CHAPTER 3 MATERIALS AND METHODS

3.1 Apparatus and Instruments

Incubator shaker (Innova 4340, New Brunswick Scientific, Co.Inc, Germany) was used for incubation of *S. enterica* serovar Typhimurium. A centrifuge (model micro 120 Hettich, Germany) was used for sample preparation. EPSON flatbed scanner was used to scan the membrane. Field emission scanning electron microscopy (FESEM) analysis was conducted on a Hitachi S-4700 system (Japan) and a JOEL model JAM-2100 was used for SEM and TEM, respectively. Nitrocellulose membrane was from GE healthcare.

3.2 Materials and Chemicals

Tetracloroauric acid ($HAuCL_4.3H_2O$), tween-20, protein A and streptavidin were purchased from Sigma. Sodium borohydride ($NaBH_4$) were purchased from VWR (England). Sodium citrate dihydrate, Silver Enhancer solution (A and B) and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich (Germany). Sodium chloride (NaCl), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), nutrient broth media, skim milk and glycerol were purchased from Merck. Sodium acetate anyhydrous ($C_2H_3O_2N_2$), 1-ethyl-3(-3dimethylaminopropyl carbodimide (EDC) were purchased from Fluka. Bovine serum albumin (BSA) was from PAA (Austria). Goat anti *Salmonella,* rabbit anti-goat IgG (H&L), Horseradish Peroxidase (HRP) conjugate secondary antibody, goat anti *Salmonella sp* functionalized with biotin were purchased from Genway (USA), Multiwalled carbon nanotubes (MWCNTs) carboxylic acid fungtionalized (MWCNTs-COOH) was purchased from Cheap Tube Inc, (USA) and Ted Pella Inc (USA), Streptavidin functionalized magnetic beads was from Invitrogen (USA). All reagents were of analytical grade and were used as received. All solutions were prepared with Milli-Q water.

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3.3 Procedures

3.3.1 Bacterial preparation

Salmonella enterica serovar Typhimurium was cultured in 20 mL of nutrient broth and incubated at 37 ^{0}C overnight with shaking at 200 rpm. 1 mL of the culture was transferred into 100 mL nutrient broth media and incubated at 37 ^{0}C for 16 h with shaking at 200 rpm. 1 mL of cell culture was centrifuged at 13 000 rpm for 15 min and supernatant was removed. Then, the pellet were washed 3 times with 0.15 M phospate buffer, pH 7.4 Finally the pellet was resuspended in 1 mL of phospate buffer and kept at 4 ^{0}C until used.

The amount of cells was estimated after enrichment by spread appropriately diluted cultures on nutrient agar to determine colony forming unit per milliliter (CFU/mL). 0.9% saline solution was prepared for 10-fold serial dilution. The cell numbers were determined by spread plating of 0.1 mL of a proper dilution on nutrient agar. After incubation for 24 h at 37 ^{0}C, the numbers of colony were counted to determine colony forming units per millilitre (CFU/mL).

1 mL of culture was heated at 100 ^{0}C for 10 min to break bacterial cell wall with addition of 10 µL of 1 mM of disodium-tetra diamine acetic acid (EDTA) pH 8.0 to increase

accessible the outer core LPS. After heat killed, cells were cooled down and kept at -20 ^{0}C until used. The culture was sonicated for 10 minutes before used.

3.3.2 Preparation of multiwall carbon nanotubes/gold nanoparticles (MWCNTs/AuNPs) nanocomposite

Multiwalled carbon nanotube/gold citrate nanocomposites: 3 mg of multiwall carbon nanotubes (MWCNTs) were added into 4 ml of sodium citrate solution (1% wt) and exposed to ultrasonic treatment for 5 min for well dispersion. Then, MWCNTs suspension was transferred to 96 mL of milli-Q water. The diluted suspension was heated to the boiling point while stirring, and then 0.5 mL (1 wt%) $HAuCl_4$ was injected. The suspension was kept boiling for 5 min until the color of solution turned red. After that the MWCNTs were separated by membrane filter (pore diameter is 0.2 μm) and washed 3 times by centrifugation at 10 000 rpm for 5 min each. The MWCNTs were resuspended in 500 μL milli-Q water.

Multi walled carbon nanotube/gold borohydride nanocomposite: 3 mg of multiwall carbon nanotube (MWCNTs) were added in 4 mL sodium borohydride solution (1 wt%) and exposed to ultrasonic treatment for 5 min to make MWCNTs disperse well. Then, carbon nanotube suspension was transferred to 96 mL cold milli-Q water. The diluted suspension was cooled while stirring, and then 0.5 mL (1 wt%) $HAuCl_4$ was injected. The suspension was kept stirring for 1 h until the color of solution turn dark purple. After that the MWCNTs were separated by membrane filter (pore diameter is 0.2 μm) and washed 5 times by centrifugation at 10 000 rpm for 5 min each. The MWCNTs were resuspended in 500 μL milli-Q water.

3.3.3 Immobilization of antibody on MWCNTs/AuNPs

Electrostatic attachment method: 500 μL MWCNTs/AuNPs (1.5 mg/mL in 10 mM phosphate buffer pH 6) was mixed with 0.5 μL of anti-*S. enterica* serovar Typhimurium polyclonal antibody (final concentration 5 μg/ml) and was shaken at RT for 1 h. Then, 100 μL of 10% BSA (in milli-Q water) was added to block the MWCNTs/AuNPs, and the solution was shaken at RT for 30 min. After that, the MWCNTs/AuNPs were separated and washed 3 times by centrifugation at 10 000 rpm for 5 min each and the pellet was resuspended in 500 μL phosphate buffer pH 6 with 1 % BSA.

Electrostatic interaction was used to immobilize antibody on MWCNTs/AuNPs surface. A fundamental property of antibodies is the isoelectric point (pI), which is defined as the pH at which the antibodies carries no net electrical charge. When the pH is lower than pI, the antibody has positive charge and on the other hand, when the pH is higher than pI, antibody has negative charge. Anti-*S.typhimurium* antibody has pI of 7.4 and was diluted in buffer pH 6 which made the antibody become positive charge. Carboxylic functionalized MWCNTs have negative charges from carboxylic groups that can bind to positively charged antibody [76].

Covalent attachment method: 1 mL of MWCNTs/AuNPs (2 mg/mL in 10 mM phosphate buffer pH 7) was mixed with 1 mL EDC and NHS at 400 mM and 100 mM, respectively, in phosphate buffer pH 7. The solution was shaken at RT for 2 h to activate carboxylic acid group on the surface of MWCNTs. Then, the solution was washed 1 times by centrifugation at 10 000 rpm for 5 min and pellet were resuspended in 1 mL PB pH 7.4. After that, 20 µL of polyclonal antibody was added (final concentration 0.1 µg/mL), and the solution was shaken overnight at 4 ^{0}C. Then, the solution was washed 5 times with 10 mM PB buffer pH 7.4 and the pellet were dispersed in 1 mL of 10 mM PB pH 7.4 and stored at 4 ^{0}C for further use.

Covalent linking was used to immobilize antibody on MWCTNs/AuNPs surface. In this study, carboxylic functionalized MWCNTs were activated by using 1-ethyl-3(-3dimethylaminopropyl carbodimide (EDC) in the precence of *N*-hydroxysuccinimide (NHS), the stable active ester was performed to allow the cross linked protein onto MWCNTs.

The carboxyl group on MWCNTs surface were reacted with EDC, forming an amine reactive *O*-acylisourea intermediate that is very unstable and susceptible to hydrolysis. Addition of NHS stabilizes the intermediate by converting it to a semistable amine reactive NHS ester. Then, the activated CNTs were reacted with antibody allowing the cross linked of protein onto MWCNTs. The schematic drawing of protein linked onto MWCNTs is shown in Figure 3.1.

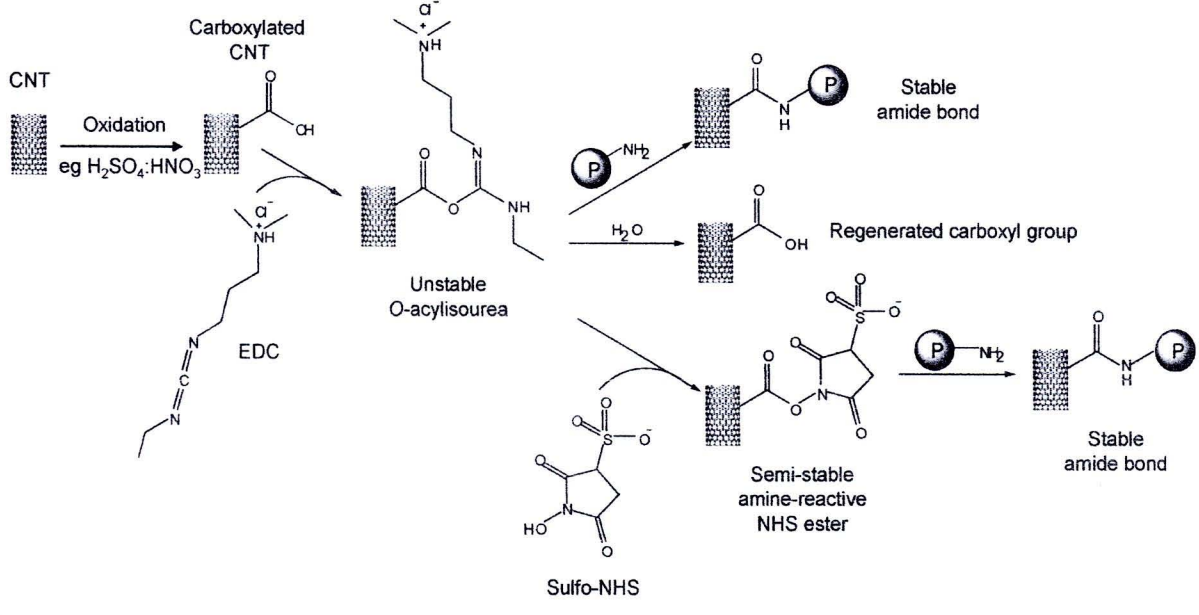

Figure 3.1 Theoretical conjugation of proteins to carboxylated CNTs using EDC in the presence and absence of sulfo-NHS [77].

3.3.4 Immunoassay on Nitrocellulose Membrane

Platform I : The immunoassay was performed by spotting 0.5 µL of capture antibody (1 mg/mL in 10 mM PBS pH 7.2) on nitrocellulose membrane for 1 h at RT, then the membrane were washed 3 times with 10 mM PBST pH 7.4 for 5 min each. After that membrane were blocked with 3% skim milk or 2% BSA (dillute in 10 mM PBST pH 7.4) for 1 h at RT. The membrane were washed with PBST buffer 3 times for 5 min each, and then 50 µL aliquot of various 10-fold serial dilutions of heat-killed *S. enterica* serovar Typhimurium in 1% skim milk or 1% BSA dilute in PBST were added and incubated for 1 h at RT. Afterwards, MWCNTs/AuNPs/Ab in 10 mM PB pH 6 containing 1% BSA or 1 % skim milk was added and incubated for 1 h at RT. After washing with 10 mM PB+0.05% tween pH 6, 3 times and 5 min each, the final washing step was carried out in 0.1M $NaNO_3$ for 1 min. Then, silver enhancer solutions A and B were mixed at 1:1 ratio and added onto each membrane for 10 min. Afterwards, membrane were washed with milli-Q water and then scanned by a flatbed scanner. The obtained image was analyzed by using an image processing software imageJ.

Platform II : The immunoassay was performed by coating nitrocellulose membrane with 50 µL of 100 ng/mL streptavidin or protein A for 2 h at RT and then the membrane were washed 3 times with 10 mM PBST pH 7.4 for 3 min each. After that, anti-*Salmonella* antibody (0.5 mg/mL in 10 mM PBS pH 7.4) were immobilized on membrane for 1 h at RT and then the membrane were washed 3 times with 10 mM PBST pH 7.4 for 3 min each. Subsequently, membrane were blocked with 3% skim milk or 2% BSA (dillute in 10 mM PBST pH 7.4) for 1 h at RT. The membrane were washed with PBST buffer 3 times for 5 min each, and then 50 µl aliquot of various 10-fold serial dilutions of heat-killed *S. enterica* serovar Typhimurium in 1% skim milk or 1% BSA dilute in PBST were added and incubated for 1 h at RT. Afterwards, MWCNTs/AuNPs/Ab in 10 mM PB pH 6 containing 1% BSA or 1 % skim milk was added and incubated for 1 h at RT. After washing with 10 mM PB + 0.05% tween pH 6, 3 times and 5 min each. The final wash was carried out in 0.1M $NaNO_3$ for 1 min. Then, silver enhancer solutions A and B were mixed at 1:1 ratio and added onto each membrane for 10 min. Afterwards, membrane were washed with milli-Q water and then scanned by a flatbed scanner. The obtained image was analyzed by using an image processing software imageJ.

Platform III :

Preparation of reaction I (Magentic bead/Ab complex): 10 µL of streptavidin functionalized magnetic bead (0.1 mg/mL in 10 mM PBST pH 7.4) were mixed with 5 µL of biotinylated antibody (2 µg/mL in 10 mM PBS pH 7.4) and 85 µL of 10 mM PBST pH 7.4. The mixture was incubated by rolling for 1 h at RT, washed 3 times with 10 mM PBST pH 7.4 and resuspended in 50 µL of 10 mM PBST pH 7.4.

Prepareation of reaction II (MWCNTs/AuNPs/Ab/Ag complex): 10 µL of various concentrations of heat-killed *S. enterica* serovar Typhimurium (in 10 mM PBST pH 7.4) and 4 µL of MWCNTs/AuNPs/Ab (the final concentration is 0.125 mg/mL) were mixed in 86 µL of 10 mM PBST pH 7.4. The mixture was incubated by rolling for 1 h at RT, washed 3 times with 10 mM PBST pH 7.4 and resuspended in 50 µL of 10 mM PBST pH 7.4.

Immunoassay: Reaction I and II were mixed together in 10 mM PBST pH 7.4 (final volume 100 µL) using a roller shaker for 1 h at RT. Subsequently, the pellet and supernatant were separated using a magnetic bar and the pellet was washed 3 times with milli-Q water for 15 min each. After that, the pellet was resuspended in 10 µL milli-Q water and 1 µL were used to spot on nitrocellulose membrane. Then, silver enhancer solutions A and B were mixed at 1:1 ratio and added onto each membrane for 5 min. Afterwards, membrane were washed with milli-Q water and then scanned by using a flatbed scanner. The obtained image was analyzed by an image processing software imageJ. After that, graphpad prism software was used to fit the data and draw the graph.

Data analysis: Data were obtained by measure the gray intensity of the spot using imageJ software. The program automatically measure the grayscale. After determine of gray scale value of each spot, the data were analyzed by Prism software. Limit of detection (LOD) were calculated with the equation :

LOD = (3*SD blank) + mean of blank

Where, SD blank is standard deviation of blank (without *S. enterica* serovar Typhimurium), and mean of blank is average of value blank intensity.

To fit a sigmoidal curve with Prism, the X values must be logarithm of concentration. If entered concentrations, Prism can transform those values. The data were normalized to obtain standard dose-response curve. A standard dose-response curve is defined by four parameters: the baseline response (bottom), the maximum response (top), the slope and the *S. enterica* serovar Typhimurium concentration that provokes a response halfway between baseline and maximum (EC_{50}). EC_{50} is defined as the concentration of agonist (*S. enterica* serovar Typhimurium) that provokes a response half way between the baseline (bottom) and maximum response (top). Response or Y ($\%B/B_0$) is percentage of binding in the presence (B) and the absence (B_0) of *S. enterica* serovar Typhimurium. Prism software fits the data to the equation below to fit the curve.

Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

Bottom	: Baseline response
Top	: Maximum response
LogEC50	: Logarithm concentration of agonist (*S. enterica* serovar Typhimurium) that provokes a response half way between the baseline (bottom) and maximum response (top)
X	: Logarithm of concentration of agonist (*S. enterica* serovar Typhimurium)
HillSlope	: The steepness of the curve, HillSlope is fixed to 1.0