Supporting information

GAG mimetic functionalised solid and mesoporous silica nanoparticles as viral entry inhibitors of herpes simplex type 1 and type 2 viruses

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Experimental section

Materials

Tetraethylorthosilicate (TEOS) (98%), ammonium hydroxide (NH_4OH) (28% NH_3 w/w), cetyltrimethylammonium bromide (CTAB), Pluronic® F127 and sodium benzenesulfonate (97%) were purchased from Sigma Aldrich. Ethanol absolute (EtOH), methanol (MeOH) and toluene were purchased from Merck. Dry toluene was prepared by storing toluene on activated 3Å sieves (Sigma Aldrich) overnight. 2-(4-Chlorosulfonylphenyl)ethyltrimethoxysilane in dichloromethane (DCM) (50% w/w) was purchased from Acros Organics. Hydrochloric acid (HCl) 32% w/w was purchased from Ajax Finechem. Phosphate buffered saline (PBS) tablets were purchased from Amersco and dissolved with water to prepare 1× concentration (pH 7.3-7.5) containing 137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer. The media used for antiviral and cytotoxicity assays, purchased from Invitrogen, was Dulbecco's modified eagle medium (DMEM) which was supplemented with penicillin, L-glutamine with either 2% or 10% v/v fetal calf serum (FCS). The HSV-1 used was the H129 CSB strain, and the HSV-2 was a clinical isolate. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2*H*-tetrazolium, inner salt) reagent was purchased from Promega.

Synthesis of solid silica nanoparticles (SSNs)

The method to prepare SSNs was modified from Yu et al.[1] A mixture of 88.8 mL absolute EtOH and 11.2 mL TEOS was added dropwise to a second solution containing 70.4 mL absolute EtOH, 27.2 mL type 1 ultrapure water and 2.8 mL NH_4OH (28% NH_3 w/w). The final solution was stirred at room temperature (20 ºC) for 13 h. The silica nanoparticles were separated by centrifuging the suspension at 32,055 relative centrifugal force (rcf) for 20 min and washed twice with absolute EtOH. The nanoparticles were dried overnight at 50 ºC.

Synthesis of mesoporous silica nanoparticles (MSNs)

The method to prepare MSNs were modified from Kim et al.[2] CTAB (1.0 g) and Pluronic® F127 (4.0 g) were dissolved in a solution of EtOH and NH_4OH (2.8% NH_3 w/w) (2:5 v/v, 298 mL). The mixture was stirred for approximately 1 h to ensure complete dissolution followed by the addition of 3.6 g of TEOS. The subsequent reaction was stirred at room temperature briefly for 60 s, then kept in static condition for 24 h. The resulting nanoparticles were collected by centrifugation at 28,174 rcf for 15 min, followed by washing twice with type 2 pure water and once with EtOH. The nanoparticles were then dried overnight at 60-80 ºC. The surfactant was removed through an extraction step using MeOH and HCl. Approximately 300 mg of the unextracted MSNs were stirred in 32 mL MeOH at 60 ºC followed by the addition of 2 mL of aqueous HCl (32% w/w). This mixture was stirred at 60 ºC for 48 h with the MeOH/HCl solution replaced after 24 h. The MSNs were separated by centrifugation at 28,174 rcf for 15 min, then washed four times with type 2 pure water and four times with MeOH. Finally, the MSNs were dried at 60-80 ºC overnight.

Functionalisation of solid and mesoporous silica nanoparticles

Silica nanoparticles were dispersed in dry toluene in a 1:100 (w/v) ratio. 2-(4-Chlorosulfonylphenyl)ethyltrimethoxysilane (CSPTMS) in 50% (w/w) dichloromethane (DCM) was added to the stirring suspension at 2-3 times excess volume per weight of silica. The reaction proceeded at reflux under a N_2 environment for 20 h. The intermediate compound was separated by centrifugation at 18,031 rcf for 20 min and washed sequentially with toluene, EtOH, and acetone. The compound was then dried at 50 ºC overnight to form **2**.

The intermediate compound (**2**) was dispersed in type 2 pure water and stirred at room temperature for 24 h. The suspension was centrifuged at 32,055 rcf for 15 min and washed twice with type 2 pure water. The compound was then stirred in aqueous 10% (w/v) NaCl solution twice for 3 h. The compound was washed again with type 2 pure water twice and then dried overnight at 50 ºC to afford the final compound (**3**).

The method described above afforded a functionalisation density of 22.0% w/w (MSN-SO_3). To prepare analogues of MSN-SO_3 with functionalisation density of 1.1% (MSN-SO_3-1%) and 4.3% (MSN-SO_3-4%) w/w, CSPTMS in 50% (w/w) DCM was added to unfunctionalised MSNs (**1**) in dry toluene at 0.22 and 0.55 times volume per weight of silica, respectively. All other reaction conditions including the hydrolysis reaction, remained constant.

Cell viability assay

The cytotoxicity of the nanoparticles were evaluated using a MTS assay. Vero cells (African green monkey kidney epithelial cell line) or HEK293 cells (human embryonic kidney cell line) were seeded at 2.4×10^4 cells/well or 2×10^3 cells/well, respectively and grown overnight in 96-well plates to achieve confluent monolayers. Two-fold serial dilutions of nanoparticle suspensions were prepared from a stock suspension of 2000 µg/mL. The 96 well plates were aspirated and replaced with 50 µL DMEM 10% FCS and 50 µL nanoparticles in PBS (DMEM 10% FCS for HEK293 assay). Nanoparticle suspensions and DMEM 10% FCS were added to wells without cells to record absorbance due to the nanoparticles which is a source of intereference. Cells were incubated at 37 ºC with 5% CO_2 for 48 h. MTS reagent (20 µL/well) was added to each well and incubated for 2 h. The absorbance was measured at 490 nm. The readings from wells containing cells were subtracted from wells without cells to correct for particle interference.

Antiviral assays

Concentration-response assay: Vero cells (1.2×10^5 cells/well) were grown in 24-well plates overnight. From a 500 µg/mL stock nanoparticle suspension in PBS for the HSV-1 assay and 250 µg/mL for the HSV-2 assay, two-fold dilutions were prepared in PBS. A mixture of 250 µL of nanoparticles and 50 µL of HSV-1 (approx. 120 plaque forming units (pfu)/well in untreated control) or HSV-2 (approx. 80 pfu/well in untreated control) diluted in DMEM 2% FCS were incubated at room temperature for 1 h under agitation. The cell culture medium was aspirated and replaced with 250 µL of nanoparticle/virus mixture and incubated for 1 h at 37 ºC with 5% CO_2. The nanoparticle/virus suspension was aspirated and overlayed with CMC (1×) in DMEM 2% FCS. After 72 h, cells were fixed with formaldehyde (0.4% w/v) in PBS, stained with toluidine blue (0.1% w/v) in water and dried before plaques were counted. IC_{50} values were calculated by fitting a four-parameter dose-response equation (variable slope model) to a plot of log(inhibitor) vs normalised response using Graphpad Prism version 6 software. The R^2 values for SSN-SO_3 were: HSV-1 $\geq$ 0.95; HSV-2 $\geq$ 0.94; and for MSN-SO_3 were: HSV-1 $\geq$ 0.97; HSV-2 $\geq$ 0.92.

Functionalisation density assay: Vero cells (1.2×10^5 cells/well) were grown in 24-well plates overnight. From a 250 µg/mL stock nanoparticle suspension in PBS (MSN-SO_3 with 1.1% and 4.3% w/w functional group), 250 µL of nanoparticles and 50 µL HSV-1 (approx. 49 pfu/well in untreated control) diluted in DMEM 2% FCS was incubated at room temperature for 1 h (final concentration of nanoparticle: 208 µg/mL). The cell culture medium were aspirated and replaced with 250 µL of nanoparticle/virus mixture and were incubated for 1 h at 37 ºC with 5% CO_2. The nanoparticle/virus suspension was aspirated and overlayed with CMC (1×) in DMEM 2% FCS. After 72 h, cells were fixed , stained and plaques counted as described above.

Control assay: Vero cells (1.2×10^5 cells/well) were grown in 24-well plates overnight. From a 500 µg/mL nanoparticle and benzene sulfonate suspension in PBS, 250 µL of nanoparticles and 50 µL of either HSV-1 (approx. 98 pfu/well in untreated control) or HSV-2 (approx 61 pfu/well in untreated control) diluted in DMEM 2% FCS was incubated at room temperature for 1 h (final concentration of nanoparticle: 417 µg/mL). The cell culture medium were aspirated and replaced with 250 µL of nanoparticle/virus mixture and were incubated for 1 h at 37 ºC with 5% CO_2. The nanoparticle/virus suspension was aspirated and overlayed with CMC (1×) in DMEM 2% FCS. After 72 h, cells were fixed , stained and plaques counted as described above.

Viral penetration assay: Vero cells (1.2×10^5 cells/well) were grown in 24-well plates overnight. Vero cells in 24 well plate were cooled under ice. The cell culture medium was aspirated and replaced with either cooled HSV-1 (approx. 69 pfu/well in untreated control) or HSV-2 (approx. 71 pfu/well in untreated control) diluted in DMEM 2% FCS. The cells were incubated with the virus under ice for 2 h. An experiment was run in parallel where cells were incubated with HSV-1 (approx. 103 pfu/well in untreated control) or HSV-2 (approx. 111 pfu/well in untreated control) at 37 ºC for 2 h. The virus was aspirated and the cells washed with cooled PBS three times and treated with nanoparticles (417 µg/mL final concentration). Equivalent viral titres were used for both the 'cold' (4 °C) and 'warm' (37 °C) penetration assays but lower plaques numbers were observed for the 'cold' assay possibly due to a higher amount of virus removed during the washing step. The temperature was increased to 37 ºC to allow viral penetration and was maintained under this condition for 1 h. The nanoparticles were aspirated and overlayed with CMC (1×) in DMEM 2% FCS. After 72 h, cells were fixed , stained and plaques counted as described above.

Time-of-addition assay: Vero cells (1.2×10^5 cells/well) were grown in 24-well plates overnight and infected with HSV-2 (approx. 35 pfu/well in untreated control) diluted in DMEM 2% FCS. For the t = 0 h time point, 200 µL nanoparticle suspensions (final concentration of 400 µg/mL) were mixed with 50 µL virus immediately prior to infection. After 1 h, the virus/nanoparticle suspensions were aspirated and the cells were washed once with PBS and overlayed with either CMC (1×) in DMEM 2% FCS (t = 0 h), or DMEM 2% FCS (t = 2, 4, and 6 h). At different time points post infection (t = 2, 4, 6 h), media was aspirated and the cells were treated with nanoparticle suspensions for 1 h at 37 ºC with 5% CO_2. The nanoparticles were then removed and the cells were overlayed with CMC (1×) in DMEM 2% FCS. After 72 h, cells were fixed , stained and plaques counted as described above.

Cell susceptibility assay: Vero cells (1.2×10^5 cells/well) were grown in 24-well plates overnight. The cell culture medium were aspirated and replaced with 208 µL nanoparticle suspensions (417 µg/mL final concentration) and 42 µL DMEM 2% FCS. Cells were incubated at 37 ºC with 5% CO_2 for 1 h. Wells were aspirated, washed three times with PBS and the cells were then infected with HSV-1 or HSV-2 (approx. 100 pfu/well in untreated control) for 2 h at 37 ºC with 5% CO_2. Virus suspension was removed from the wells and overlayed with CMC (1×) in DMEM 2% FCS. After 72 h, cells were fixed , stained and plaques counted as described above.

Characterisation

Dynamic light scattering (DLS) and zeta potential were measured using a Malvern Nano ZS Zetasizer The suspension was prepared in type 2 pure water. TEM images were taken with JEOL 1010 instrument operated at 100 kV with samples prepared on 200 mesh copper coated grids**.** To prepare suspensions to image the interaction of the functionalised nanoparticles (**3**) and virus, 250 µL of MSN-SO_3 at (final concentration: 417 µg/mL) and 50 µL of HSV-1 (2.5×10^6 pfu) were incubated at room temperature for 1 h under agitation. To obtain nitrogen adsoption-desorption isotherms, a Tristar II 3020 system was used at 77 K. Surface area was determined based on the Brunauer-Emmett-Teller theory and the pore size distribution was calculated using the Barrett-Joyner-Halenda (BJH) method.

Thermogravimetric analysis (TGA) data were obtained using a Mettler Toledo TGA/DSC 2 STAR system. Samples were weighed onto alumina (aluminium oxide) crucibles and loaded onto the instrument. The sample was then heated from 50-900 ºC with heating rate of 5 ºC/min. Compressed air was used as a protective gas with flow rate of 50 mL/min. A FLASH 2000 CHNS/O analyser was used to determine the elemental composition of the functionalised nanoparticles. The percentage weight value of carbon was determined by CO_2, and sulfur by SO_2.

X-ray photoelectron spectroscopy (XPS) was conducted using a Kratos Axis ULTRA X-ray photoelectron spectrometer incorporating at 165 nm hemispherical electron energy analyser. The incident radiation source was a monochromatic Al Kα X-ray (1486.6 eV) at 225 W (15 KV, 15 ma). Survey (wide) scans were taken at analyser pass energy of 160 eV and multiplex (narrow) high resolution scans at 20 eV. Survey scans were carried out over 1200-0 eV binding energy range with 1.0 eV steps and a dwell time of 100 ms. High-resolution scans were run with 0.05 eV steps and 250 ms dwell time. Atomic concentrations were calculated using the CasaXPS version 2.3.14 software and a Shirley baseline with Kratos library relative sensitivity factors. ^{13}C solid state NMR (SSNMR) was conducted using a Bruker Avance III spectrometer 300 MHz.

Characterisation

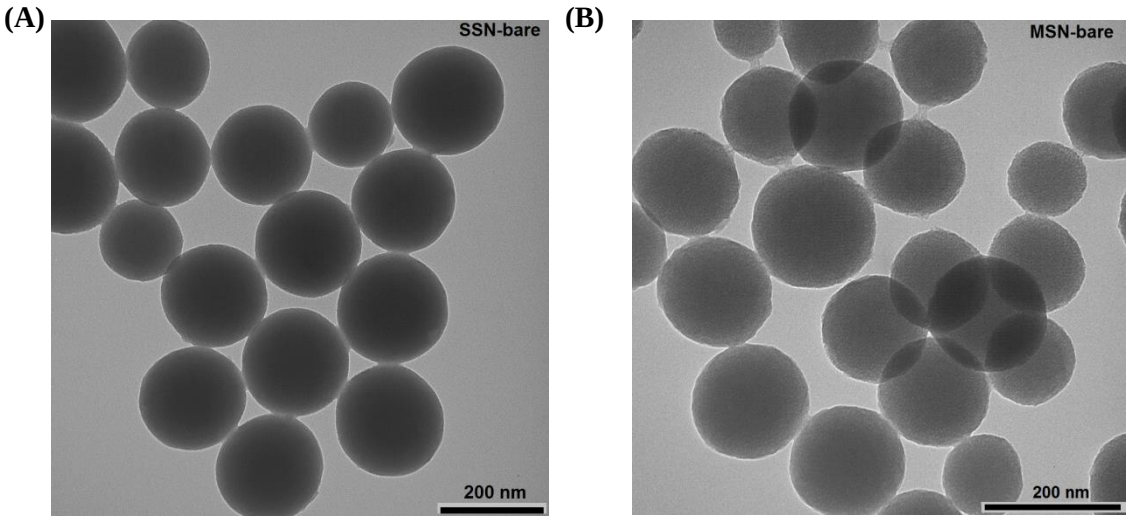


Fig. S1. TEM images of: (A) SSN-bare; and (B) MSN-bare. The scale bar represents 200 nm.

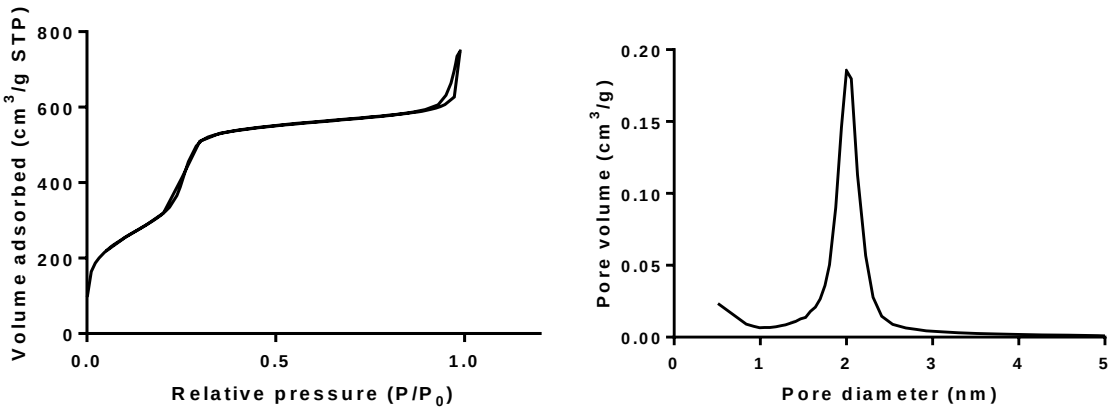


Fig. S2. Nitrogen adsorption-desorption isotherm of MSN-bare and BJH pore size distribution.

Table S1. Characteristics of nanoparticles estimated by DLS.[a]

Compound	Hydrodynamic diameter (nm)	PDI	Zeta potential (eV)
MSN-bare	163.2	0.380	-39.6
MSN-SO_3	205.0	0.256	-29.5
SSN-bare	234.0	0.205	-52.9
SSN-SO_3	306.0	0.295	-30.8

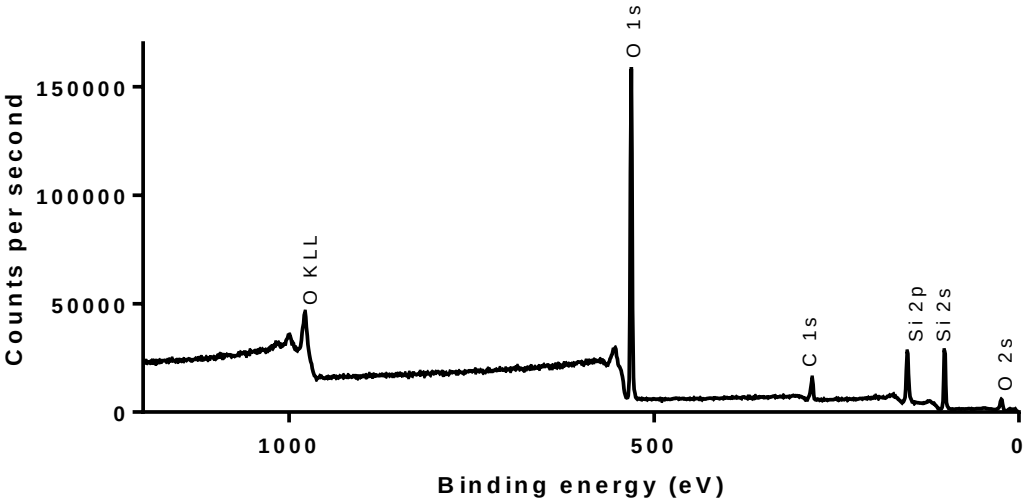
[a] Nanoparticles were dispersed in type 2 pure water at approximately 1000 µg/mL.

Table S2. Colloidal stability of functionalised nanoparticles (**3**) in biological medium.[a]

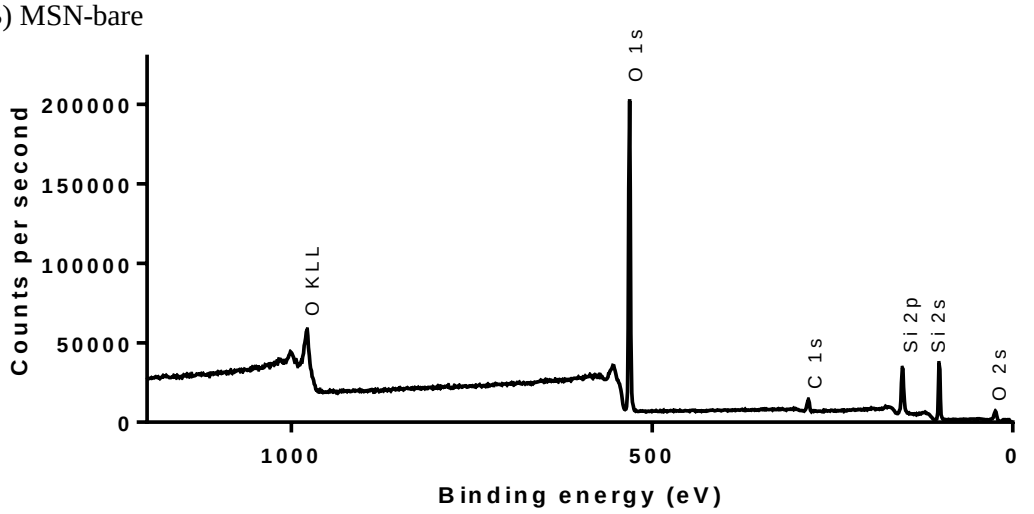
Compound	Hydrodynamic diameter (nm)	PDI
MSN-SO_3	199.3	0.278
SSN-SO_3	300.8	0.116

[a] Nanoparticles were dispersed in DMEM at approximately 1000 µg/mL

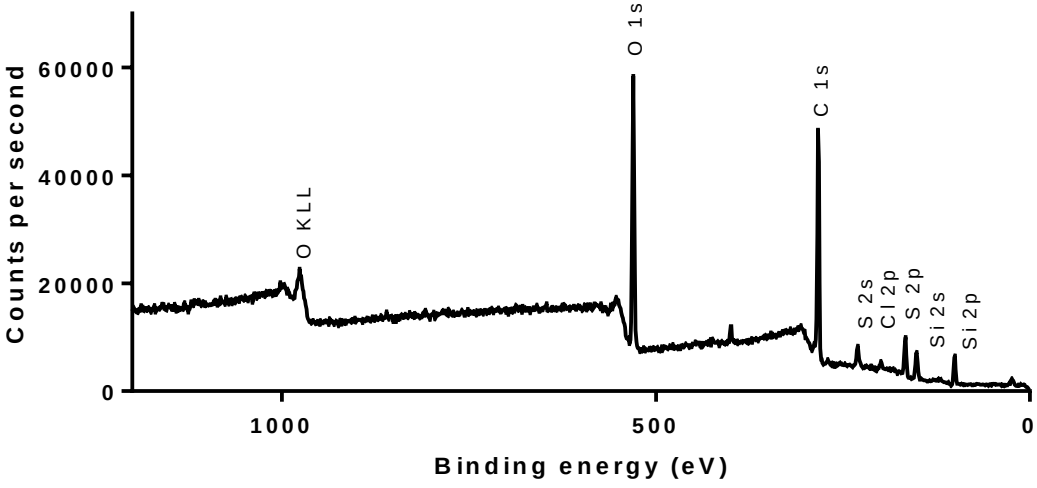
(A) SSN-bare



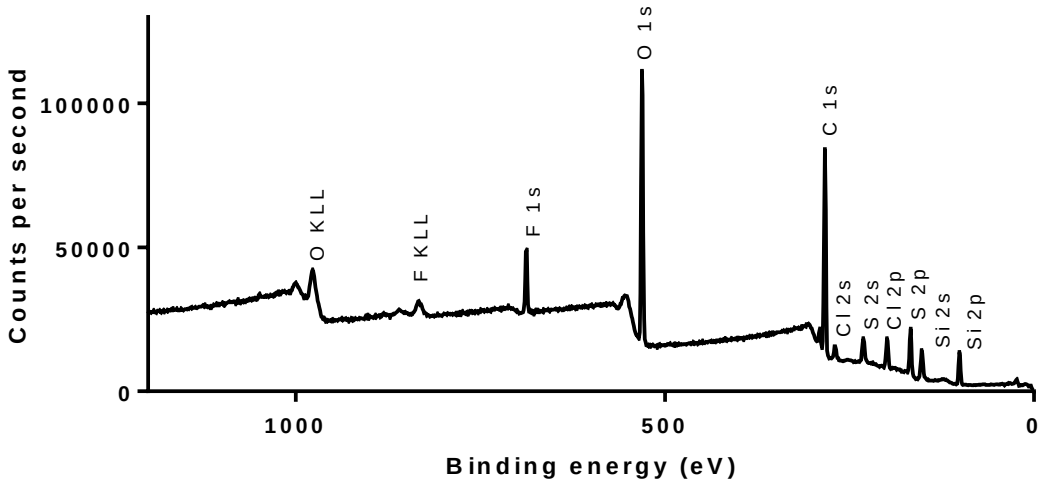
(B) MSN-bare



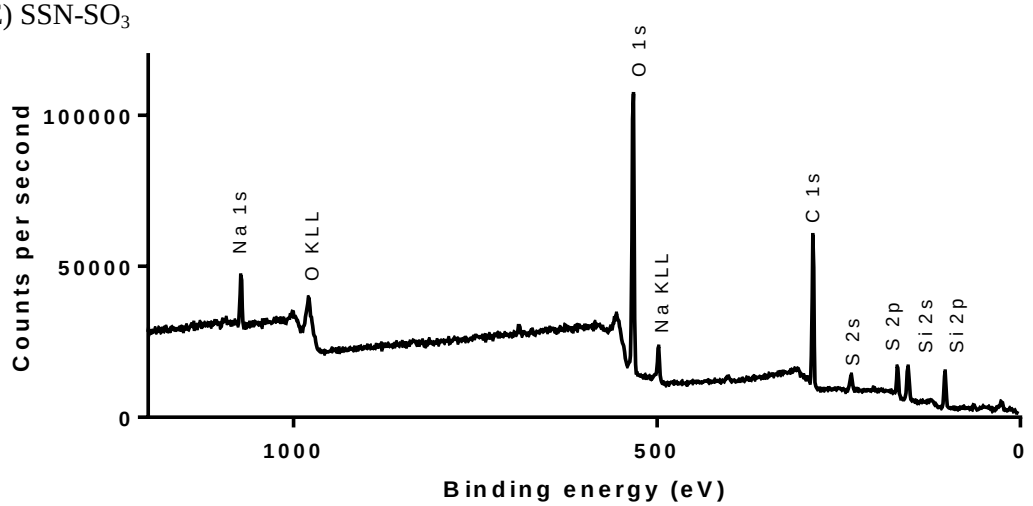
(C) SSN-SO_2Cl



(D) MSN-SO_2Cl



(E) SSN-SO_3



(F) MSN-SO_3

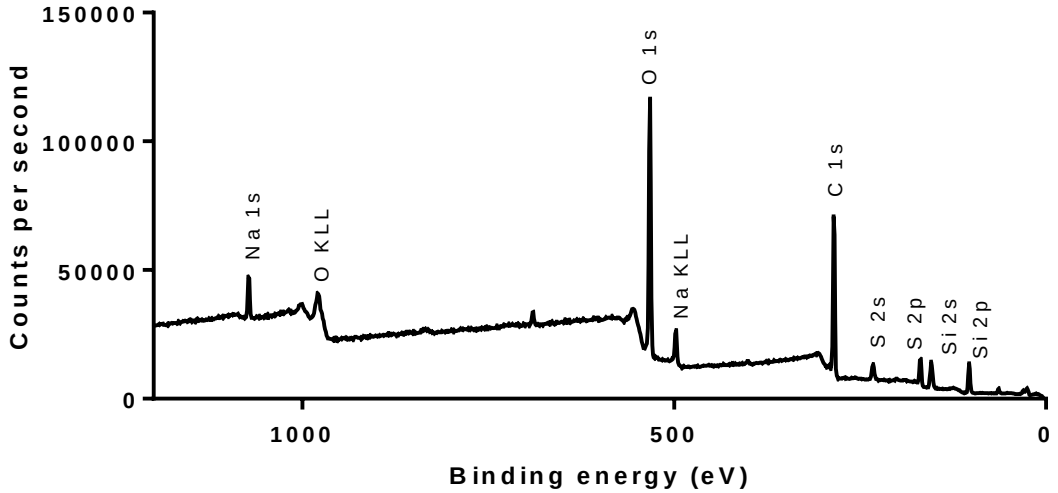
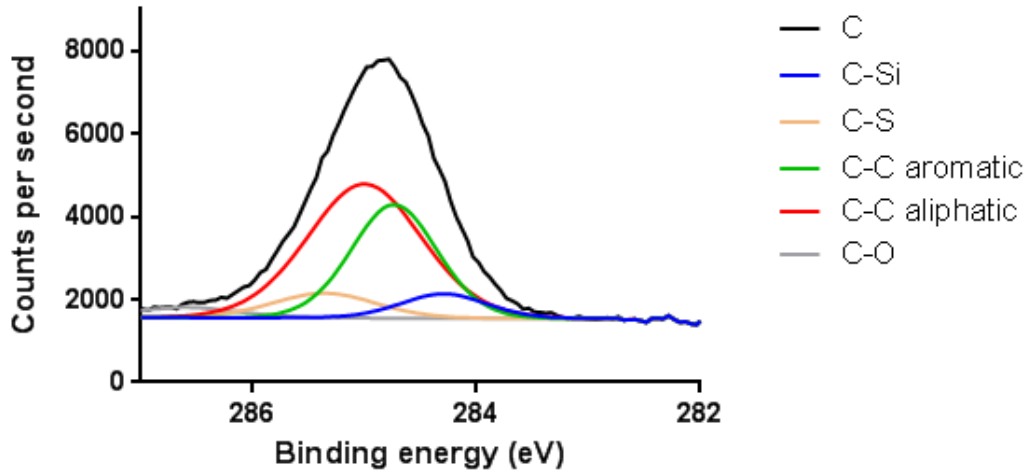


Fig. S3. XPS survey scan of: (A) SSN-bare; (B) MSN-bare; (C) SSN-SO_2Cl; (D) MSN-SO_2Cl; (E) SSN-SO_3; and (F) MSN-SO_3.

(A)



(B)

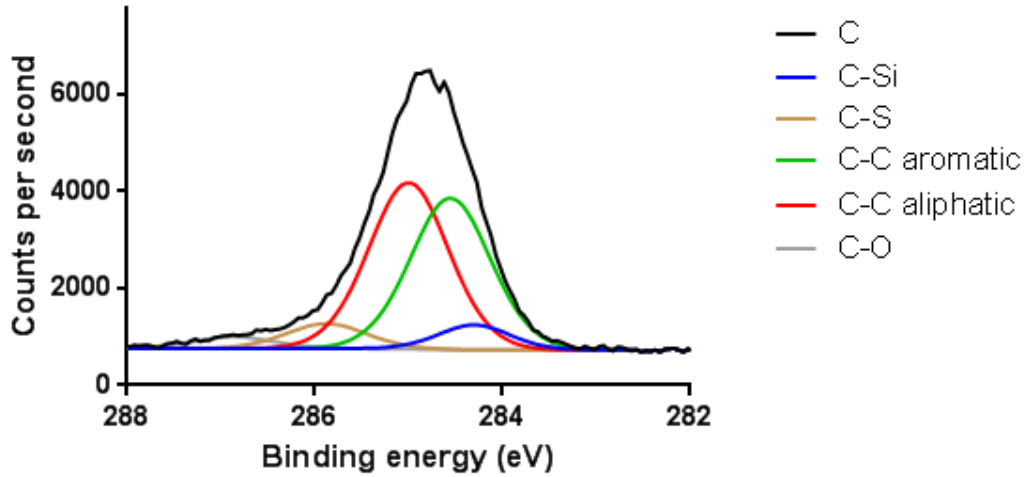
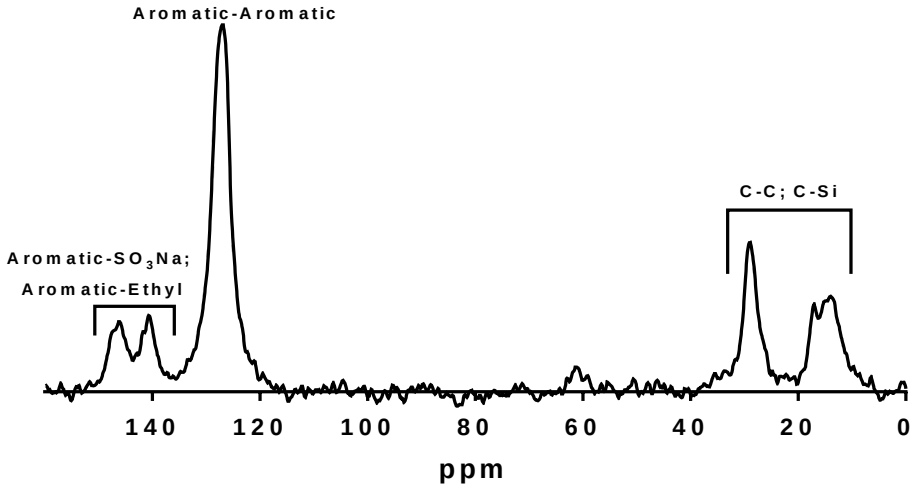


Fig. S4. Curve fitted high resolution XPS C 1s spectra of: (A) SSN-SO_3; and (B) MSN-SO_3.

(A)



(B)

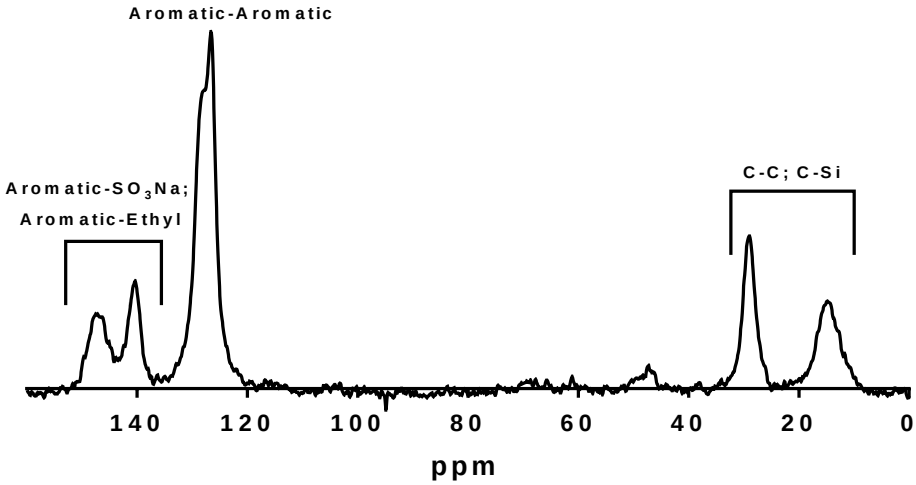


Fig. S5. ^{13}C SSNMR spectrum of: (A) SSN-SO_3; and (B) MSN-SO_3.

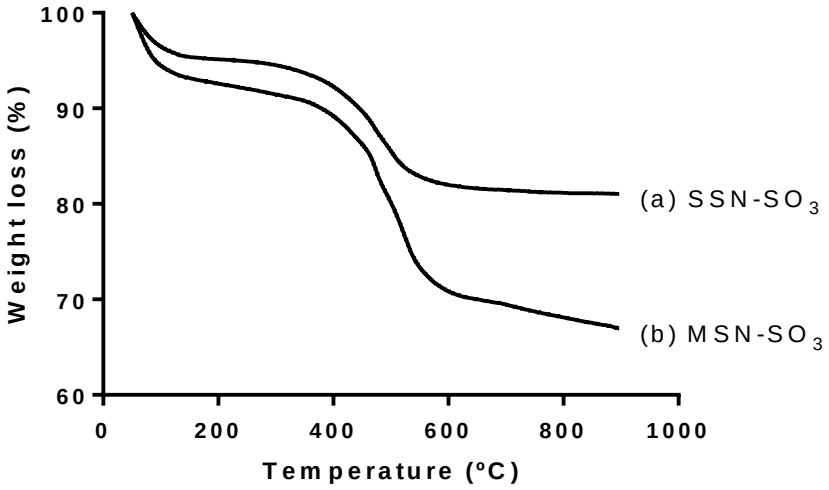


Fig. S6. TGA graphs of (a) MSN-SO_3 and (b) SSN-SO_3 expressed as % weight loss over temperature (ºC).

Table S3. Summary of TGA and elemental analysis results for the functionalised nanoparticles (**3**).

	Thermogravimetric analysis (% w/w)[a]	**Elemental analysis (% w/w)**			
		C	**S**	**C/S theoretical[b]**	**C/S observed[b]**
SSN-bare	5.2%	Not conducted.			
MSN-bare	4.3%				
SSN-SO_3	14.3% (9.1%)[c]	7.88	3.09	8.0	7.3
MSN-SO_3	26.3% (22.0%)[c]	16.95	5.96	8.0	7.6

[a] Calculated by subtracting weight loss between 150-900 ºC to exclude weight loss due to water.

[b] C/S based on molar ratio.

[c] Functionalisation density was calculated by subtracting the weight loss of **3** (between 150-900 ºC) with the loss of SSN-bare and MSN-bare (**1**).

Cytotoxicity assays

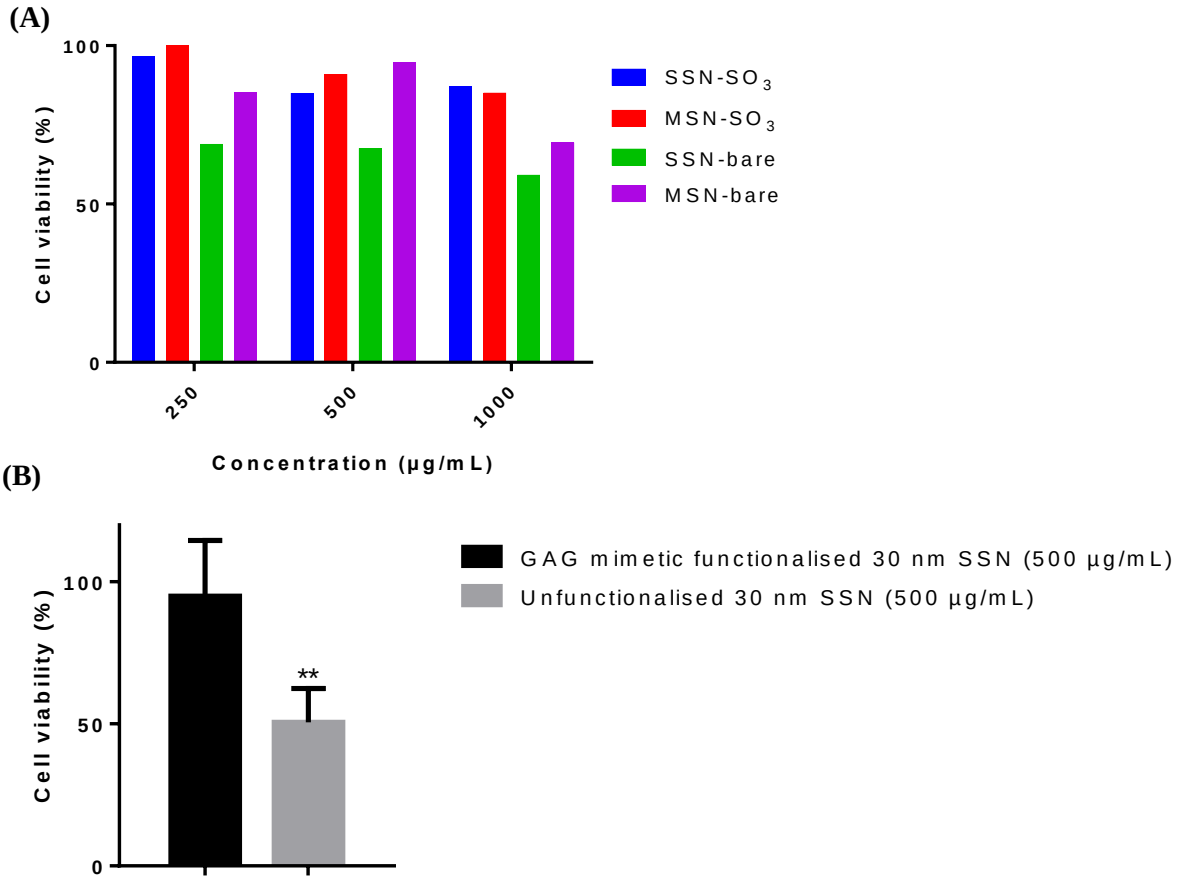


Fig. S7. MTS cell viability assay was used to determine cytotoxicity. (A) SSN-SO_3 and MSN-SO_3 (**3**) caused unsubstantial toxicity to Vero cells at the highest concentration of 1000 µg/mL after 48 h incubation (n = 1 independent experiment; single replicate) which indicates that conducting antiviral assays below this concentration is appropriate. As the cell exposure time to functionalised (**3**) and unfunctionalised (**1**) nanoparticles did not exceed 1 h for all antiviral assays, the possibility of observing cytotoxic effects are further reduced. In general, functionalising the nanoparticles with sodium benzene sulfonate appeared to reduce toxicity. This is supported by (B), which shows that 500 µg/mL of unfunctionalised 30 nm SSNs were significantly more toxic than the same size and concentration of SSNs functionalised with sodium benzene sulfonate on HEK293 cells after 48 h incubation. Error bars represent SD of three independent experiments (n = 3) as a single replicate. P-values were determined by unpaired two-tailed t-test (Student's t-test). **$p < 0.01$ (n = 3). Cell viability (%) was determined by the % absorbance of nanoparticle wells (corrected for nanoparticle interference) relative to untreated control wells.

Antiviral assays

Table S4. Characteristics of analogues of MSN-SO_3 (1.1% and 4.3% w/w functional group) estimated by DLS. Measurements were performed by preparing nanoparticle suspensions in type 2 pure water at approximately 1000 µg/mL. Functionalisation density (% w/w) were corrected for the weight loss of unfunctionalised MSNs.

Compound	Hydrodynamic diameter (nm)	PDI	Zeta potential (eV)
MSN-SO_3-1% (1.1% w/w)	196.3	0.261	-50.8
MSN-SO_3-4% (4.3% w/w)	203.0	0.212	-41.0

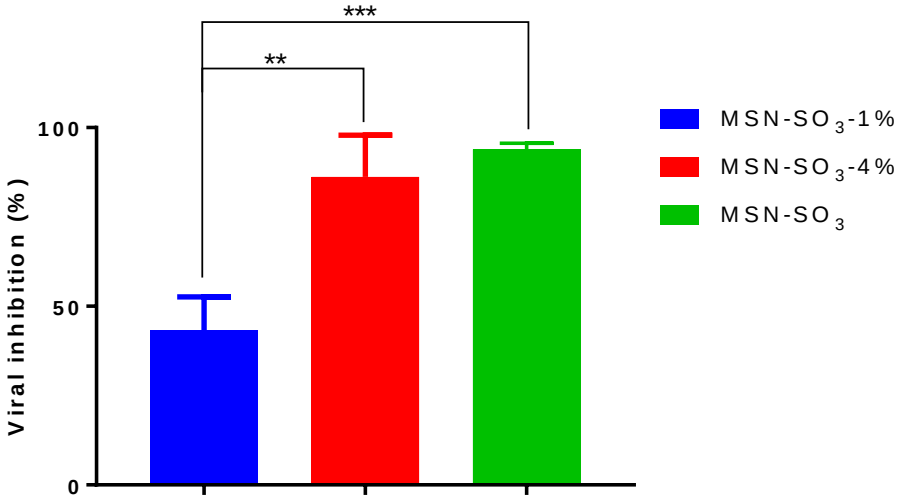


Fig. S8. The anti-HSV-1 activity of MSN-SO_3 analogues with lower functionalisation densities were evalulated using a plaque reduction assay. MSN-SO_3, MSN-SO_3-1%, and MSN-SO_3-4% had functionalisation densities of 22.0%, 1.1%, and 4.3% w/w, respectively. Each MSN-SO_3 was used at a concentration of 250 µg/mL (final concentration 208 µg/mL). The data of MSN-SO_3 is the same as is depicted in Fig. 2B. The results reveal that increasing the functionalisation density of sodium benzene sulfonate onto MSNs increases the antiviral activity against HSV-1. However, the antiviral potency appears to plateau at relatively low functionalisation density. It should be noted MSN-SO_3 were incubated with a higher viral concentration (approx. 120 pfu/well in untreated control) compared to MSN-SO_3-1% and MSN-SO_3-4% (approx. 49 pfu/well in untreated control). However, since MSN-SO_3 reached approximately 100% activity at this concentration, the differences in viral concentration is unlikely to affect the results. The result is expressed as % viral inhibition which was determined by the % reduction of plaque number compared to untreated control wells. Error bars represent SD of duplicate wells (n = 3 independent experiments). The differences in antiviral activity were significant based on one-way ANOVA. **$p < 0.01$; *** $p < 0.001$.

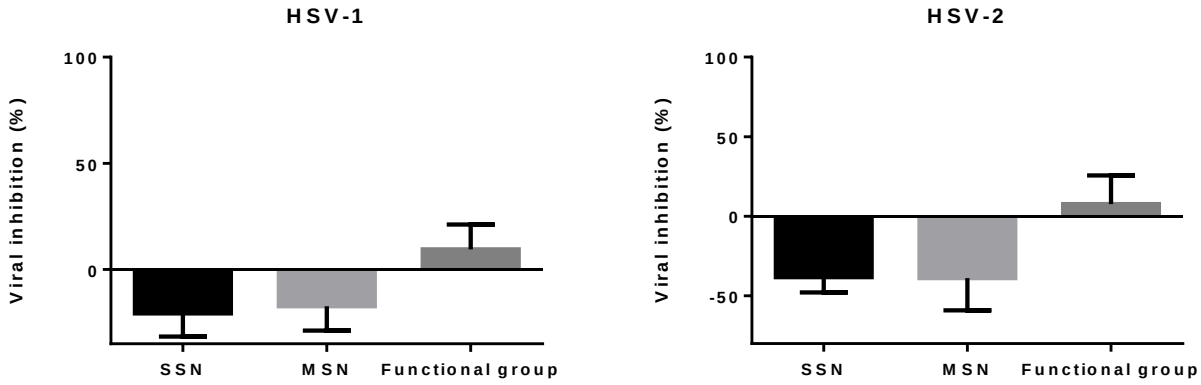


Fig. S9. The antiviral activity of unfunctionalised solid (SSN-bare) and mesoporous (MSN-bare) nanoparticles (**1**) and the functional group sodium benzene sulfonate alone, were investigated using the plaque reduction assay. The compounds were incubated with HSV-1 or HSV-2 for 1 h at a final concentration of 417 µg/mL and inoculated to Vero cells. The result is expressed as % viral inhibition which was determined by the % reduction of plaque number compared to untreated control wells. Error bars represent SD of triplicate wells (n = 1 independent experiment).

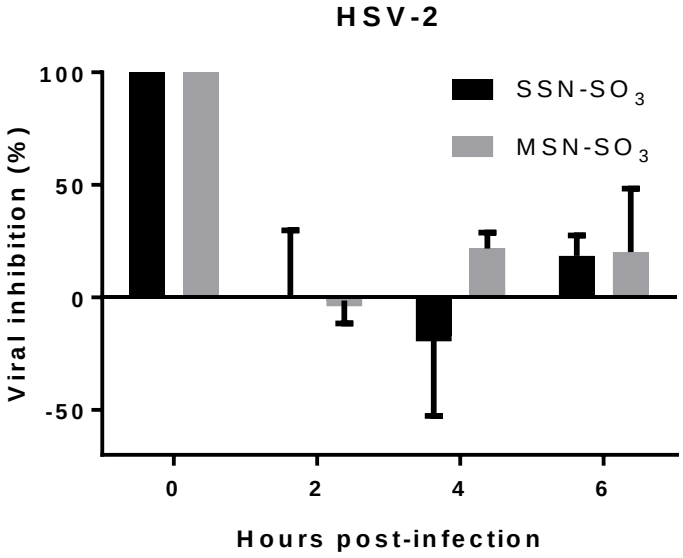


Fig. S10. A time-of-addition assay investigated the effect of nanoparticles (**3**) post-viral entry. Three time points post-viral entry were investigated. When nanoparticles (**3**) were added to cells at the same time with virus at a final concentration of 400 µg/mL, antiviral effect was observed. However, when the functionalised nanoparticles were added at t = 2, t = 4 and t = 6 h post-infection, minimal response was observed indicating an inability of the nanoparticles to inhibit viral infection post-entry. This supports their mechanism of **3** as a viral entry inhibitor. The result is expressed as % viral inhibition which was determined by the % reduction of plaque number compared to untreated control wells. Error bars represent SD of triplicate wells (n = 1 independent experiment).

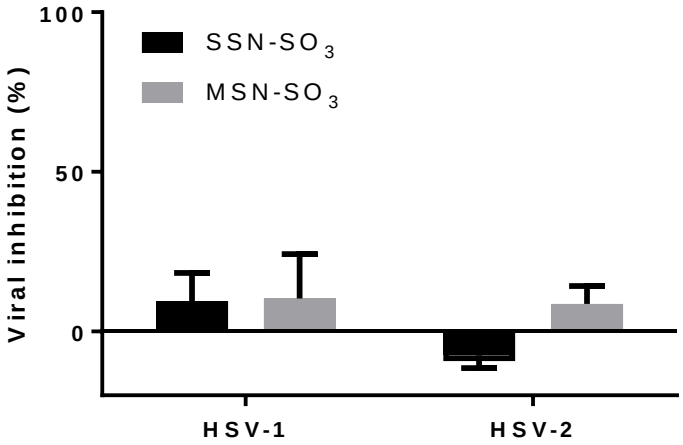


Fig. S11. A plaque reduction assay was conducted to determine if the functionalised nanoparticles (**3**) affect the ability of the cells to become infected with virus. Vero cells were incubated with nanoparticles at a final concentration 417 µg/mL SSN-SO_3 or MSN-SO_3 for 1 h at 37 ºC which resembles the protocol of previous antiviral assays. The nanoparticles were then removed by washing with PBS three times. The cells were infected with either HSV-1 or HSV-2 for 2 h at 37 ºC. Plaques were counted 72 h post infection. The results are expressed as % viral inhibition which was determined by the % reduction of plaque number compared to untreated control wells. Error bars represent SD of triplicate wells (n = 1 independent experiment).

References

1 M. Yu, Y. Niu, J. Zhang, H. Zhang, Y. Yang, E. Taran, S. Jambhrunkar, W. Gu, P. Thorn and C. Yu, *Nano Res*., 2016, **9**, 291-305.

2 T. Kim, P. Chung and V. S. Lin, *Chem. Mater*., 2010, **22**, 5093-5104.