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Detection of single-digit foodborne pathogens with the naked eye using carbon nanotube-based multiple cycle signal amplification

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Experimental

Materials and reagents.

1-(3-(Dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS), horseradish peroxidase (HRP), 2-morpholineethanesulfonic acid monohydrate (MES), and concanavalin A (Con A) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). EDC and NHS were dissolved in water immediately prior to use. Anti-*E.coli* O157:H7 monoclonal antibody (mAb) as capture antibody was obtained from Kirkegaard & Perry Laboratories, Inc.(U.S.A.), and biotin-anti-HRP antibody were purchased from Abcam (Hong Kong) Ltd. Avidin-HRP was purchased from Life Technologies Co. (U.S.A.). Multiwalled carbon nanotubes (MWCNTs, chemical-vapor deposition (CVD) method, purity ≥ 97%, diameter 20-40 nm, length 1-2 μm, and special surface area 100～120 m^2/g) were purchased from Nanoport Co. Ltd. (Shenzhen, China). Bacteria DNA magnetic purification kits were purchased from Land-Bridge Technology Co.,Ltd. (Beijing, China).

Ultrapure water obtained from a Millipore water purification system (≥ 18 MΩ, Milli-Q, Millipore) was used in all assays. All other reagents, including H_2O_2 and

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tetramethylbenzidine (TMB) were of analytical grade and used as received. Phosphate buffered saline (PBS, 0.1 M, pH 7.4) was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4.

Bacteria and Culture Plating Methods. *E.coli* O157:H7 (ATCC 43888), *Salmonella* (ATCC 1111), were purchased from American Type Culture Collection (ATCC, Rockville, MD). The lyophilized cells were rehydrated with tryticase soy broth (TSB, BD Diagnostics), plated on the tryticase soy agar (TSA, BD Diagnostics) and grown at 37 ºC for 24 h prior to use. The viable cell number was determined by a microbial plate count method. 1 mL of dilutions were plated on the TSA and incubated at 37 ºC for 24 h, and the resulting colonies were counted to determine the number of colony forming units per milliliter (CFU/mL). The bacterial suspensions were individually prepared by picking the colonies on TSA and serially diluted to concentrations from 10^1 to 10^6 CFU/mL with physiological saline solution for the specificity and sensitivity study. The concentration of dilution was regarded as the level of 10^2 CFU/mL when 80-120 colonies were grown on the TSA plates.

Preparation of HRP-CNTs-ConA conjugates. The HRP-CNTs-ConA bioconjugates were prepared based upon a modified literature protocol.[1-4] 40 mg of MWCNTs were functionalized and treated with 50 mL H_2SO_4/HNO_3 (3:1, v/v) for 6 h with continuous ultrasonication at 70 ºC. The resulting dispersion was centrifuged, washed with ultrapure water several times until its pH adjusting to 7.0, followed by drying to obtain functionalized MWCNTs with hydrophilic carboxylate groups. A 0.2 mg of the oxidized, shortened MWCNTs was resuspended in a 2 mL of 100 mM MES buffer (pH 6.0), containing 100 mM NHS, and 100 mM EDC, 0.2% Trition-X100 solution, and sonicated for 1 h at room temperature. The resulting mixture was centrifuged at 12,000 rpm for 5 min, and the supernatant was discarded, followed by washing twice with PBS buffer to the unreacted materials. The final precipitate was resuspended in 2.0 mL of 10 mM pH 8.0 Tris-HCl solution. Afterward, the ConA and HRP were added to a final concentration of 5 μM and 5 U/mL, respectively. The reaction was

allowed to continue for 4 h at room temperature, and then kept in a refrigerator at 4 ºC overnight. After this step, the reaction mixture was washed with Tris-HCl solution during several centrifugation cycles at 12,000 rpm at 4 ºC to remove free ConA and HRP. Subsequently, the as-prepared bioconjugates precipitate was dispersed in Tris-HCl buffer and stored at 4 ºC before usage.

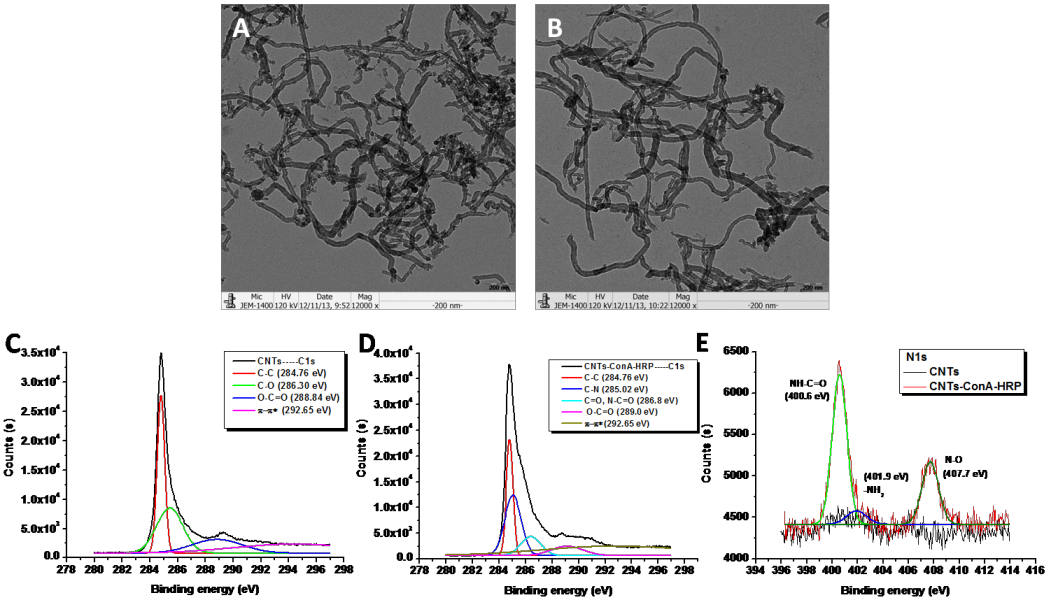

Fig S1. TEM images of carboxylic CNTs (A) and ConA-CNTs-HRP bioconjugate (B); (C-E) XPS spectra of carboxylic CNTs and ConA-CNTs-HRP bioconjugate: C1s spectrum of carboxylic CNTs (C) and ConA-CNTs-HRP bioconjugate (D); N1s spectrum of carboxylic CNTs and ConA-CNTs-HRP bioconjugate (E).

Figure S1C shows C1s XPS of carboxylic CNTs. The spectrum shows one large peak at 284.8 eV which can be ascribed to the bulk carbon, and one smaller peak at 288.8 eV which can be ascribed to carboxylic group. The corresponding N(1s) spectrum shows no signal above the detection limit of the instrument, even with extensive signal averaging (black curve in Fig S1E). After conjugation with proteins, the modified tubes were characterized by XPS after briefly warming to 75°C in an ultrahigh vacuum to remove any residual physically adsorbed proteins. Compared to the main bulk C1s at 284.8 eV, the resulting C1s photoelectron spectrum shows some broadening of the bulk peak (Figure S1D). We notice that there is no significant intensity near 288.8 eV. The absence of intensity at 288.8 eV is important because the C1s binding energy of carboxylic groups is expected to decrease significantly when a

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carboxylic acid group is converted to a carbonyl amide, which is at 286.8 eV. Therefore, the changes observed in the C1s spectrum support the formation of a carbonyl amide linkage to the nanotubes. The N1s spectrum shows a peak with a binding energy of 400.6 eV which is consistent with the formation of the amide bonds between proteins and carboxylic CNTs (red curve in Figure S1E).

Multiple cycle immunoassays for pathogens. The immunoassay of *E.coli* O157:H7 using HRP-CNTs-ConA conjugates were performed as follows: 96-well polystyrene plates (Costar) were modified with 50 μL of anti-*E.coli* O157:H7 monoclonal antibody (diluted to 5 μg/mL in 0.02 M Tris-HCl buffer at pH 7.4) at 4 ºC overnight. After washing the plates three times with wash buffer (0.01 M PBS buffer and 0.05% Tween 20 at pH 7.4), the plates were blocked with blocking buffer (5 mg/mL BSA in PBS) for 1 h at 37 ºC. Then, the plates were washed three times with wash buffer, and 50 μL of sample solution was added to each well. After 1 h at 37 ºC, the plates were washed three times with wash buffer. Subsequently, 50 μL of HRP-CNTs-ConA bioconjugates (diluted 10-fold with 0.01 M pH7.4 PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}) was added to each well, and incubated at 37 ºC for 1 h and washed three times with wash buffer. Then, 50 μL of biotin-anti-HRP antibody diluted 1:2000 in PBS buffer was added for 30 min at room temperature. The plates were then washed twice, and 50 μL of avidin-HRP diluted 1:10000 in PBS buffer was added for 10 min at room temperature. The multiple cycle experiments repeatedly using the biotin-anti-HRP antibody and avidin-HRP were performed in a same fashion. Finally, color development was produced using H_2O_2 and tetramethylbenzidine (TMB). The reaction was stopped with 2 M H_2SO4, and absorbance was determined at 450 nm by a Multiskan Ascent 354 microplate reader (Thermo, USA).

Detection of *E.coli* O157:H7 using Real time-PCR.[5] 25 g of meat sample was stomached for 2 min and cultured overnight at 37 ºC in 225 mL of TSB medium. DNA purification from medium was performed following the protocol of bacteria DNA magnetic purification kits. Then, the real time-PCR (RT-PCR) for detection of

E.coli O157:H7 was performed according to SN/T 1870-2007 using Applied BiosystemsViiA™ 7 Real-Time PCR System (Life Technologies, USA).[5]

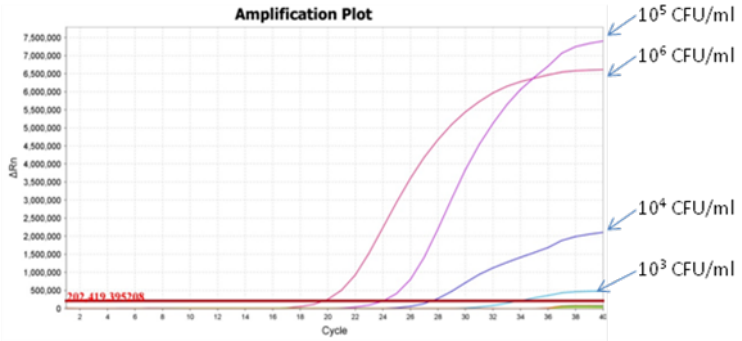

Fig S2. Amplification curve of RT-PCR assay for *E.coli* O157:H7 detection.

Table S1. Analytical performance of typical detection methods for *E. coli* O157:H7

Detection technique	Sample matrix	Analysis time	Detection limit (CFU/ml)	Ref
ELISA	Ground beef	~24h	10^3	6
PCR-ELISA	Milk	5h	10^3	7
RT-PCR	Ground beef	~4h	10^3 cells/g	8
SPR sensor	Culture	<60min	10^3	9
Electrochemical sensors	Milk	~30min	10^3	10
QCM sensors	PBS	~50min	10^3	11
Cantilever sensors	Ground beef	Not given	50 cells/ml	12
Our methods	Meat	~3h for 30 samples	10^2	

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