Supporting Information

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SI Text

SI Materials and Methods. ***Synthesis of nanozymes, gold nanoparticle-oligonucleotide conjugates DNA-NPs, and gold nanoparticle-RNase A conjugates RNase-NPs. Materials.*** Thiol-modified antihepatitis C virus (anti-HCV) DNA oligonucleotides were purchased from Bio-synthesis Inc. RNase A (ribonuclease A from bovine pancreas), RNase-free buffers, and chemicals were ordered from Sigma-Aldrich.

Gold nanoparticle synthesis. Citrate-stabilized gold nanoparticles (13 ± 1 nm in diameter Fig. S1*A*) were prepared according to literature procedures (1, 2).

Synthesis of DNA-NPs. Gold nanoparticles (10 nM, 13 ± 1 nm in diameter, see Fig. S1) were mixed with alkylthiol-modified oligonucleotides with the anti-HCV or control sequence (Fig. 1 in main text, 6.4 nmol), and phosphate buffer (1.0 M, pH 7.4) was added to bring the mixture solution to 10 mM phosphate. After 8 h, a sodium chloride solution (NaCl, 1.5 M solution in RNase-free water) was added to bring the NaCl concentration gradually to 0.3 M during a period of 32 h. The solution was further shaken for another 8 h. Then the resulting DNA-NPs were purified using centrifugation (13,000 rpm, 20 min, three times), and were redispersed in RNase-free water for use.

Synthesis of RNase A-NPs. Gold nanoparticles (10 nM, 13 ± 1 nm in diameter, see Fig. S1) were mixed with RNase A (1.5 μM) in a carbonate buffered solution (2 mL; carbonate, 10 mM; pH 9.6.) (3, 4). The mixture solution was shaken for 30 min, and then the resulting RNase-NPs were purified using centrifugation (13,000 rpm, 15 min, two times) and were redispersed in RNase-free water.

Dynamic light scattering (DLS) analysis. The DLS assays of 13-nm gold nanoparticles, 12-RNase anti-HCV nanozymes, 12-RNase anti-HCV nanozymes with 29-DNA oligonucleotides (denoted as NZ-Ls), DNA-NPs, and RNase-NPs were carried out using a DLS spectrometer (Brookhaven Instruments Corporation). The results are shown in Table S1.

Determination of the number of RNase A and oligonucleotide loaded onto gold nanoparticles. RNase A loading determination. The average number of RNase molecules loaded onto a single nanozyme was determined by a subtraction method. The total amount of RNase molecules loaded onto gold nanoparticles in a synthesis batch was determined by subtracting the amount of unloaded RNase molecules from the amount of RNase molecules added initially. This total loading amount was then divided by the total number of nanozymes in the solution, yielding the average number of RNase A per single nanozyme. The number of nanozymes was determined by using UV-Vis absorption spectroscopy ($\lambda = 524$ nm, $\varepsilon = 2.0 \times 10^8\ M^{-1}\,cm^{-1}$). The amount of unloaded RNase in a reaction solution was determined by measuring the RNase activity of the supernatant after removal of nanozymes. A typical RNase activity measurement was performed according to the literature method, in which cytidine-2′,3′-phosphate was used as the substrate (5). Then the amount of RNase molecules was obtained using a standard RNase activity curve. Initial reaction rate as a function of RNase concentration: 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, and 4.0 μg/mL were determined experimentally; see Fig. S7. Note that all the vials and tubes used in this experiment were modified by silane for minimizing the nonspecific binding of RNase A onto the surface of these glass containers.

DNA loading determination. The number of nanozymes or DNA-NPs were determined using UV-Vis spectroscopy ($\lambda = 524$ nm, $\varepsilon = 2.0 \times 10^8\ M^{-1}\,cm^{-1}$) (6). DNA oligonucleotides were released from nanozymes or DNA-NPs by dissolving their gold nanoparticle backbones in 0.1 M KCN solution. The number of DNA molecules per nanoparticle was determined using an oligonucleotide quantification kit (Oligreen, Invitrogen) following the manufacturer's recommendations. The DNA loading number was calculated by dividing the concentration of oligonucleotides by the concentration of gold nanoparticles (Fig. S8).

Determination of nanozyme activity in vitro. Synthesis of RNA substrates using in vitro transcription. The pJFH1 plasmid was a gift from Dr. Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan). (7). The human α-1 antitrypsin (AAT) gene (GenBank: BT019455.1) was amplified from a patient liver tissue and cloned into the pEF6/V5-His-TOPO vector (Invitrogen). The expression vector pTOPO-AAT was sequenced using the BigDye Terminator V3.1 Kit from Applied Biosystems. The pJFH1 was cut by using Cla I, and the pTOPO-AAT was cut by Xba I. The resulting linearized DNA plasmids were purified and used as the templates for in vitro transcription to make the HCV RNA segment (nucleotides 1–1149) or the 1,257-nt AAT RNA using a MEGAscript T7 kit (Ambion).

Proteinase K resistance tests. In a typical proteinase K resistance test, nanozymes (0.034 nM) or particle-free RNase A (0.408 nM) were first incubated with proteinase K (10 nM) in a PBS buffer (pH 7.4) at 37 °C for 1 h. Then the product of this proteinase K treatment was divided into two parts and further incubated with the HCV (or AAT) RNA (0.12 μM) in a PBS buffer (11 μL; pH 7.4) at 37 °C for 15 min. The products were analyzed using electrophoresis in a 2% formaldehyde agarose gel as described in the section of *Materials and Methods* in the main text.

Evaluation of the toxicity and cellular uptake of nanozymes. Cell proliferation assay. FL-Neo cells were dispensed into 96-well plates at a final concentration of 3×10^3 cells/well in a culture medium (100 μL), and incubated overnight before treatment. The culture medium was then removed and replaced with new medium with the anti-HCV nanozyme of varying concentrations (0.034, 0.068, 0.14, 0.27, and 0.54 nM). After 72 h treatment, cell viability was measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] cell proliferation assay (Promega). The cells were incubated for 3 h after addition of MTS/PMS (phenazine methosulfate) solution to allow for color development, and then the absorbance values were read at 492 nm using a Multiscan plate reader.

Cellular uptake. In a typical experiment, anti-HCV nanozymes (0.034, 0.14, and 0.54 nM) with Dulbecco's modified Eagle medium (DMEM, 2 mL) were added into the cells (30% confluence) cultured in two six-well plates, respectively. After incubation for 3 d, culture media were removed, and the treated cells were washed with phosphate buffered saline (1 × PBS) three times. Afterwards, the cells were detached from the six-well plates using trypsin (1.0 mL, 0.25%) and transferred into glass vials. The number of cells in each well was counted using a hemocytometer (8).

Then, the cells were lysed under sonication (Branson 2510, 37 °C) for 1 h, and clear solutions were obtained. Au nanoparticles in these solutions were dissolved using freshly made aqua regia (0.3 mL), and resulting solutions were then diluted by 50-fold using a solution containing 2% HNO_3, 1% HCl, and 8 ppb Rhenium (internal standard), and the concentrations of gold ions in these solutions were determined using inductively coupled plasma mass spectrometer (ICP-MS, Element 2 ICP-MS, Thermo Scientific, USA). The concentration of Au nanoparticles in each well was calculated using the Au ion concentration and the size of gold nanoparticles determined using TEM. Finally, the number of gold nanoparticles per cell in each well was calculated using the number of cells in the corresponding well (9, 10).

Analysis of the HCV and G1P3 RNAs, and HCV-NS5A proteins. RNA extraction and quantitative reverse transcribed real-time PCR (qRT-PCR) analysis for the RNA levels of HCV, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and G1P3. RNA samples were extracted from FL-Neo cells or mouse tumor tissues using an RNA isolation reagent (TRIzol; Invitrogen). To prevent DNA contamination, total RNA was treated with RNase-free DNase II (Invitrogen). Total RNA samples (2 μg per reaction) were reversely transcribed into cDNAs by RT II reverse transcriptase (Invitrogen). Then, the cDNAs were used as templates in quantitative real-time PCR with HCV 3′ NTR gene-specific primers, [i.e., forward primer (FP): 5′-CCTTCTTTAATGGTGGCTCCAT-3′, nucleotides 9538-9559; reverse primer (RP): 5′-GGCTCACGGACCTTTCACA-3′, nucleotides 9582-9600; Probe 5′-TTAGCCCTAGTCACGGCT-3′, nucleotides 9561-9578]. The amplification reactions were performed using TaqMan RT-PCR on a StepOne Plus real-time PCR system (Applied Biosystems). The primers for G1P3 gene were 5′-CAAGCTTAACCGTTTACTCGCTGCTGT-3′ (Forward) and 5′-TGCGGCCGCTGCTGGCTACTCCTCATCCT-3′ (Reverse, see ref. 11). The human GAPDH was used as an internal control in PCR amplification, and its primers were 5′-TCACCAGGGCTGCTTTTA-3′ (FP) and 5′-TTCACACCCATGACGAACA-3′ (RP). The PCR conditions were as follows: 10 min at 95 °C and 40 cycles of 15 s at 95 °C, 30 s at 58 °C (GAPDH or G1P3) or 60 °C (HCV), and 30 seconds at 72 °C, with an extension for 10 min at 72 °C.

Western blot analysis of HCV-NS5A proteins. Cell lysates were prepared by treating samples with ice-cold lysis Tris-HCl buffer (20 mM, pH 7.8; NP40, 10%; glycerol, 10%; NaCl, 137 mM; EDTA, 10 mM), and a protease inhibitor cocktail (Roche Applied Science) for 20 min on ice followed by centrifugation at 4 °C for 15 min to sediment particulate materials. Proteins then were separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose membranes, and then blocked with 5% skim milk in phosphate-buffered saline. Mouse anti-HCV NS5A antibody was used as the primary antibody (1:250) and peroxidase-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) was used as the secondary antibody (1:1,000). Beta-actin was detected by an antibody (1:8,000, clone AC-74, Sigma-Aldrich) and used as a loading control. Signals were detected by using the Supersignal West Pico chemiluminescent substrate (PIERCE) according to the manufacturer's directions (12).

Immunofluorescence imaging of HCV-NS5A proteins. FL-Neo cells were transferred onto glass cover slips and fixed with 5% acetic acid in ethanol. The cells were washed with phosphate-buffered saline and incubated with monoclonal antibody to HCV NS5A protein for 1 h. The secondary antibody was FITC-labeled goat anti-mouse immunoglobulin G antibody. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc.), followed by examination under a fluorescence microscope (Nikon TE-2000 microscope, Nikon (13).

Antiviral effects of nanozyme in FL-Neo cells on both the HCV RNA and viral protein expressions. FL-Neo cells were grown in DMEM, supplemented with 10% fetal bovine serum, 200 μmol/L L-glutamine, 10 mM nonessential amino acids, and antibiotics at 37 °C in 5% CO_2. FL-Neo cells were seeded onto 35-mm wells of a six-well cell culture plate and cultured overnight. The FL-Neo cells were treated on day 1, day 3, and day 5 with fresh DMEM containing 0.068 nM nanozymes (or DNA-NPs) when growth media were changed. Control cells were incubated only with the culture medium. Treatment ended on day 7 when the cells were harvested, divided into three parts, and then processed for qRT-PCR analyses, Western blot analyses, and single-cell level immunofluorescence imaging (see below for technique details on these analyses).

Evaluation of nanozyme activity in vivo. Antiviral effects of nanozyme in the HCV-Infected xenotransplantation mouse model. When tumors reached 300–500 mm^3, mice (each mouse approximately 22 g) were randomly divided into four groups. One group was set up as controls without any treatment. Another three groups were injected subcutaneously on day 1, day 3, and day 5 with nanozymes, control nanozymes, or DNA-NPs (sterile PBS, 100 μL; 0.34 pmol). Treatment ended on the seventh day when the mice were sacrificed and processed for HCV RNA assay using qRT-PCR. The human GAPDH was used as an internal control for HCV RNA and G1P3 mRNA levels in PCR amplification (12). In these experiments, mice were bred and maintained in microisolators under pathogen-free conditions. All experimental procedures were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the committee on the Animal Care Service of the University of Florida (Permit Number: 200801081).

Toxicity of nanozyme in the xenotransplantation mouse model. Toxicity in vivo was evaluated using the following two methods: The first method was the observation of mouse body weight and general appearance (such as activities, fur, and appetite, etc.) every 12 h (14). There was no sign of toxicity during the treatment with anti-HCV nanozymes and DNA-NPs. The second one was to examine the liver tissue by histology (14). No appearance of hepatocellular damage nor cholestasis was observed in the mice treated three times with anti-HCV nanozymes and DNA-NPs after 7 d at the dose of 0.34 pmol per injection. Taken together, these results show that anti-HCV nanozymes did not display toxicity to the xenotransplantation mouse model under treatment conditions used in this study.

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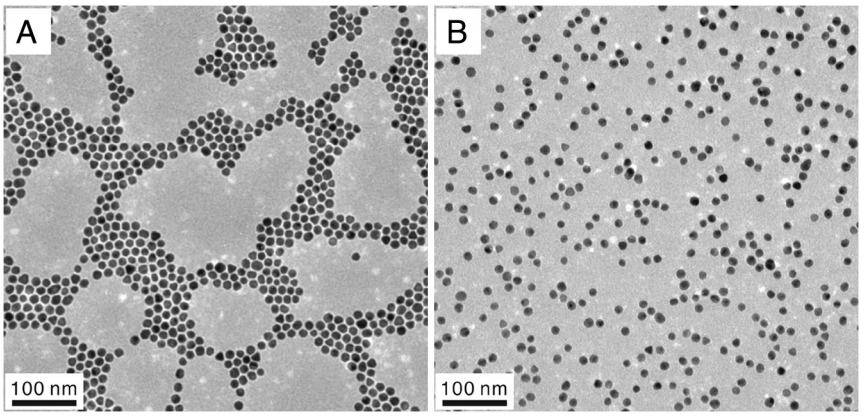

Fig. S1. TEM images of citrate-stabilized gold nanoparticles (*A*) and nanozymes (*B*) taken on a JEOL 200CX operated at 200 kV.

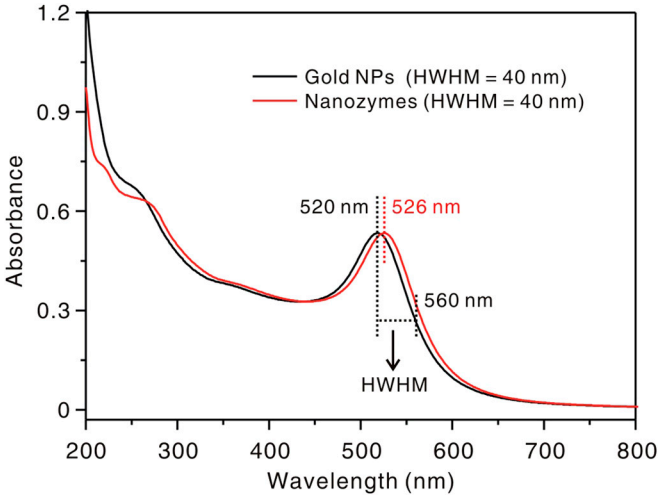

Fig. S2. UV-Vis spectra of citrate-stabilized gold nanoparticles (black) and nanozymes (red). HWHM, half width at half maximum.

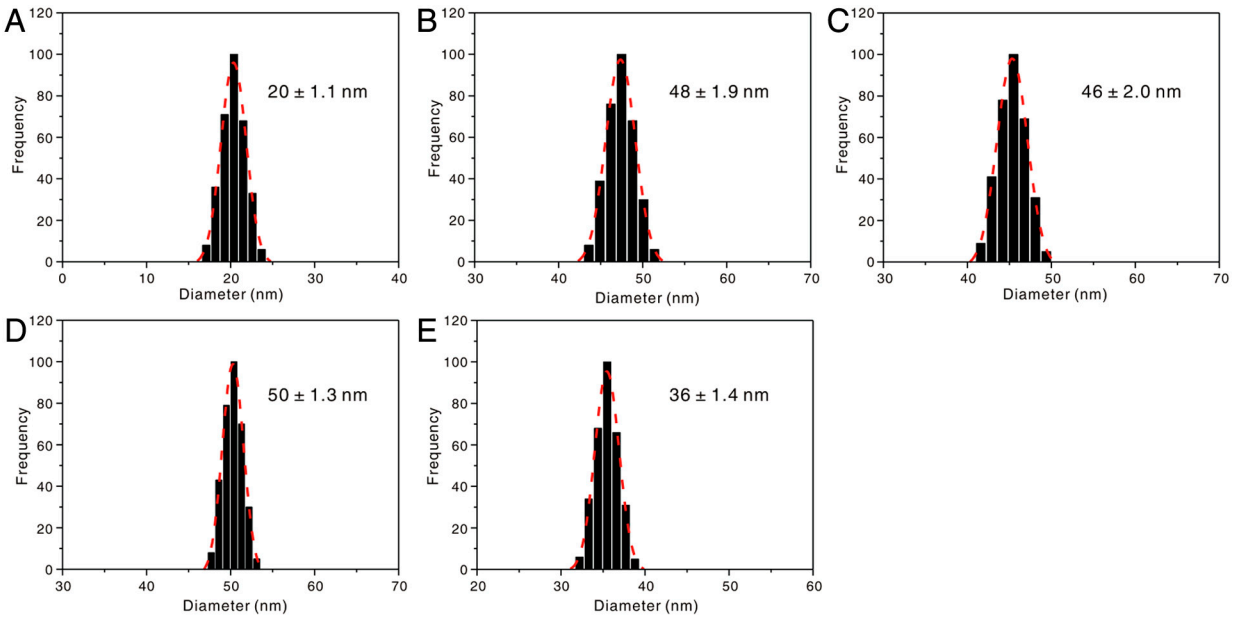

Fig. S3. Dynamic light-scattering data of nanoparticles: (*A*) unmodified gold nanoparticles, (*B*) nanozymes, (*C*) NZ-Ls, (*D*) DNA-NPs, and (*E*) RNase-NPs.

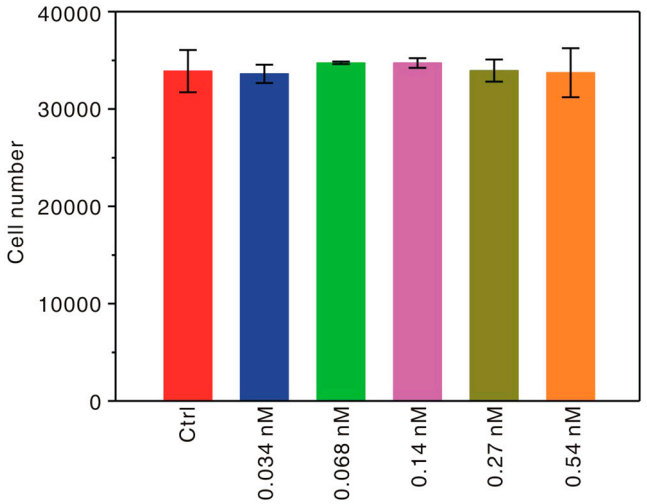

Fig. S4. Viability of FL-Neo cells treated with anti-HCV nanozymes at different concentrations: 0, 0.034, 0.068, 0.14, 0.27, and 0.54 nM (see *SI Materials and Methods* for details). Ctrl, blank control.

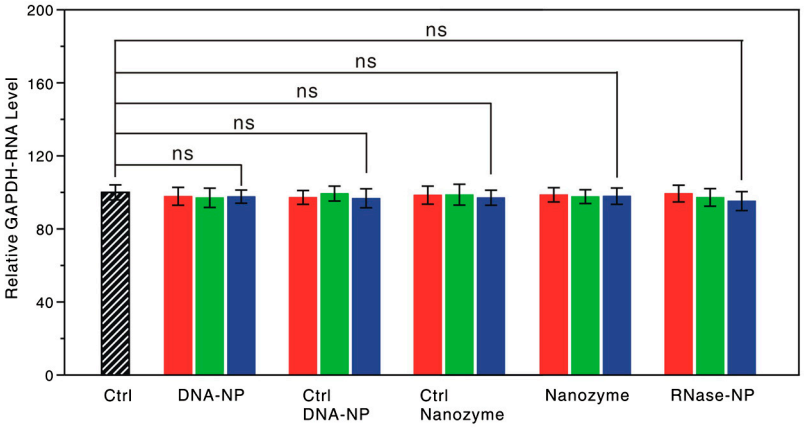

Fig. S5. GAPDH RNA levels in the FL-Neo cells treated with the DNA-NPs, control DNA-NPs, control nanozymes, anti-HCV nanozymes, and RNase-NPs at concentrations of 0.034 (in red), 0.14 (in green), and 0.54 nM (in blue, also see *SI Materials and Methods* for details). Ns, nonsignificance, $P > 0.30$.

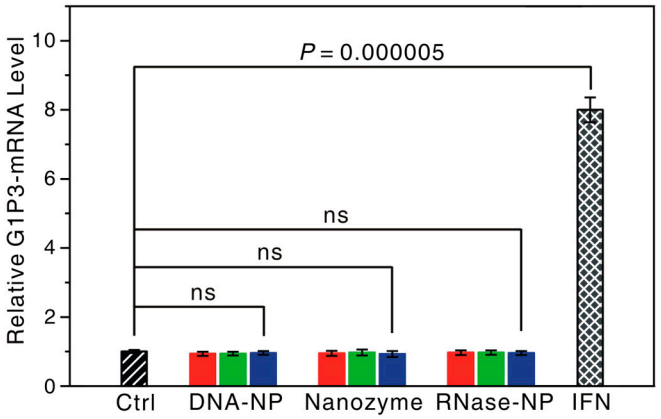

Fig. S6. G1P3 mRNA levels in the FL-Neo cells treated with the DNA-NPs, nanozymes, and RNase-NPs at concentrations of 0.034, 0.14, and 0.54 nM, and IFN α at concentrations of 10 U/mL. G1P3 mRNA levels were relative to that of human GAPDH. Please see *SI Materials and Methods* for details. Ns, nonsignificance, $P > 0.38$.

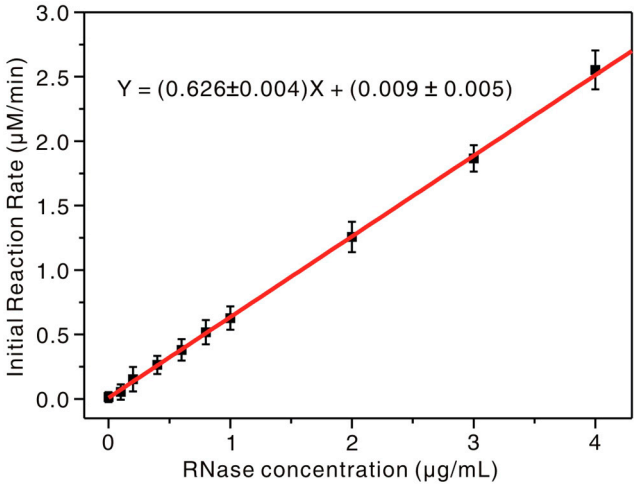

Fig. S7. Plot of initial reaction rate as a function of RNase A concentration for hydrolysis of cytidine 2′,3′-phosphate. The substrate concentration was 0.1 mg/mL.

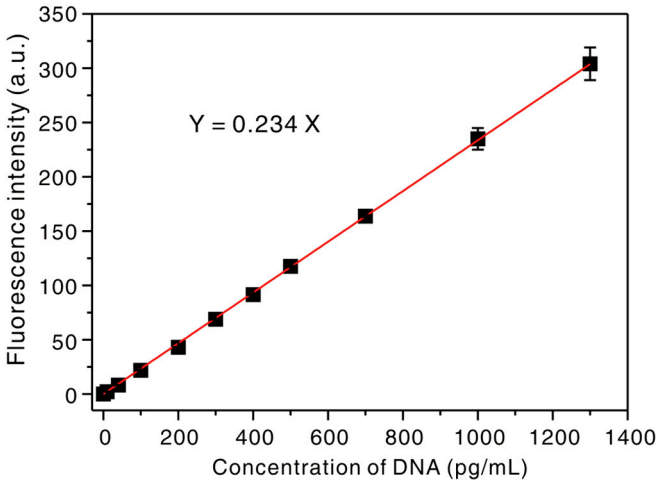

Fig. S8. Plot of fluorescence intensity as a function of DNA concentration.

Table S1. Zeta potentials and hydrodynamic diameters of Au nanoparticles (NPs), nanozymes, DNA-NPs, and RNase-NPs

Sample	RNase number on each NP	DNA number on each NP	Zeta-potential (mV)	Hydrodynamic diameter (nm)
Au NPs	0	0	−15 ± 2.3	20 ± 1.1
Nanozymes	12.1 ± 0.5	78 ± 4	−14 ± 1.1	48 ± 1.9
NZ-Ls	12.3 ± 0.8	29 ± 3	−11 ± 2.3	46 ± 2.0
DNA-NPs	0	110 ± 4	−28 ± 1.7	50 ± 1.3
RNase-NPs	44.3 ± 0.9	0	−7 ± 1.9	36 ± 1.4

Table S2. Determination of the average RNase A and DNA loading number per nanozyme synthesized at varying concentration ratios of Au NPs, RNase A, and alkylthiol-functionalized DNA strands (see the section of determination of the number of RNase A and oligonucleotide loaded on gold nanoparticles in *SI Materials and Methods* for details)

Sample	Initial mole ratio NP:RNase:DNA	DNA sequence	The number of RNase A per NP	The number of DNA strands per NP
Nanozyme-1	1:10:300	Anti-HCV	2.4 ± 0.3	100 ± 2
Nanozyme-2	1:30:300	Anti-HCV	7.3 ± 0.2	90 ± 3
Nanozyme-3	1:50:300	Anti-HCV	12.1 ± 0.5	78 ± 4
NZ-L	1:40:60	Anti-HCV	12.3 ± 0.8	29 ± 3
Control nanozyme	1:50:300	Control DNA	12.0 ± 0.5	80 ± 3

Table S3. Determination of the average DNA and RNase A loading number per Au NPs at varying concentrations of RNase A and DNA used in syntheses of the DNA-NPs and RNase-NPs (see the section of determination of the number of RNase A and oligonucleotide loaded on gold nanoparticles in *SI Materials and Methods* for details)

Sample	Initial mole ratio	DNA sequence	The number of RNase A per NP	The number of DNA strands per NP
DNA-NPs	NP:DNA = 1:300	Anti-HCV	0	110 ± 4
Control DNA-NPs	NP:DNA = 1:300	Control	0	108 ± 5
RNase-NPs	NP:RNase = 1:150	—	44.3 ± 0.9	0

Table S4. Cellular uptake of anti-HCV nanozymes at different concentrations added to cell growth media (see the section of evaluation of the toxicity and cellular uptake of nanozymes in *SI Materials and Methods* for details)

Concentration of nanoparticles added to cell growth media	0.034 nM	0.14 nM	0.54 nM
Average no. of DNA-NPs found in each cell	$(3.0 \pm 0.2) \times 10^3$	$(1.5 \pm 0.1) \times 10^4$	$(5.1 \pm 0.3) \times 10^4$
Average no. of nanozymes found in each cell	$(2.9 \pm 0.1) \times 10^3$	$(1.3 \pm 0.1) \times 10^4$	$(4.6 \pm 0.2) \times 10^4$
Average no. of control DNA-NPs found in each cell	$(3.2 \pm 0.1) \times 10^3$	$(1.4 \pm 0.2) \times 10^4$	$(5.0 \pm 0.2) \times 10^4$
Average no. of control nanozyme found in each cell	$(2.8 \pm 0.1) \times 10^3$	$(1.3 \pm 0.2) \times 10^4$	$(4.8 \pm 0.3) \times 10^4$
Average no. of RNase-NPs found in each cell	$(3.2 \pm 0.2) \times 10^3$	$(1.4 \pm 0.1) \times 10^4$	$(4.9 \pm 0.3) \times 10^4$