Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B

Supplementary information

Preventing viral infections with polymeric Virus Catchers: A novel nanotechnological approach to anti-viral therapy

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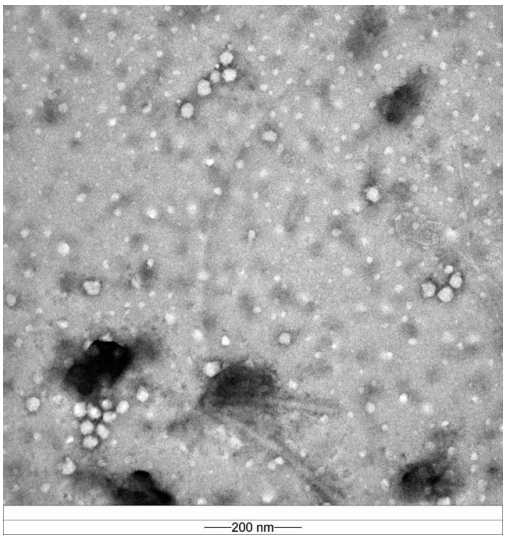
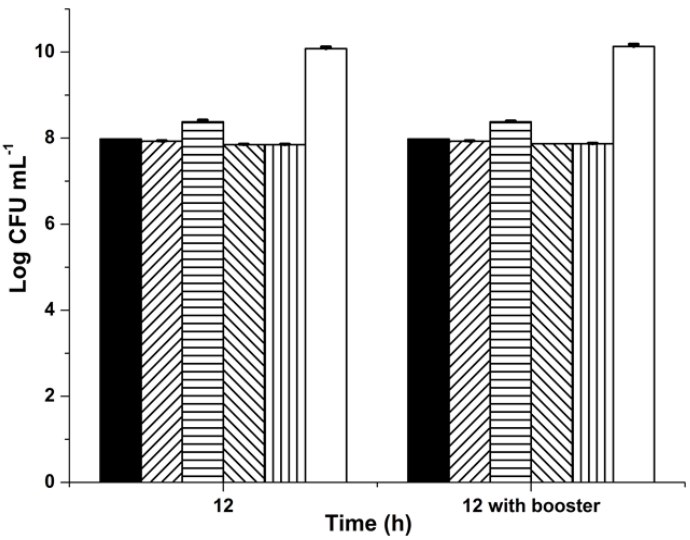
Supplementary Figures



Supplementary Figure 1. TEM image of the fr bacteriophages



Supplementary Figure 2. Antiviral effect of particles in a host virus system after a booster dose. Differences between the samples with and without booster dose were not statistically significant. (■ - $vMIP_{MAA}$; ◪ - NIP_{MAA} ▤ - viMIP; ▨ - iNIP; ▥ - Phage-infected bacteria; □ - Non-infected bacteria).

Supplementary Tables

Table 1 Surface elemental composition (%) of particles obtained from XPS

Polymer particles	C	O	N
Before template removal			
$vMIP_{MMA}$	63.96	35.64	0.35
$vMIP_{MAA}$	62.38	37.02	0.60
After template removal			
$vMIP_{MMA}$	68.65	31.32	0.03
$vMIP_{MAA}$	70.02	29.83	0.05
Control particles			
NIP_{MMA}	72.35	27.57	0.08
NIP_{MAA}	69.19	30.66	0.05
Surface modification and shell synthesis			
Core	67.66	32.34	0
Amine functionalized	67.54	31.36	1.10
Aldehyde functionalized	66.11	27.24	0.75
Phage immobilized	73.53	24.79	1.68
viMIP after alkaline hydrolysis	64.06	35.86	0.05
iNIP	61.27	38.73	0

Table 2 Zeta potential measurements of all particles

Polymer particles	Zeta potential (mV) [a] pH: 6.11; Temperature: 23.9 °C
$vMIP_{MMA}$	-32.80 ± 0.87
NIP_{MMA}	-28.37 ± 1.03
$vMIP_{MAA}$	-24.67 ± 1.96
NIP_{MAA}	-33.40 ± 0.40
viMIP	-53.73 ± 0.63
iNIP	-49.70 ± 0.06

a. Presented as mean ± standard error

Supplementary Materials and Methods

Materials

Sodium bicarbonate (99.7 – 100.3%), sodium bisulphite (minimum 99%), methyl acrylic acid (MAA), boric acid (≥ 99.5%) and glutaraldehyde (50%), were purchased from Sigma (USA). Methyl methacrylate (MMA, 99%), ethylene glycol dimethacrylate (EGDMA, 98%), oleic acid (90%), sodium dodecyl sulphate (SDS, minimum 98.5%), sodium bicarbonate (99.7 - 100.3%), ammonium persulfate (APS, 98%), hydrochloric acid, cetyl alcohol (CA, 95%), ethylene diamine (99%), and trifluoro acetic acid (TFA, 99%) were from Aldrich (USA). Ammonia solution (25%), ethanol, N, N- dimethylformamide (DMF), sodium chloride (NaCl), iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$), and iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) were obtained from Merck (USA). Sodium hydroxide pellets were from J.T. Baker, acetic acid from Fisher Chemicals (UK), and acetonitrile (ACN) from Tedia. Bacto™ tryptone, bacto™ yeast extract, bacto™ beef extract, and bacto™ agar was purchased from Becton, Dickinson and Company (BD, USA), and polyvinyl alcohol, 80 mol% hydrolysed, molecular weight (MW) 6000 was obtained from polysciences. All chemicals were used directly without further purification.

The *Escherichia coli* bacteriophage (fr) ATCC® 15767-B1™ and bacterial host *Escherichia coli* ATCC® 19853™ used in this study were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Instrumental methods of analysis

Field-emission scanning electron microscope (FESEM, JSM-6700F, JEOL) and Transmission electron microscope (TEM, JEM-2010, JEOL) were used to observe the morphology of the polymeric particles and the phages respectively. The sizes and distribution of the polymeric particles were determined using Dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd, USA). X-ray photoelectron spectroscopy (XPS, AXIS His-165 Ultra, Kratos Analytical, Shimadzu) was employed to determine the surface elementary composition of the support particles after each surface functionalization reaction.

Host bacteria

The solid culture medium was prepared using tryptone, yeast extract, agar and NaCl with DI water and autoclaved for the purpose of sterilization. Liquid medium was also prepared in a similar fashion without the addition of agar. Stock bacterial cultures were maintained on tryptone agar plates. Prior to the experiments, a host culture was prepared by inoculating a 10 ml medium with E.coli and incubated for 18 h at 37 ^{o}C and 300 rpm. For experimental tests appropriate serial dilutions were made in broth medium.

Bacteriophage propagation and purification

The fr phage stocks were prepared on the host strain by a plate lysis procedure[1]. Briefly, the method of propagation of fr phages was accomplished by the double-layer soft agar technique as follows. An aliquot of the phage sample was mixed with an

overnight host culture in sterile conditions and incubated for 5 min to facilitate attachment of the phage to the host cells. The phage host mixture was then mixed with soft agar that had been melted and cooled to 45 ^{o}C in a water bath. The contents were then poured over the surface of an agar plate and allowed to set at room temperature. Finally, the plates were incubated for 18 h at 37 ^{o}C. After incubation at 37 ^{o}C, the plates showing confluent plaques were chosen. The top agar was removed, mixed with propagating medium and centrifuged to sediment the cellular debris and agar. The supernatant was separated and further filtered through 0.22µm filter units (Millipore). The phage stocks were maintained in sterile tubes at 4 ^{o}C until further use.

Bacteriophage enumeration assay or plaque assay

The concentration (infectivity titer) of the fr phage samples were determined by a plaque assay. The phage sample to be assayed was serially diluted in Escherichia medium broth. The solution from each dilution was mixed with host bacteria. The soft agar layer technique described previously was adopted for determining the titer (concentration) of samples. The prepared agar plates were incubated for 18 h at 37 ^{o}C. Upon completion, only those plates with countable numbers of plaques (from 30 to 300 per plate) were used and hence the concentration was reported as plaque forming units per ml (pfu mL^{-1}). In our batches of propagation, the infectivity titer of the fr phages prepared were 10^{10} pfu mL^{-1}.

Preparation of the virus surface imprinted nanoparticles (vMIP)

A one-stage miniemulsion polymerization was used for the virus imprinting process. A 1.88 w/v% of polyvinyl alcohol, 0.01M SDS and 0.01 mM of sodium bicarbonate was mixed with 20 mL of DI water to form the first aqueous phase. The functional, 0.8 mL of MMA or 0.47 mL of MAA, and cross-linking monomers, 4.2 mL of EGDMA, in the molar ratio of 1:4 were mixed to form the oil phase. The oil phase was added drop-wise into the first aqueous phase and homogenized at 24000 rpm with a homogenizer (T25B, IKA Labortechnik, Germany) to create a miniemulsion. The template virus, fr phages were added into the miniemulsion. The mixture was then added drop-wise into the second aqueous phase (400 mL, 0.05 w/v% of SDS). This reaction mixture was transferred to a 1 L airtight, three-neck, round bottom reactor and purged with nitrogen gas for 15 min to displace the dissolved oxygen thus forming a nitrogen gas atmosphere above the reaction solution. The temperature of the reactor was maintained at 40 °C using a temperature-controlled water bath. The redox-initiated polymerization was started by injecting 0.25 g of APS initiator and 0.23 g of sodium bisulphite into the reaction mixture which was left to proceed for 24 h. The polymer product was then washed with different washing medium. Firstly, the particles were washing five times with DI water, followed by five times with a solution of 10% w/v SDS: 10% v/v acetic acid to remove the template virus, four times with excess ethanol to remove the surfactant or any unreacted monomer and initiator, and finally six times with DI water. The solution was centrifuged (Universal 32R, Hettich Zentrifugen, Germany) at 9000 rpm for 40 min after each washing step,

to isolate the polymeric product from the washing medium. The corresponding non-imprinted polymers (NIPs) to the virus imprinted polymers (vMIPs) were prepared and washed in a similar manner, except without the addition of the template viruses. The product particles were stored as suspensions in liquid culture medium for subsequent studies.

Preparation of surface imprinted nanoparticles using immobilized virus template (viMIP)

(a) Preparation of superparamagnetic core particles

Iron oxide (Fe_3O_4) magnetite was prepared by the coprecipitation method using 0.8 M $FeCl_3.6H_2O$, 0.4 M $FeCl_2.4H_2O$, and 3% hydrochloric acid. The above reactants were mixed, forming a clear, yellowish green solution that was then added into 250 mL of 5.23% ammonia solution. The resultant solution turned black and was then stirred magnetically at 1000 rpm for 1 h. The Fe_3O_4 magnetite was then washed three times with deionized (DI) water before being suspended in DI water. The Fe_3O_4 magnetite (1 g) prepared above was mixed with 1 mL of oleic acid. MMA (1.28 mL) and EGDMA (9.05 mL) in the molar ratio of 1:4 were mixed with oleic acid coated Fe_3O_4 magnetite particles forming the oil phase and ultrasonicated at 65% power for 80 s (Sonics Vibracell VCX 130). After homogeneity had been achieved, the oil phase containing Fe_3O_4 nanoparticles was added drop-wise to a 50 mL solution of 0.01 M SDS and 0.03 M CA (first aqueous phase) that was stirred at 300 rpm. The oil-first aqueous phase mixture was then ultrasonicated a second time at 65% power for 90 s

to create a miniemulsion. This miniemulsion was added drop-wise to a 600 mL solution containing 0.3 g SDS which was concurrently stirred at 300 rpm for 15 min. This reaction mixture was transferred to a 1 L airtight, three-neck, round bottom reactor and purged with nitrogen gas for 15 min to displace the dissolved oxygen thus forming a nitrogen gas atmosphere above the reaction solution. The temperature of the reactor was maintained at 80 °C using a temperature-controlled water bath. The polymerization reaction was initiated by injecting 0.5 g of APS initiator into the reaction mixture which was left to proceed for 24 h. Upon completion, the polymeric core particles were washed thrice with DI water, followed by thrice with 50% ethanol and finally thrice with DI water. The solution was centrifuged (Universal 32R, Hettich Zentrifugen, Germany) at 9000 rpm for 40 min after each washing step, to isolate the polymeric product from the washing solvent.

(b) Aminolysis

One gram of the polymeric core particles was washed twice with DMF and redispersed in 20 mL of DMF. Next, 20 mL of ethylene diamine was added to the DMF-washed core particles and was refluxed at 400 rpm at 110 °C for 12 h. The surface amine-functionalized core particles were then washed once with DI water, twice with 50% ethanol and finally twice with DI water to remove the excess amine.

(c) Aldehyde functionalization

Acetic acid and sodium acetate were used to prepare a pH 5 acetate buffer solution. One gram of the amine-functionalized polymeric core particles was incubated in 10

mL of acetate buffer solution and degassed for 10 min under room temperature. The supernatant was removed and redispersed in 10 mL of the same buffer with 5% glutaraldehyde. The reaction mixture was magnetically stirred at 400 rpm for 12 h under room temperature. In post treatment, the surface aldehyde-functionalized core particles were washed thrice with DI water.

(d) Template virus immobilization on core particles

The aldehyde-functionalized polymeric particles were then washed once with 0.1 M borate buffer (pH 9.33). A 15 mL aliquot of the fr phage solution (10^{10} pfu/ml) was then added to 1.0 g of the particles. The mixture was magnetically stirred at 300 rpm for 4 h at 4°C for the coupling to occur. The virus-immobilized support particles were then washed four times with DI water upon completion of the reaction.

(e) Shell layer synthesis

MMA and EGDMA were employed as the functional and cross-linking monomers, respectively. MMA (1.28 mL) and EGDMA (9.05 mL) in the molar ratio of 1:4 was added to 1.0 g of the template virus-immobilized core particles. The mixture was then ultrasonicated at 45% power for 90 s to ensure that it was mixed thoroughly. It was then added drop-wise into a 50 mL solution of 0.01 M SDS and 0.03 M CA which was concurrently stirred at 300 rpm. The mixture was ultrasonicated again at 45% power for 110 s to generate a miniemulsion. The miniemulsion obtained was then added drop-wise into a 600 mL solution containing 0.3 g SDS which was stirred at 300 rpm for 15 min. This reaction mixture was transferred to a 1 L airtight, three-

neck, round bottom reactor and purged with nitrogen gas for 15 min to displace the dissolved oxygen thus forming a nitrogen gas atmosphere above the reaction solution. The temperature of the reactor was maintained at 40 °C using a temperature-controlled water bath. A redox-initiated polymerization reaction was started by injecting 0.25 g sodium bisulphite followed by 0.25 g of APS into the reaction mixture which was left to proceed for 24 h. Upon completion, virus imprinted polymeric particles (viMIPs) were obtained which were washed thrice with DI water, followed by thrice with 50% ethanol and finally thrice with DI water.

(f) Template removal

After the formation of the shell layer, the immobilized template viruses were removed by hydrolysis. A 10 mL aliquot of a 1.0 M sodium hydroxide solution was added to 1.0 g of the core-shell virus imprinted nanoparticles. The hydrolysis mixture was stirred at 300 rpm and allowed to react for 5 h under reflux at 35 °C. The template-immobilized molecularly imprinted core-shell particles (viMIPs) obtained were washed three times with DI water.

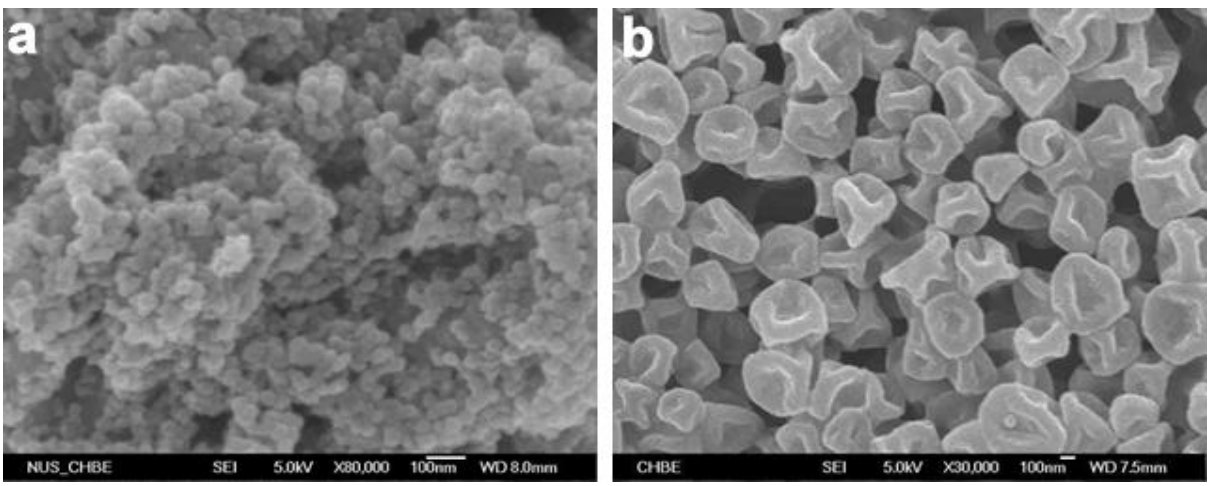
The corresponding non-imprinted particles (iNIPs) were prepared through the same procedures as the imprinted particles, but without the template immobilization step. These particles served as control samples in the characterization and adsorption studies.

Supplementary Results

Virus surface imprinted nanoparticles (vMIP)

Nanoparticles were prepared using a hydrophobic cross-linker ethylene glycol dimethacrylate (EGDMA) mixed with different functional monomers with a fixed molar ratio of 1:4. Virus imprinted nanoparticles (vMIP) were obtained by the addition of the whole viral template virus (fr phages) into the monomer phase prior to the polymerization step. Non-imprinted particles were also prepared without the addition of the template virus and were used as controls in all the experiments. FESEM images showed (see Supplementary Figure 3(a)) mono-dispersed nanoparticles having a narrow size distribution. The presence of the template molecules did not affect the size and there were no significant morphological differences between the resulting vMIPs and the non-imprinted nanoparticles (NIPs). X-ray photoelectron spectroscopy (XPS) was used to confirm the inclusion of the template during miniemulsion polymerization and template extraction by SDS/acetic acid. Owing to the abundant amine groups on the template viral protein shell, a significant increase in the nitrogen composition was noted for all of the imprinted nanoparticles indicating that the virus template interacted with the functional monomers during the pre-polymerization step and was embedded during polymerization (see Supplementary Table 1). It is hypothesized that, the viral particles interacted non-covalently with the functional monomers MMA & MAA through hydrophobic and electrostatic interactions respectively. Interestingly, the nitrogen composition obtained for $vMIP_{MAA}$ was substantially higher than those obtained for

$vMIP_{MMA}$ indicating that a higher percentage of the viral templates were imprinted for $vMIP_{MAA}$. Finally, the viral template was removed with a template removal washing procedure, commonly the most challenging step in molecular imprinting, and a significant reduction in nitrogen composition was observed for both $vMIP_{MMA}$ and $vMIP_{MAA}$ particles. During the template removal, 91.4% and 91.7% of the initial template imprinted were extracted from the $vMIP_{MMA}$ and $vMIP_{MAA}$ particles respectively, indicating an effective template removal.



Supplementary Figure 3. FESEM images of virus imprinted particles prepared using (a) one-stage (b) two-stage miniemulsion polymerization.

Surface imprinted nanoparticles using immobilized virus template (viMIP)

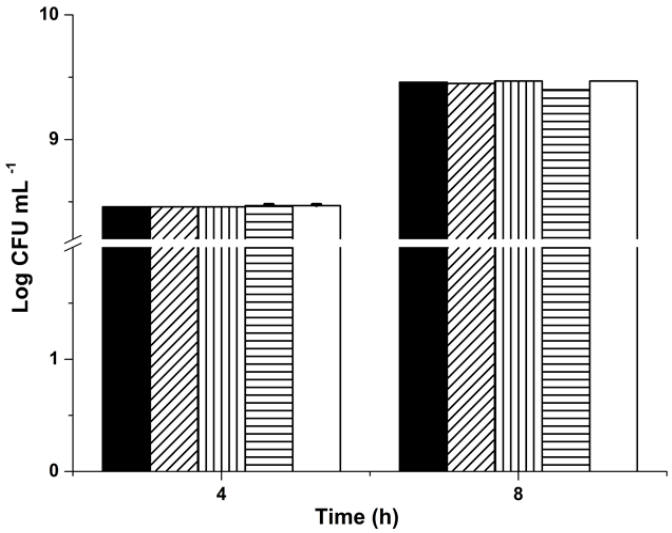
Support beads or core particles were prepared using MMA and EGDMA as monomers in the first stage of an oil-in-water miniemulsion polymerization reaction. Subsequently, the support beads were subjected to a series of surface functionalization reactions to immobilize the template virus while XPS was used to monitor each

surface modification step by comparing the surface nitrogen composition as before (see Supplementary Table 1). A second stage of miniemulsion polymerization was performed on the virus immobilized support beads using MMA and EGDMA as the functional and cross-linking monomers respectively. Finally, the imine bond formed between the surface and template virus was base hydrolyzed for template removal, resulting in the formation of a virus imprinted shell layer over the support particles. A significant reduction in nitrogen composition 1.68% to 0.05% was observed after this hydrolysis step. Overall, the virus immobilized molecularly imprinted polymeric nanoparticles (viMIP) after template removal, had no observable difference in nitrogen composition as compared to the non-imprinted polymers (iNIP), confirming the success of the hydrolysis reaction. Particle sizing, performed using DLS, showed the sizes of the support beads, viMIP and iNIP to be 352.6 ± 6.9 nm, 591.2 ± 2.7 nm and 574.9 ± 2.3 nm respectively. It should be noted that the nanoparticles increased significantly in size from 350 nm to 590 nm after the formation of the imprinted shell layer over the support beads, and the viMIPs and iNIPs had a unique red-blood-cell-(RBC) like morphology (see Supplementary Figure 3(b)). As reported in previously[2], the larger 'RBC'-like core-shell particles has aseptic surface area that is nearly equivalent to those prepared by the one-stage miniemulsion polymerization at 18 m^2 g^{-1}. The high specific surface areas and the unique concave surface morphology enabled the imprinted binding sites to be very close to the core particle surface for enhanced smooth mass transfer. It should also be noted that the virus immobilized imprinted and control particles had similar morphologies and sizes. An advantage of this core-shell nanoparticles is that additional functional characteristics such as

magnetic susceptibility or viral degradation capabilities can be added to the core for applications such as cell tracking, contrast agents for imaging and magnetic purification. Finally, the zeta-potential of all nanoparticles were measured and summarized in Supplementary Table 2.

Adsorbed virus infectivity study using bacterial growth assay

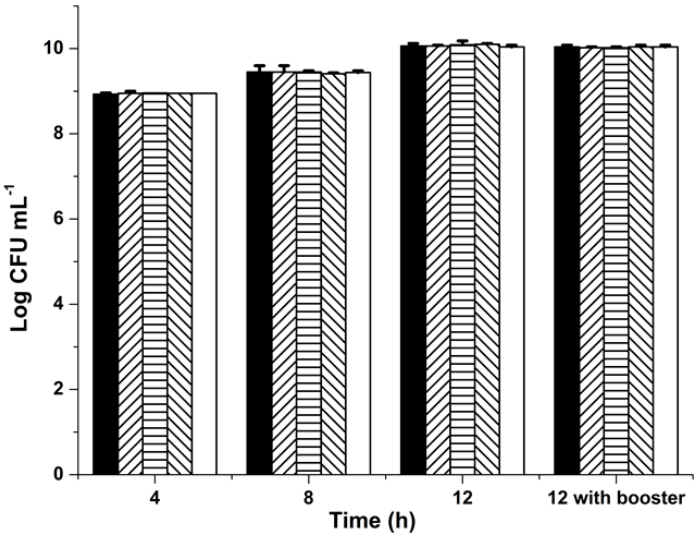
To determine if the virus-adsorbed particles exhibit infection behavior, non-infected bacterial growth was monitored in the presence of imprinted particles after the binding process. The bacterial growth profiles were measured by counting live bacterial colonies on agar plates for each sample. Imprinted particles before adsorption and non-imprinted particles with adsorbed viruses were used as controls. Under identical assay conditions, no significant difference in bacterial concentration was observed among the virus-adsorbed particles, non-adsorbed particles and the controls (see Supplementary Figure 4). This suggested that the viruses were inactivated by the binding process and viral desorption is impeded even in the presence of the host cells.



Supplementary Figure 4. Adsorbed virus infectivity study using bacterial growth assay. (■ - $vMIP_{MAA}$; ◪ - NIP_{MAA}; ▥ - virus-adsorbed $vMIP_{MAA}$; ▤ - virus-adsorbed NIP_{MAA}; □ - Bacteria).

Influence of particles on bacterial growth

Additional experiments were performed to investigate if the particles alone had an effect on the growth of the host bacteria. Non-infected bacteria were cultured in the presence of equal concentrations of imprinted or non-imprinted particles for 12 h and non-infected bacteria without particles were used as controls. There were no significant statistical differences in bacterial concentration between the different samples, thus showing that the imprinted particles do not have any positive or negative effect on the host cell viability (see Supplementary Figure 5).



Supplementary Figure 5. Influence of particles on bacterial growth. (■ - $vMIP_{MAA}$; ▨ - NIP_{MAA} ▤ - viMIP; ▩ - iNIP; □ - Non-infected bacteria).

References

1. M. H. Adams, *Bacteriophages*, Interscience Publishers, New York, 1959.

2. C. J. Tan, M. G. Chua, K. H. Ker and Y. W. Tong, *Anal. Chem.*, 2008, **80**, 683-692.