorescence intensity profile of regions of interest (white line) across the lines from left to right.