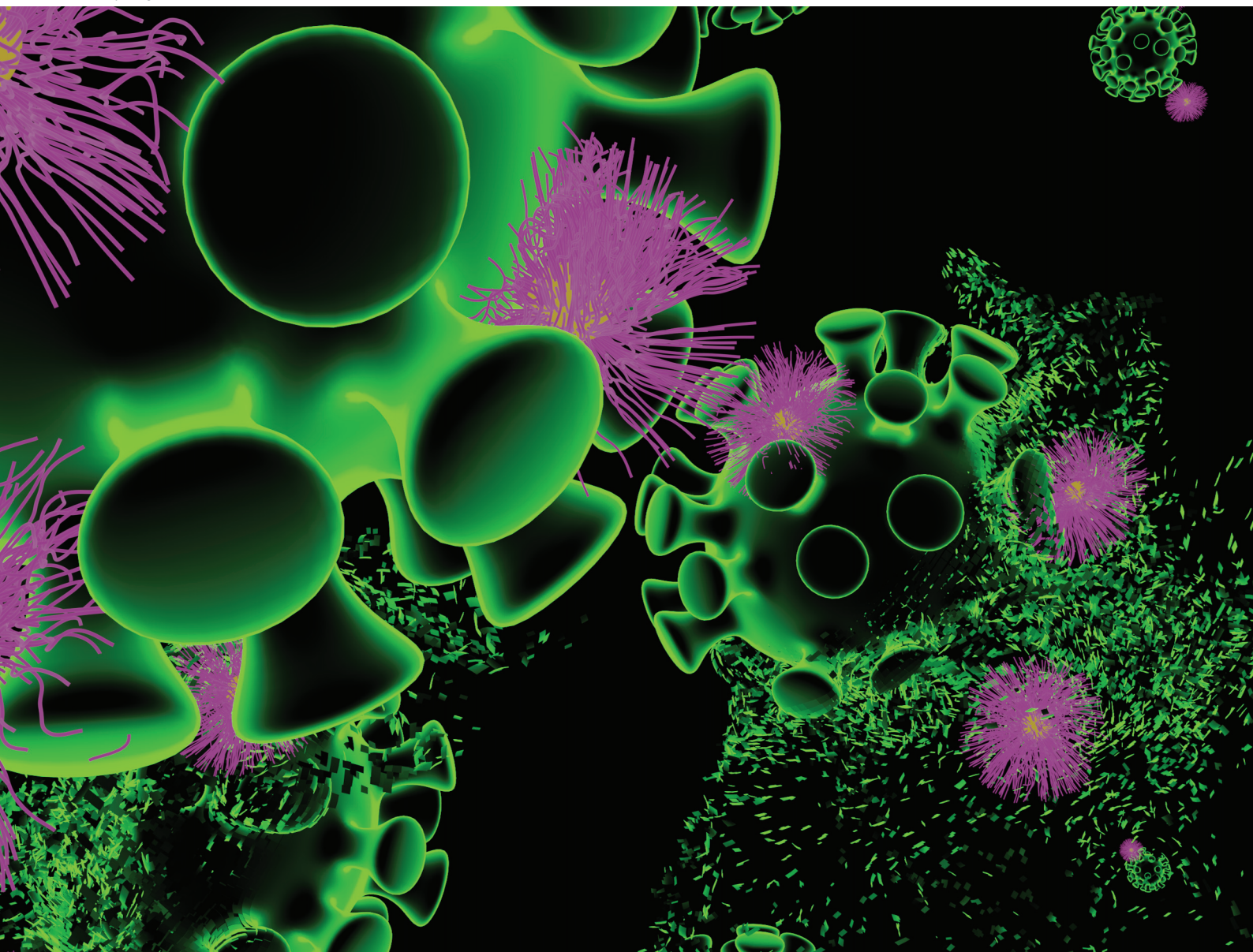


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## Antiviral mechanism change of poly(styrene sulfonate) through gold nanoparticle coating†

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Viruses are pathogens capable of causing serious global health problems and therefore the development of interventions against them is of paramount importance. One strategy towards designing broad-spectrum antivirals is through the mimicking of sulfonated glycopolymers on the cell surface so that the virion/cell interaction is inhibited by the antiviral material. A number of natural and synthetic polymers have been investigated, however, most show a virustatic mechanism, which is reversible and non-destructive. Herein we present a facile route to virucidal materials by attaching a previously known virustatic polymer, poly(styrene sulfonate), onto gold nanoparticles. We show that it is possible to alter the polymer's mode of action whilst maintaining its low IC<sub>50</sub> by changing the macromolecular architecture.

### Introduction

Viral infections continue to be a significant threat to global health.<sup>1</sup> There are over two hundred viruses currently known to be pathogenic to humans, and up to four new species being discovered every year.<sup>2</sup> In addition, viral outbreaks tend to occur without warning and therefore a rapid, as well as broad-spectrum, response is desired to overcome the barriers associated with other treatments, such as virus specificity or laborious development processes.

A key characteristic of current broad-spectrum antivirals is their extracellular mode of action.<sup>3</sup> One of the ways in which this can be achieved is through the mimicking of cell surface receptors, such as heparan sulfate proteoglycans (HSPGs),<sup>4</sup> or silicic acids.<sup>5</sup> Many viruses, through their viral attachment

ligands (VALs), use HSPGs as initial attachment sites.<sup>6</sup> Their role in viral attachment and cell entry for a wide range of viral families is well studied,<sup>4,7–16</sup> including for viruses such as: herpes,<sup>8</sup> papillomaviruses,<sup>9,11</sup> SARS-CoV-2,<sup>17</sup> and HIV-1.<sup>18</sup>

Both natural and synthetic sulfated/sulfonated materials have been shown to act as HSPG-mimics and bind to viruses; their antiviral properties has been known for many years.<sup>19–24</sup> Consequently, a wide array of materials, such as sulfonated nanoparticles,<sup>22,25,26</sup> both natural (such as sulfonated polysaccharides from seaweed) and synthetic polymers,<sup>27–29</sup> have been demonstrated as being broad-spectrum antivirals.

Whilst these materials have displayed broad-spectrum antiviral activity towards HSPG-binding viruses, their application as *in vivo* antivirals remains a challenge. The interaction of these materials has been shown to be reversible and prone to detachment from a virion upon dilution, rendering the virus active again; this has been termed as being virustatic.<sup>30–32</sup>

Recently, Groß and co-workers demonstrated the use of poly(styrene sulfonate) (PSS) decorated gold nanoparticles (AuNP) that showed broad-spectrum antiviral activity against a range of enveloped viruses, such as: SARS-COV-2, Zika, respiratory syncytial virus (RSV) and HIV-1.<sup>26</sup> They showed that over a broad range of PSS molecular weights, the nanoparticles showed: broad-spectrum antiviral activity, biocompatibility, and that the antiviral activity was not dependent on the size of the AuNP core itself but rather the size of the polymer. However, they reported that all of the PSS coated AuNPs they studied were virustatic.<sup>26</sup>

An alternative extracellular antiviral approach, termed virucidal, is one where interaction with the material results in an irreversible conformational change in the virion, which permanently prevents viral entry (*e.g.* bleach). This is regarded as the more favourable mode of action, as the virus is destroyed on contact, but it is often associated with greater cytotoxicity limiting its *in vivo* application. However, there have been reports, more recently, of materials with broad-spectrum virucidal properties with low cytotoxicity including cyclodextrin,<sup>25</sup> cucurbit[*n*]urils,<sup>22</sup> dendrimers,<sup>28</sup> and nanoparticles.<sup>30</sup>

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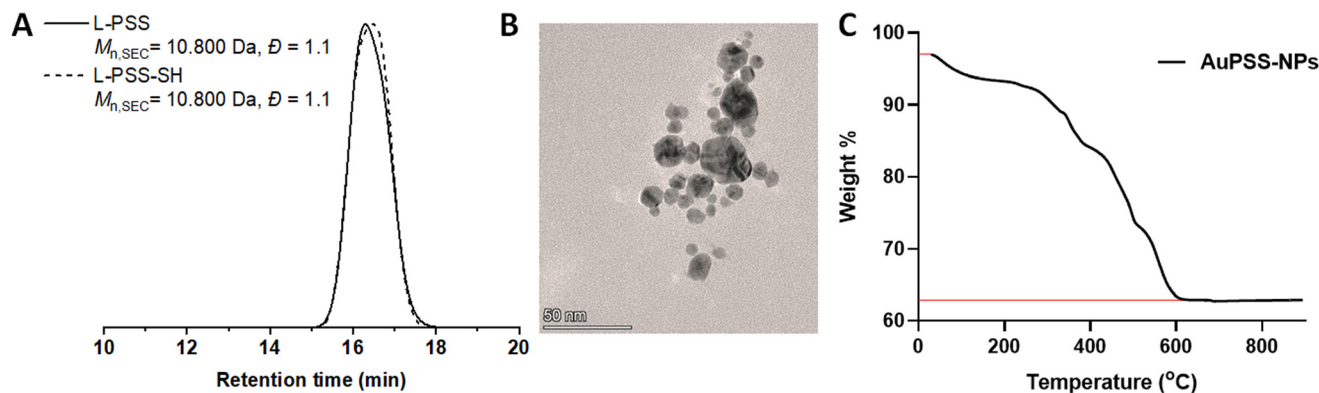
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† Electronic supplementary information (ESI) available: <sup>1</sup>H NMR of L-PSS, UV/vis spectrum of PSS-AuNP, DLS data for PSS-AuNP. See DOI: <https://doi.org/10.1039/d3py01217d>

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**Fig. 1** (A) Normalised SEC traces of L-PSS and L-PSS-SH measured in aqueous eluent (pH = 9 water : MeOH 70 : 30), (B) TEM image of PSS-AuNP (scale bar = 50 nm), and (C) TGA of PSS-AuNP showing a mass loss of 31%.

herpes simplex virus-2 (HSV-2), which has been used in previous studies to determine antiviral activity of polymeric and sulfonated materials.<sup>25,30,34</sup>

The  $IC_{50}$  values for PSS-AuNPs and L-PSS on HSV-2 were shown to be  $IC_{50} = 89.91 \text{ ng mL}^{-1}$  and  $IC_{50} = 27.90 \text{ ng mL}^{-1}$ , respectively (Fig. 2B); both of which is significantly (ten-fold) lower than previously reported virucidal materials,<sup>25,34</sup> as well as the previously reported virustatic PSS-coated AuNPs.<sup>26</sup> This highlights the advantages of utilising inherently multivalent polymers during the formation of a nanoparticle core for producing antiviral materials. A weight adjusted  $IC_{50}$  was also calculated by using the loss in polymer mass, as calculated from the TGA data; this showed that the  $IC_{50}$  value for PSS-AuNPs, not including the weight of the gold core, was  $IC_{50} = 28.02 \text{ ng mL}^{-1}$  (Fig. 2C). This shows that the antiviral efficacy of the PSS attached to the gold core had not been altered and remains a potent extracellular antiviral. In addition, fluorescence microscopy imaging confirmed that addition of antiviral to HSV-2, in a 1 : 1 volume ratio, showed a decrease in infection when compared to the no treatment control (Fig. S6†).

In order to show that attachment to AuNPs does not alter the broad-spectrum efficacy of PSS, we used a median tissue culture infectious dose ( $TCID_{50}$ ) assay against respiratory syncytial virus (RSV), which is a single stranded RNA virus with distinct differences to HSV-2, yet is well known to use HSPG during its infection cycle.<sup>35</sup> This confirmed that PSS-AuNPs were antiviral against RSV further supporting a potential broad-spectrum application of the material (Fig. S5†).

#### Antiviral mode of action check through serial dilution

Previous studies on PSS-coated AuNPs have reported a virustatic mode of action,<sup>26</sup> in order to determine the mode of action of PSS-AuNP a viral plaque reduction assay was performed.

Here the viral plaque count was monitored over a serial dilution of both PSS-AuNP and L-PSS; an assay used previously to determine virucidal activity.<sup>25,34,36</sup> Briefly, the antiviral is incubated with virus after which the mixture is incubated onto a cell line followed by serial dilution and the antiviral activity

is measured by counting the number of viral plaques formed. In general, if there is a reduction in the number of viral plaques, even with dilution (greater than 99% reduction, or 2log reduction as indicated by the red line in Fig. 3), the mode of action is considered virucidal, since the antiviral activity of the material remains intact over multiple dilutions.

Both L-PSS and PSS-AuNP were incubated with HSV-2 after which the mixture was diluted up to six-fold and incubated on a Vero cell line. Comparison of the plaque forming units (PFUs) of L-PSS and PSS-AuNP against the no treatment control (NTC), there was a greater reduction in PFU for PSS-AuNP than for L-PSS. Plotting the PFUs on a logarithmic scale showed that for PSS-AuNP the number of PFUs decreased by over 99% (equivalent to a greater than 2log reduction in PFU) whereas for L-PSS, the PFU decrease was only 66% (Fig. 3). These results would indicate that the bound PSS has a virucidal mode of action whereas the unbound PSS has a virustatic mode of action.

Further confirmation of change in mode of antiviral activity, for example by a DNA exposure assay, was performed but with inconclusive results as the highly anionic nature of the polymer interfered with the key component of the assay, DNase (Fig. S7†).

In conclusion, we demonstrate the switching of antiviral activity of PSS from virustatic to virucidal by changing the conformation of the polymer in solution through coating onto AuNPs. Linear PSS was synthesised by aqueous RAFT polymerisation and cleaved to the thiol, which was then used as a ligand for an *in situ* gold nanoparticles formation *via* a modified Brust-Schiffrin approach. Viral plaque assays with HSV-2 showed that whilst the linear polymer on its own showed virustatic activity, the PSS coated gold nanoparticles showed virucidal activity, indicating that the antiviral mode of action could be altered through PSS architecture with an  $IC_{50}$  value that was 10-fold lower than previously reported materials of similar composition. Previous PSS-coated AuNPs have shown significant potential as broad-spectrum antivirals for use *in vivo*, yet their mode of action limits their scope. Here, by accessing a virucidal mode of action, while maintaining low toxicity and



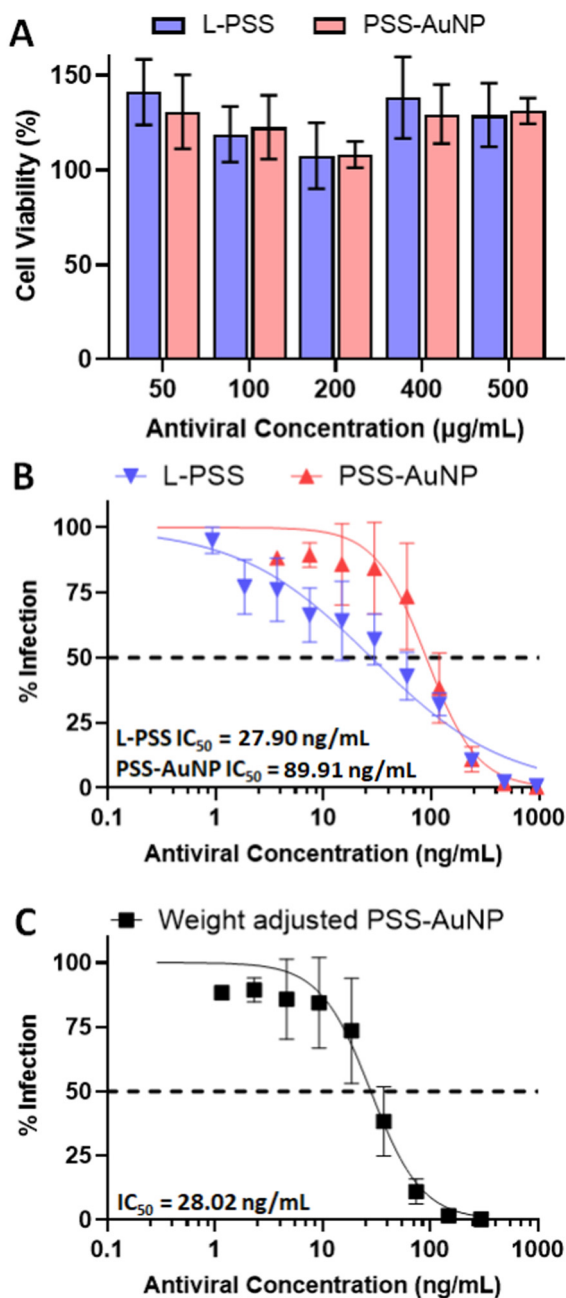


Fig. 2 (A) Metabolic activity of vero cells incubated with PSS-AuNP and L-PSS (B) dose response curves of L-PSS and PSS-AuNP against HSV-2 (C) AuNP weight adjusted dose response curve of PSS-AuNP.

broad-spectrum efficacy, these materials have significant potential as broad-spectrum *in vivo* virucides.

## Materials and methods

### Materials

4-(((2-Carboxy-ethyl)thiol)-carbono-thioyl)thio)-4-cyano-pentanoic acid (CTA) was purchased from Boron Pharmaceuticals. Sodium 4-vinylbenzenesulfonate (NaSS), 4,4'-azobis(4-cyano-

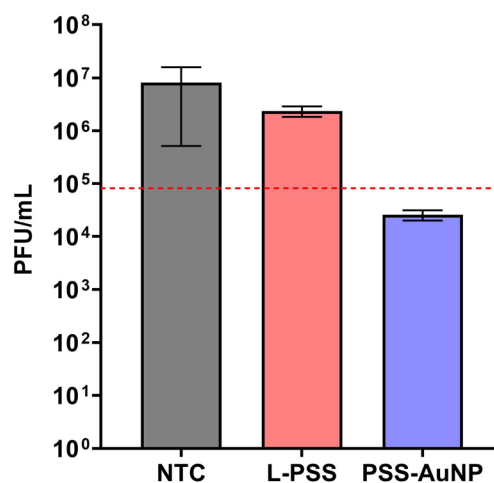


Fig. 3 Viral titer reduction of L-PSS and PSS-AuNP compared to a no treatment control (NTC) against HSV-2 where the red dotted line indicates a 2log reduction, or greater than 99% reduction, compared to the NTC.

leric acid) (ACVA), sodium borohydride (NaBH<sub>4</sub>), hydrazine solution, MEM Non-Essential Amino Acids were purchased from Sigma-Aldrich. 1 kDa and 10 kDa MWCO RC dialysis tubing were purchased from Spectrum. High glucose Dulbecco's Modified Eagle Medium (DMEM) media, Minimum Essential Medium (MEM), Fetal Bovine Serum (FBS), and L-glutamine, 99% were purchased from ThermoFisher Scientific. CellTiter 96<sup>®</sup> Aqueous MTS Reagent was purchased from Promega.

The cell line used in viral culture was Vero (African green monkey fibroblast kidney cells, ATCC CCL-81) kindly donated by Professors Pamela Valley and Paul Klapper in the University of Manchester Faculty of Biology, Medicine and Health (UoM, FBMH). The cell line used in cytotoxicity studies was HepG2 (a human liver hepatocellular carcinoma cell line, HB-8065) donated by the UoM Biomaterials research group.

Isolates of respiratory syncytial virus (RSV) and herpes simplex virus (HSV-2) were provided by Professors Pamela Valley and Paul Klapper (University of Manchester, UK), and propagated in vero cells, generating stocks of known log (TCID<sub>50</sub>) per mL.

### Cell culture

Vero cells were cultured in high glucose DMEM media supplemented with 2% FBS and 1% L-glutamine. HepG2 cells were cultured in MEME media supplemented with 10% FBS, 1% L-glutamine and 0.1% non-essential amino acids.

### Instrumentation

**Dynamic light scattering (DLS).** DLS and zeta potential measurements were performed using a Malvern Zetasizer Nano equipped with a He-Ne solid-state laser operating at 633 nm and back-scattered light at a scattering angle of 173° at a concentration of 2 mg mL<sup>-1</sup>.



**Thermogravimetric analysis (TGA).** TGA was performed using a TGA Q500 under a nitrogen flow of 25 mL min<sup>-1</sup> from 25 °C to 900 °C at a rate of 10 °C min<sup>-1</sup>. The sample (31.5 mg) was prepared on an aluminium pan.

**Gel permeation chromatography (GPC).** PSS was analysed with PSS SEC instrument equipped with a differential refractive index (DRI). The system was equipped with 2X PL aquagel-OH MIXED-M (400 × 7.5 mm) and a PL aquagel-OH guard column. The eluent is 70% Water (pH = 9), 0.2 M NaNO<sub>3</sub>:MeOH 30% Narrow poly(ethylene oxide) standards were used for calibration between 100–1 300 000 g mol<sup>-1</sup>. All samples were filtered through a 0.2 μm nylon filters before injection.

**Transmission electron microscopy (TEM).** TEM images were recorded using a Thermo Fisher Scientific Talos F200A AEM instrument operating at an accelerating voltage of 200 kV. Samples were prepared by depositing 3 μL of diluted PSS-AuNPs onto 400 mesh carbon-coated copper grids for 30 min and then carefully blotted with filter paper to remove excess solution. Mean nanoparticle diameters were determined by ImageJ software.

## Experimental

### RAFT polymerisation of linear poly(styrene sulfonate), L-PSS

NaSS (2.00 g, 9.70 mmol), CTA (59.6 mg, 0.194 mmol), and ACVA (5.44 mg, 0.019 mmol) were dissolved in deionised water and degassed with N<sub>2</sub>. The mixture was then left to stir in a oil bath set to 70 °C for 2.5 hours. The reaction was then quenched in an ice bath and dialysed against water.

### RAFT agent cleavage to thiol

L-PSS (725 mg, 0.079 mmol) was dissolved in water (2 mL) and hydrazine was added (19.8 mg, 0.396 mmol) and left to stir for 3 hours upon which the colour of the solution changed from clear yellow to colourless. The cleaved L-PSS was dialysed against water in 1 kDa MWCO RC dialysis tubing for 3 days.

### PSS-AuNP formation

PSS-AuNPs were synthesised using a modified Brust-Schiffin method.<sup>37</sup> Briefly, a 1 mM solution of HAuCl<sub>4</sub> was added to a stirring solution of L-PSS (9 mM) NaBH<sub>4</sub> (1.24 mL of 1 mg mL<sup>-1</sup> solution) was then added and left to stir for 1 hour, upon which the solution changed colour from pale yellow to amber. The solution was then dialysed against water in 10 kDa MWCO RC dialysis tubing for 3 days to remove any unreacted L-PSS.

### MTS assays

HepG2 cells were incubated with L-PSS and PSS-AuNPs at concentrations of 500 mg/mL–50 mg mL<sup>-1</sup> for 24 hours (37 °C, 5% CO<sub>2</sub>). The cells were then washed with PBS and fresh MEME media was added. 20 μL of MTS reagent was added and incubated with the cells for four hours, as per the manufacturer's instructions, and the absorbance at λ = 490 nm was

recorded using a plate reader. The significance of differences in absorbance values in cells treated with the materials as compared to NTCs was calculated using the unpaired *t*-test.

### HSV-2 dose response assays

A 1:100 dilutions of HSV-2 virus stock was treated with a range of concentrations of L-PSS and PSS-AuNPs for 1 hour (37 °C, 5% CO<sub>2</sub>). These samples were then incubated with vero cells (24-well plate of >99% confluency) and incubated for a further hour. The samples were then removed and the cells overlaid with a 3:7 ratio of methylcellulose (1.5 wt% methylcellulose in DI) and 2% FBS DMEM overlay (MTC overlay) and incubated for 24–27 h (37 °C, 5% CO<sub>2</sub>). After incubation the cells were fixed and stained with crystal violet. Plaque-counting was performed using a standard light microscope.

### RSV TCID<sub>50</sub> assay

The virus stock samples were treated with a concentration range of L-PSS and PSS-AuNPs for 1 hour (37 °C, CO<sub>2</sub>). The samples were then serially diluted across 96 well plates which had been seeded with 6000 vero cells per well the previous day. The plates were incubated for 5 days and then fixed and stained with crystal violet. RSV infectivity across each plate was measured using the Spearman-Kärber formula and compared back to NTC plates.

### HSV-2 virucidal assay

An inhibitory concentration achieving IC<sub>90</sub>–IC<sub>99</sub> within the virucidal assay was identified for both L-PSS and PSS-AuNPs. Virus stocks were treated with these concentrations for 1 hour (37 °C, 5% CO<sub>2</sub>). The viral samples were then serially diluted across 96 well plates of confluent vero cells (monolayer) resulting in a post-treatment dilution range of 1:30–1:2.187 × 10<sup>2</sup>. The plates were incubated for 1 hour (37 °C, 5% CO<sub>2</sub>) before the diluted samples were removed from the plate and replaced with MTC overlay. The plates were then further incubated for 24 hours then fixed and stained with crystal violet. Plaque-counting was performed using a standard light microscope and the pfu mL<sup>-1</sup> of the viral samples treated with L-PSS and PSS-AuNPs was calculated and compared to the NTC. As previously described, a difference of 2log or greater was necessary to confirm either material as virucidal, otherwise the material was considered virustatic.<sup>25,30</sup>

## Author contributions

L. B. performed all the experiments and wrote the original draft, J. K. wrote the final manuscript, L. M. J. performed the microscopy and contributed in the original manuscript draft, E. S. performed additional assays and contributed in the original manuscript draft, and S. T. J. was involved in conceptualisation, writing of manuscript, and project supervision.



## Conflicts of interest

There are no conflicts to declare.

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