Electronic Supplementary Material (ESI) for Nanoscale Horizons.

Mixed-Monolayer Glyconanoparticles for the Detection of Cholera Toxin by SERS

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Electronic Supporting Information

1) Reagents

Silver nitrate, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), sodium hydroxide (anhydrous), calcium nitrate tetrahydrate, manganese nitrate tetrahydrate, glucosamine hydrochloride, N-acetylneuraminic acid (sialic acid), concanavalin A (ConA), cholera toxin B-subunit (CTB), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC*),* N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), DL-dithiothreitol (DTT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) powder, 2-(N-morpholino)ethanesulfonic acid (MES) powder, magnesium chloride hexahydrate, calcium chloride hexahydrate, calcium nitrate tetrahydrate, calcium carbonate, sodium sulfate, potassium bicarbonate and sodium bicarbonate were purchased from Sigma-Aldrich (Gillingham, U.K.). C18 Spin Columns, galactosamine hydrochloride and heterobifunctional thiol/carboxy polyethylene glycol ($CTPEG_{12}$) were purchased from Thermo Fisher Scientific (Loughborough, UK). Heterobifunctional thiol/amine polyethylene glycol ($ATPEG_{12}$ and $ATPEG_{18}$) and thiol/carboxy polyethylene glycol ($CTPEG_{18}$) was purchased from Nanocs, (New York, USA). Doubly distilled and deionised water (dH_2O) was prepared in-house.

2) Instrumentation

Aggregation measurements were obtained using two instruments. Extinction changes were measured using a Varian, Cary Win-UV 300; a dual beam scanning UV-visible spectrophotometer with 190 – 900 nm range. Changes in SERS intensity were measured using a 200 mW Argon ion laser radiation source with an excitation wavelength of 514.5 nm coupled with a Renishaw InVia Raman microscope. A 20x long working distance objective lens was used for laser focusing. Spectral range of 0-3000 cm^{-1} was recorded with 3 accumulations of 1 second per sample. In all instances 3-5 replicates were measured for each concentration and the spectra were normalised against the 800 cm^{-1} peak intensity obtained from a cyclohexane sample which was freshly obtained and measured on the day the experiment was performed on. For all Raman spectra, data handling was carried out using GRAMS/AI software for spectra baseline correction.

3) Nanoparticle Preparation

EDTA reduced/capped silver nanoparticles were prepared as follows. All glassware was cleaned with aqua regia before use and rinsed thoroughly with distilled water. A 500 mL, 0.12 mM solution of EDTA (23 mg) was prepared. This was heated and prior to boiling, 80 mg of sodium hydroxide was added (giving a concentration of 4 mM). Once boiled, 22 mg of silver nitrate in 5 mL of dH_2O was added slowly in 1 mL aliquots with stirring to give a final silver nitrate concentration of 0.26 mM. The water was boiled further with stirring for 15 minutes before the heat was removed and stirring continued until the silver EDTA colloid (AgNP) reached room temperature. Each batch of nanoparticles were used for 2 months.

4) Lectin/Toxin Solution Preparation

Solutions of lectin/toxin were prepared at a stock concentration of 2 mg/mL in 10 mM HEPES with 0.2 mM $Ca(NO_3)_2$ and $Mn(NO_3)_2$, pH 7.4 buffer (HB2). These were aliquoted and stored at 2 °C. Subsequent dilutions of the lectin stocks were made in HB2 or synthetic freshwater, the preparation of which is described below.

5) Synthetic Freshwater (Soft Water)

5.1 Preparation

Three solutions containing a variety of ions and at varying concentrations were prepared using the volumes indicated in table 1. After combining the components (S1, S2 and S3), the solution was mixed for 6 hours to ensure sufficient dissolution and mixing of all components. The pH was measured as 7.5.

Table S1. Ionic content of the constituent solutions (S1, S2 and S3) of synthetic freshwater (soft water) including the volumes of each component solution required.

Stock solution	Final required concentration (mg L^{-1})	Volume of Stock Solution Required (µL)
S1		50
$MgCl_2 \cdot 6H_2O$	1.458	
$CaCl_2 \cdot 6H_2O$	3.206	
$Ca(NO_3)_2 \cdot 4H_2O$	0.601	
S2		45450
$CaCO_3$	6.814	
S3		50
Na_2SO_4	5.288	
$KHCO_3$	0.977	
$NaHCO_3$	0.458	
dH_2O		4450

Table S2. Ion type and related concentration found in the prepared synthetic freshwater (soft water).

Ion	Concentration (µM)
Na^+	250
Ca^{2+}	264
Mg^{2+}	60
K^+	25
Cl^-	280
NO_3^-	30
SO_4^{2-}	114.5
HCO_3^-	385

5.2 Particle Stability in Synthetic Freshwater

Table S3. Extinction data for GSNPs 1 day after resuspension in HB2 or synthetic freshwater (SF)

Sample	λmax. (nm)	600 nm : Plasmon extinction
GSNP (HB2)	413	0.166
GSNP (SF)	413	0.166

6) Preparation of SERS-active glyconanoparticles

6.1 Raman Reporter Molecule

4-((1*H*-benzo[*d*][1,2,3]triazol-6-yl)diazenyl)-3,5 dimethoxyphenol (RB1) was used as the Raman reporter molecule for SERS detection of CTB.

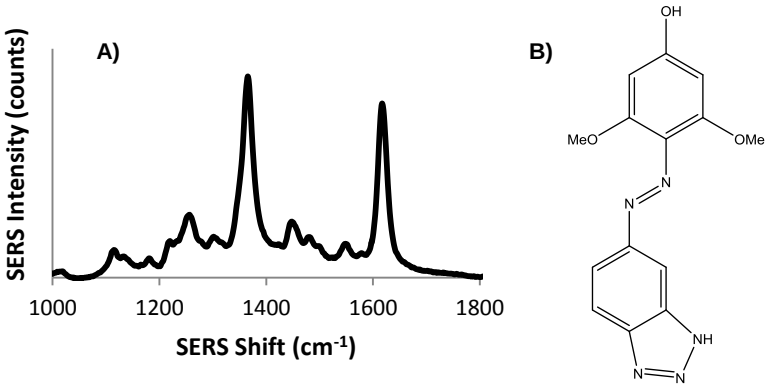

Figure S1. A) SERS spectrum of RB1 obtained at an excitation wavelength of 514.5 nm, 1 second acquisition, 3 accumulations. The 1364 cm^{-1} peak was monitored when measuring an increase in SERS intensity B) Structure of RB1.

6.2 PEG Linker Preparation

The PEG-carbohydrate linkers were prepared using two methods; on and off nanoparticle coupling. Both methods were used depending on the type of carbohydrate used in nanoparticle functionalisation. All concentrations stated are final.

6.2.1 On-Nanoparticle Coupling - used for the preparation of glucose nanoparticles

10 µM RB1 and 40 µM $CTPEG_{12}$ were added to the appropriate volume of AgNP (for example 100 µL of 0.1 mM RB1 and 40 µL of 1 mM $CTPEG_{12}$ were added to 860 µL of AgNP, this procedure was scaled up as required). A 10 mM glucosamine solution was prepared in 100 mM HEPES buffer @ pH 7.4 (HB1). These conjugates were centrifuged at 4000 rpm for 10 minutes. The pellet was kept and the supernatant removed and centrifuged at 7000 rpm for 15 minutes. The supernatant from the second centrifugation step was subsequently removed and discarded and the pellets from each centrifugation step combined. 5.4 mM EDC and 4.6 mM sNHS solutions were prepared in 10 mM MES, pH 6.0 buffer (MB). Separate 100 µL EDC and 240 µL sNHS solutions were mixed (340 µL total) and added to each pellet and mixed for 15 minutes at room temperature.
After 15 minutes 1.1 mM of carbohydrate solution in HB1 was added to the coupling mixture (for 1mL of particles 100 µL of the 10 mM solution), with sufficient HB1 added to ensure a pH of 7-7.4 (400 µL for 1 mL of particles), and shaken for 16 hours at room temperature. Following mixing, the previous centrifugation steps were employed. The collected pellets were then resuspended in HB2 and used within 1 week of preparation

6.2.2 Off-Nanoparticle Coupling (aminated carbohydrates) - used for the preparation of the galactose-based linkers

A 1.7 mL scale reaction batch was prepared. A 10 mM solution of thiol-carboxyl $CTPEG_n$ was prepared in 100 µL MB. A 156 mM solution of aminated carbohydrate was prepared in 500 µL 10 mM HEPES buffer @ pH 7.4 (HB3). 200 mM EDC and 225 mM sNHS MB solutions (200 µL each) were combined. This mixture was added to the 10 mM $CTPEG_n$ solution and mixed for 1 hour. After which the solution of aminated carbohydrate was added and mixed for 1 hour. Following this, 700 µL of 0.01M NaOH solution was added to raise the pH to 7.5-8 and mixed for 16 hours. This same procedure can apply to any aminated carbohydrates such as galactosamine, glucosamine and mannosamine.

6.2.3 Off-Nanoparticle Coupling (carboxylated carbohydrates) - used for the preparation of the sialic acid-based linkers

A 1.7 mL scale reaction batch was prepared. A 19 mM solution of carboxylated carbohydrate was prepared in 100 µL MB. 200 mM EDC and 225 mM sNHS MB solutions (200 µL each) were combined. The 400 µL EDC and sNHS mixture was added to the 19 mM carboxylated carbohydrate solution and mixed for 1 hour. A 1.25 mM $ATPEG_n$ was prepared in 500 µL HB3. This was added, to the coupling mixture and shaken for 1 hour at room temperature. Following this, 700 µL of 0.01 M NaOH solution was added and mixed for 16 hours. This same procedure can apply to any carboxylated carbohydrate.

The off-nanoparticle coupled products were added to the nanoparticles and mixed for 16 hours. To generate 15:1 galactose:sialic acid coverage, 30µM of galactosyl-PEG_n and 2 µM sialyl-PEG_n was added to RB1 (10 µM) coated

AgNP by premixing the carbohydrate linkers together before adding to the nanoparticles. Following 16 hours mixing, the galacto-sialonanoparticles (GSNPs) were centrifuged at 4000 rpm for 15 minutes and the supernatant centrifuged at 7000 rpm for 15 minutes. The supernatant from the second centrifugation was discarded and the pellets from each centrifugation step combined and resuspended in HB2 and used within 1 week of preparation.

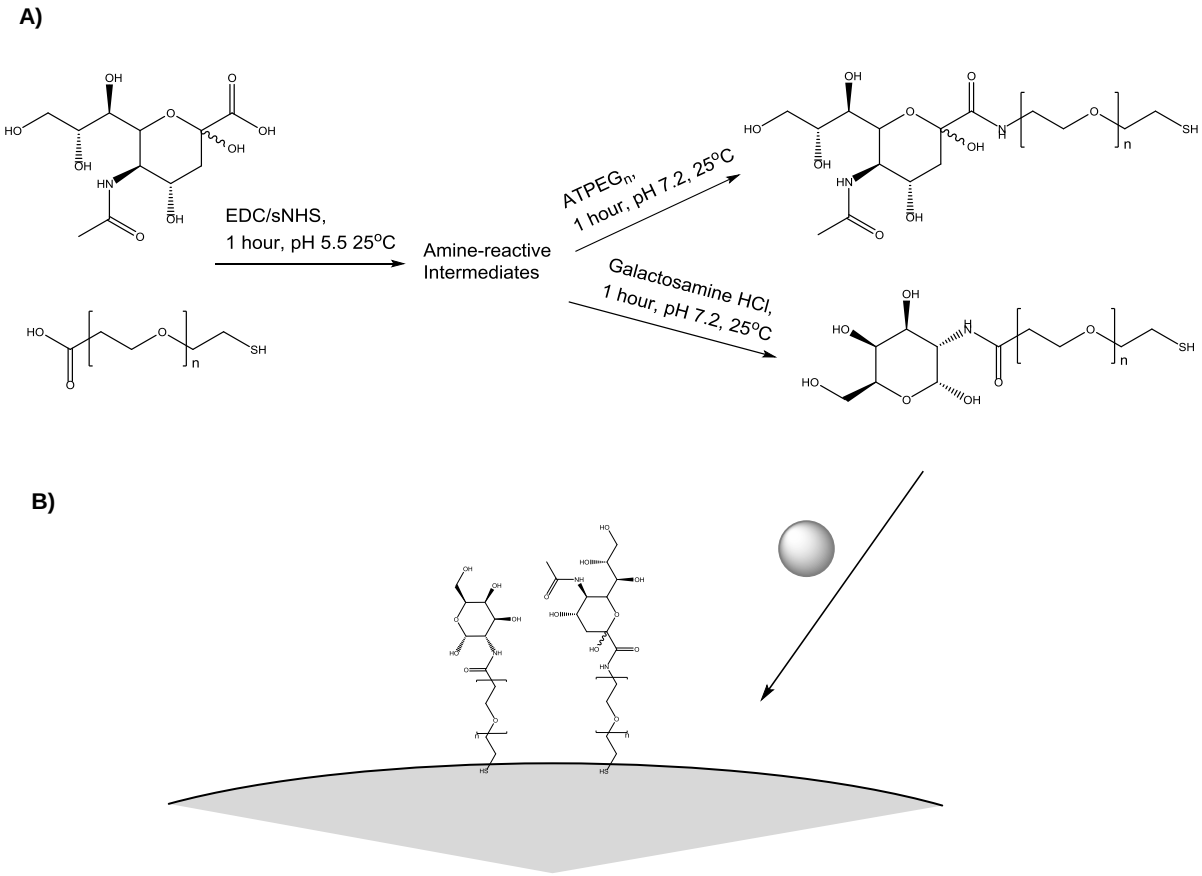

Figure S2. A) Reaction schematic of PEG_nGal and PEG_nSia linkers. B)Subsequent addition of the linkers to the nanoparticles (pre-coated in RB1 - not shown).

7) Characterisation of Linker

The prepared glyconanoparticles were analysed by UV-visible extinction spectroscopy and dynamic light scattering to evaluate the extinction and size changes at each stage of functionalisation. Size and extinction changes are indicative of successful functionalisation (see Table 1). The changes to the nanoparticle surface were also confirmed by gel electrophoresis (see Figure 1). The carbohydrate functionalisation causes the nanoparticles to interact strongly with the agarose gel which results in minimal movement in the gel.

The PEG_{12}galactose and PEG_{18}sialic acid linkers was characterised by attenuated total reflectance infra-red (ATR-IR) spectroscopy to demonstrate the success of the amide coupling reaction (see Table 2 and 3).

The linker was isolated from any excess carbohydrate, 1-(3-(dimethylamino)propyl-3-ethylurea or sulfo-NHS using C18 spin columns (Thermo Scientific™, Pierce™). The standardised procedure for using the spin columns is given at https://tools.lifetechnologies.com/content/sfs/manuals/MAN0011495_Pierce_C18_SpinCol_UG.pdf. Following preparation, the eluted sample (80 µL) was heated to remove the solvent (70% acetonitrile in dH_2O). Following evaporation, the linker was re dissolved in 20 µL dry ethanol to run on the ATR-IR instrument. The sample was added to the ATR probe so as to allow the ethanol to evaporate, leaving behind the linker residue. The entire sample was added and analysed.

Prior to using the spin columns, 10 mL of galactonanoparticle (40 µM final linker concentration) were prepared as previously described. These were centrifuged at 4000 rpm for 15 minutes and the supernatant centrifuged at 7000 rpm

for 15 minutes. The supernatant from the second centrifugation was discarded and the pellets from each centrifugation step combined and resuspended in HB3. The centrifugation steps were repeated and the pellets from the spin steps combined to give 150 µL of pellets. 152 mM of DTT was added to the pellets and heated at 50 °C for 6 hours then at 40 °C for 16 hours. After this time the resulting red-brown mixture was centrifuged at 13000 rpm for 25 minutes. The supernatant was removed and the pellet was resuspended in 100 µL of dH_2O and centrifuged a second time (with the previous conditions). The second supernatant was combined with the first and this solution was purified using the aforementioned C18 spin columns.

Table S4. Extinction and size data for bare and functionalised nanoparticles.

NP Capping	λ maximum (nm)	Size (nm) / *PdI*
EDTA	407	55.05 (± 0.30) / *0.23*
$PEG_{12}/_{18}$ Mixture	414	61.19 (± 0.56) / *0.28*
$PEG_{12}Glu$	413	77.18 (± 0.23) / *0.25*
$PEG_{12}Gal$ $PEG_{12}Sia$	409	65.39 (± 3.58) / *0.30*
$PEG_{12}Gal$ $PEG_{18}Sia$	410	66.27 (± 1.19) / *0.27*
$PEG_{18}Gal$ $PEG_{12}Sia$	411	72.08 (± 0.62) / *0.30*
$PEG_{18}Gal$ $PEG_{18}Sia$	411	82.43 (± 4.97) / *0.14*

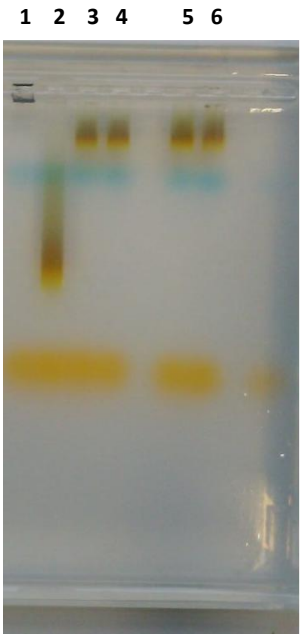

Figure S3. Agarose gel electrophoresis of AgNPs with various coatings; 1) EDTA, 2)$PEG_{12/18}$, 3) $PEG_{12}Gal/PEG_{12}Sia$, 4) $PEG_{12}Gal/PEG_{18}Sia$, 5) $PEG_{18}Gal/PEG_{12}Sia$ and 6) $PEG_{18}Gal/PEG_{18}Sia$.

Table S5. Infra-red spectrum data for the PEG_{12}-galactose linker.

Wavenumber (cm^{-1})	Functionality/Comment
3000-3500	Secondary amine stretch (weak, masked)
2500-3500	Carbohydrate-OH (strong, broad)
2912	C-H stretch (moderate)
2559	SH stretch (weak, masked)
1674	Amide C=O stretch (strong)
1184	C-N stretch (strong)
1044	C-O stretch (ether, strong)
726	N-H wag (secondary amine, moderate)
674	C-S stretch (moderate)

Table S6. Infra-red spectrum data for the PEG_{18}-sialic acid linker.

Wavenumber (cm^{-1})	Functionality/Comment
3368	Secondary amine stretch (weak, masked)
2500-3500	Carbohydrate-OH (strong, broad)
2973	C-H stretch (strong)
2700	SH stretch (weak, masked)
1673	Amide C=O stretch (strong)
1182	C-N stretch (strong)
1039	C-O stretch (ether, strong)
724	N-H wag (secondary amine, moderate)
696	C-S stretch (moderate)

8) Lectin Testing with Nanoparticle Conjugates

Glyconanoparticle samples (200 µL glyconanoparticles + lectin/toxin final volume) were tested at each lectin concentration and measured by UV-visible extinction spectroscopy or SERS after mixing with the lectin for 30 seconds with shaking. These were measured, 60 seconds, 120 seconds and 300 seconds after lectin/toxin addition.

9) CTB-induced Aggregation Measurements

9.1) Extinction/Colorimetric Data

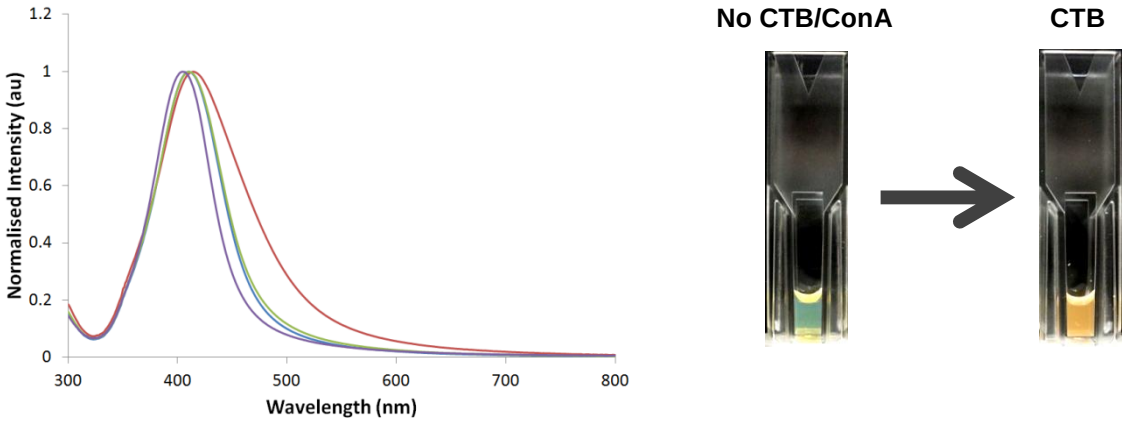

Figure S4. Extinction spectra of EDTA reduced colloid (purple), GSNPs (blue), GSNPs incubated with 80 nM ConA (green) and GSNPs incubated with 80 nM CTB (red). Photographs show cuvette containing GSNPs before and after addition of CTB (80 nM) in HB2.

9.2) Dynamic Light Scattering Data

Table S7. Size data for GSNPs (with a 15:1 gal:sia coating ratio) before and 5 minutes after addition of 100 nM CTB or ConA in HB2.

Sample	Size (nm) / *PdI*
GSNPs	53.68 (± 0.71) / *0.23*
GSNPs with 100 nM CTB	95.29 (± 2.76) / *0.18*
GSNPs with 100 nM ConA	54.37 (± 0.18) / *0.20*

10) SERS Detection of CTB and ConA

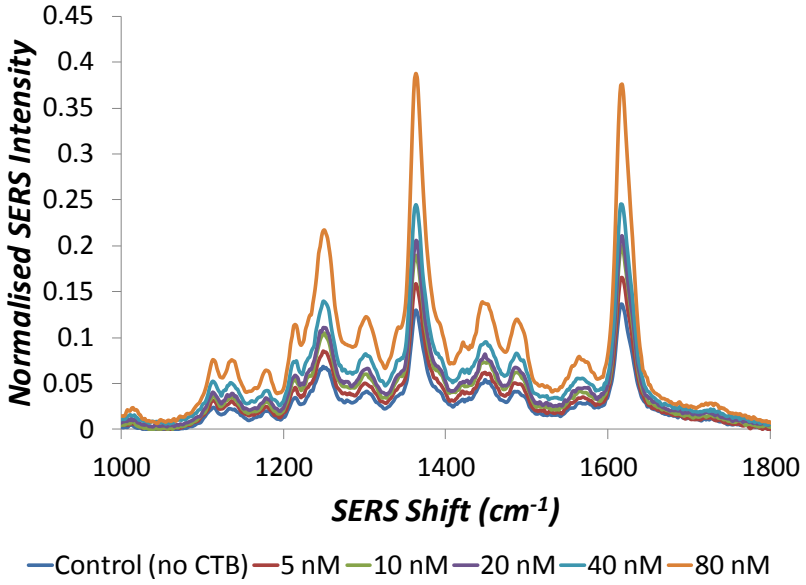

Figure S5. SERS spectra of RB1 coated GSNPs 5 minutes after incubation with CTB (in synthetic freshwater) at increasing concentrations (0-80 nM). 514.5 nm excitation wavelength used, 1 second acquisition with 3 accumulations. The laser excitation wavelength used is 514.5 nm with a 1 second acquisition, 3 accumulations and 5 replicates measured.

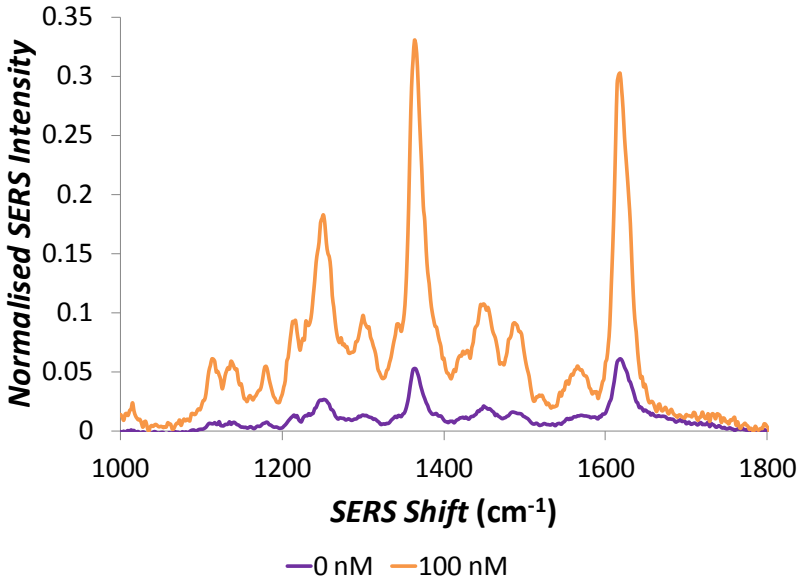

Figure S6. SERS spectra of RB1 coated gluconanoparticles 5 minutes before and after incubation with ConA in HB2. 514.5 nm excitation wavelength used, 1 second acquisition with 3 accumulations. The laser excitation wavelength used is 514.5 nm with a 1 second acquisition, 3 accumulations and 3 replicates measured.