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# Broad-spectrum Non-toxic Antiviral Nanoparticles with a Virucidal Inhibition

## Mechanism

Valeria Cagno<sup>1,2,3†</sup>, Patrizia Andreozzi<sup>4,5†</sup>, Marco D'Alicarnasso<sup>6</sup>, Paulo Jacob Silva<sup>2</sup>, Marie Mueller<sup>2</sup>, Marie Galloux<sup>7</sup>, Ronan Le Goffic<sup>7</sup>, Samuel T. Jones<sup>2,8</sup>, Marta Vallino<sup>9</sup>, Jan Hodek<sup>10</sup>, Jan Weber<sup>10</sup>, Soumyo Sen<sup>11</sup> Emma-Rose Janeček<sup>2</sup>, Ahmet Bekdemir<sup>2</sup>, Barbara Sanavio<sup>12</sup>, Chiara Martinelli<sup>4</sup>, Manuela Donalisio<sup>1</sup>, Marie-Anne Rameix Welti<sup>13,14</sup>, Jean-Francois Eleouet<sup>7</sup> Yanxiao Han<sup>11</sup>, Laurent Kaiser<sup>15</sup>, Lela Vukovic<sup>16</sup>, Caroline Tapparel<sup>3,15</sup>, Petr Král<sup>11,17</sup>, Silke Krol<sup>12,18</sup>, David Lembo<sup>1\*</sup>, Francesco Stellacci<sup>2,19\*</sup>

<sup>1</sup>Dipartimento di Scienze Cliniche e Biologiche, Univerisita' degli Studi di Torino, Orbassano, Italy.

<sup>2</sup>Institute of Materials, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland.

<sup>3</sup>Faculty of Medicine of Geneva, Department of Microbiology and Molecular medicine, Geneva, Switzerland.

<sup>4</sup>IFOM - FIRC Institute of Molecular Oncology, IFOM-IEO Campus, Milan, Italy.

<sup>5</sup>CIC biomaGUNE Soft Matter Nanotechnology Group San Sebastian-Donostia, Spain.

<sup>6</sup>Fondazione Centro Europeo Nanomedicina (CEN), Milan, Italy.

<sup>7</sup>VIM, INRA, Université Paris-Saclay, Jouy-en-Josas, France.

<sup>8</sup>Jones Lab, School of Materials, University of Manchester, Oxford Road, Manchester, M13 9PL

<sup>9</sup>Istituto per la Protezione Sostenibile delle Piante, CNR, Torino, Italy.

<sup>10</sup>Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic, Prague, Czech Republic.

<sup>11</sup>Department of Chemistry, University of Illinois at Chicago, USA.

<sup>12</sup>Fondazione IRCCS Istituto Neurologico «Carlo Besta», IFOM-IEO Campus, Milan, Italy.

<sup>13</sup>UMR INSERM U1173 I2, UFR des Sciences de la Santé Simone Veil—UVSQ, Montigny-Le-Bretonneux, France

<sup>14</sup>AP-HP, Laboratoire de Microbiologie, Hôpital Ambroise Paré, 92104, Boulogne-Billancourt, France

<sup>15</sup>Geneva University Hospitals, Infectious Diseases Divisions, Geneva, Switzerland.

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<sup>16</sup>Department of Chemistry, University of Texas at El Paso, USA.

<sup>17</sup>Department of Physics and Department of Biopharmaceutical Sciences, University of Illinois at Chicago, USA.

<sup>18</sup>IRCCS Istituto Tumori "Giovanni Paolo II", Bari, Italy

<sup>19</sup>Interfaculty Bioengineering Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland.

56 **ABSTRACT**

57 Viral infections kill millions yearly. Available antiviral drugs are virus-specific and active  
58 against a limited panel of human pathogens. There are broad-spectrum substances that prevent  
59 the first step of virus-cell interaction by mimicking heparan-sulfate proteoglycans (HSPG), the  
60 highly-conserved target of viral attachment ligands (VAL). The reversible binding mechanism  
61 prevents their use as a drug, because, upon dilution, the inhibition is lost. Known VAL are made  
62 of closely packed repeating units but the aforementioned substances are able to bind only a few  
63 of them. We designed antiviral nanoparticles with long and flexible linkers mimicking HSPG,  
64 allowing for effective viral association with a binding that we simulate to be strong and  
65 multivalent to the VAL repeating units, that generates forces (~190 pN) that eventually lead to  
66 irreversible viral deformation. Virucidal assays, electron microscopy images, and molecular  
67 dynamics simulations support the proposed mechanism. These particles show no cytotoxicity,  
68 and *in vitro* nanomolar irreversible activity against Herpes Simplex Virus (HSV), Human  
69 Papilloma Virus, Respiratory Syncytial Virus (RSV), Dengue and Lenti virus. They are active *ex*  
70 *vivo* in human cervicovaginal histocultures infected by HSV-2 and *in vivo* in mice infected with  
71 RSV.

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74 Infectious diseases account for ~20% of global mortality, and viruses are responsible for  
75 about one third of these deaths.<sup>1</sup> Lower respiratory infections and Human Immunodeficiency  
76 Virus (HIV) are among the first ten causes of deaths worldwide, and they contribute substantially  
77 to healthcare costs.<sup>2</sup> Emerging viruses (e.g. Ebola) add yearly to this death toll. The best  
78 approach to prevent viral infections is vaccination, however there exist only a limited number of  
79 vaccines and the ones that exist are not equally available in all parts of the world.<sup>3</sup> After  
80 infection, antiviral drugs are the only treatment option, but even in this case there are only a  
81 limited number of approved antiviral drugs and they are all virus specific. There is a dire need  
82 for broad-spectrum antiviral drugs that can act on a large number of existing and emerging  
83 viruses.

84 Current therapeutics can be subdivided into i) small molecules (e.g. nucleoside analogues  
85 and peptidomimetics), ii) proteins able to stimulate the immune response (e.g. interferon), and  
86 iii) oligonucleotides (e.g. fomivirsen).<sup>4</sup> They are mainly directed against HIV, hepatitis B virus  
87 (HBV), hepatitis C virus (HCV), herpes simplex virus (HSV), human cytomegalovirus (HCMV)  
88 and influenza virus. They act intracellularly, mostly on viral enzymes that are essential for viral  
89 replication but differ from any other host enzyme to allow for selectivity. Since viruses largely  
90 depend on the biosynthetic machinery of infected cells for their replication the specificity of  
91 antiviral drugs is far from ideal, resulting in a general intrinsic toxicity associated with such  
92 treatment.<sup>5,6</sup> Additionally, most viruses mutate rapidly due to error-prone replication machinery,  
93 therefore they often develop resistance.<sup>7</sup> Finally, the use of virus specific proteins as a target of  
94 antiviral drugs makes it difficult to develop broad-spectrum antivirals capable of acting on a  
95 large number of viruses that are phylogenetically unrelated and structurally different.

96           *Virustatic* substances act outside the cell by interfering with the first phases of the viral  
97 replication cycle. They can be broad spectrum and non-toxic. Their activity depends on a  
98 reversible binding event; the reversibility of the mechanism makes them medically irrelevant.  
99 For example, upon dilution the substance is detached from an unaltered viral particle allowing  
100 the virus to infect again. To achieve broad-spectrum efficacy, current virustatic materials (e.g.  
101 heparin, polyanions) target virus-cell interactions that are common to many viruses. One of these  
102 interactions is that between the VAL and its associated cell receptor responsible for the first step  
103 of the virus replication cycle. Many viruses, including HIV-1, HSV, HCMV, HPV, RSV and  
104 Flavivirus,<sup>8</sup> exploit HSPGs as the target of their VALs. HSPGs are expressed on the surface of  
105 almost all eukaryotic cell types. The binding between viruses and HSPGs usually occurs *via* the  
106 interaction of closely-packed arrangements of multiple basic amino acids on the proteins, that  
107 constitute the VAL, with the negatively charged sulfated groups of heparan sulfate (HS) in the  
108 glycocalyx of the cell surface.<sup>9</sup> A long list of HSPG mimicking materials such as heparin,<sup>10,11</sup>  
109 sulfated polysaccharides,<sup>9,12</sup> or sulfonic acid decorated polymers, dendrimers, and  
110 nanoparticles<sup>13-17</sup> have been tested and shown to exert potent virustatic activity *in vitro*, none  
111 have shown efficacy in humans. The only three polyanionic anti-HIV-1 microbicides that  
112 reached phase III clinical trial (i.e. polysulfonated PRO2000, the polysulfated Carraguard, and  
113 cellulose sulfate Ushercell) did not prevent vaginal HIV-1 transmission and in some cases even  
114 increased the rate of infection.<sup>18-21</sup> One of the possible explanations is that their effect was  
115 simply virustatic and hence vaginal and seminal fluids lead to the dilution of both the viruses and  
116 the active compounds, which resulted in the complete loss of binding and release of active virus.

117           Arguably, the ideal drug against a viral infection would be *virucidal*. Virucidal molecules  
118 cause irreversible viral deactivation, indeed their effect is retained even if dilution occurs after



119 the initial interaction with the virus.<sup>22</sup> There is a vast literature on many virucidal materials  
120 ranging from simple detergents, to strong acids, or more refined polymers,<sup>23</sup> and nanoparticles  
121 (NPs)<sup>24-27</sup> that, in some cases, are capable of releasing ions.<sup>28,29</sup> In all cases, the approaches  
122 utilized have intrinsic cellular toxicity.<sup>26</sup> Indeed, all of these materials attempt to chemically  
123 damage the virus, but it is a tall order to selectively damage a virus without affecting the host the  
124 virus replicates within.

125 An ideal drug should have all the positive properties of virustatic drugs such as broad-  
126 spectrum efficacy and low toxicity, and at the same time show a virucidal mechanism. In this  
127 paper, we show that it is possible to change the mechanism of inhibition of an antiviral  
128 nanoparticle from virustatic to virucidal by engineering its linkers in a way that we hypothesize  
129 leads to multivalent binding (i.e. the binding of multiple targets at the same time) with the  
130 consequent generation of irreversible local distortion as schematically illustrated in Figure 1A.  
131 Most VALs have binding domains composed of closely packed repeating units, hence they are  
132 ideally suited for multivalent binding to their cell receptor. All the known HSPG-mimicking  
133 NPs, polymers and dendrimers<sup>15</sup> display short linkers to expose sulfonate groups to the viral  
134 ligands, including gold NPs coated with 3-mercaptopethylsulfonate (MES)<sup>16</sup> and heparin. The  
135 relative rigidity of the sulfonate linkers should reasonably lead to the binding of only a few of the  
136 repeating units that constitute a VAL. Consequently, the resulting binding is weak and  
137 reversible.<sup>31,32</sup> On the other hand, it is known<sup>31, 32</sup> that particles, when binding strongly to a  
138 membrane (i.e. a vesicle, but we extrapolate this also to viral envelopes or capsids), can lead to  
139 significant local distortions. Hence, we replace the short linkers in MES-NPs with long ones, to  
140 achieve strong multivalent binding. We show here that strong multivalent binding leads to local  
141 distortions and eventually to a global virus deformation, with the consequent irreversible loss of

142 infectivity. We compare MES coated gold NPs, as well as heparin, with a series of NPs coated  
143 with undecanesulfonic acid (MUS) containing ligands. All NPs show *in vitro* inhibition of many  
144 HSPG dependent viruses either enveloped (HSV, RSV, Lentivirus and Dengue virus) or naked  
145 (HPV). But while the effect of the MES-NPs and heparin is lost with dilution, all MUS coated  
146 NPs show a clear irreversible effect. As expected the ‘upgrade’ from a virustatic to a virucidal  
147 mechanism adds to all of the positive traits of the former (i.e. minimal toxicity and *in-vitro* broad  
148 spectrum efficacy) a strong effect *ex-vivo* on human cervicovaginal histocultures infected by  
149 HSV-2 that is absent in the parental virustatic drugs and a strong effect *in-vivo* in mice infected  
150 with RSV.

151

## 152 **Virus and Nanoparticles Description**

153 To evaluate the inhibitory activity of our nanoparticles (NPs) we used the following  
154 viruses: HSV type 1 (HSV-1), HSV type 2 (HSV-2), pseudoviruses of human papillomavirus  
155 type 16 (HPV-16), RSV, vesicular stomatitis virus pseudo-typed lentivirus (LV-VSV-G) and  
156 Dengue virus. All of the viruses above are HSPG dependent viruses. We used adenovirus-5  
157 (AD5), a non-HSPG dependent virus, as a control. To mimic HSPG, we prepared NPs coated  
158 with MES and NPs coated with MUS. MES-NPs are reported in literature and are supposed to be  
159 virustatic, *i.e.* the sulfonic acid moieties at the end of their short linkers are effective mimics of  
160 HSPG and as a consequence they show good efficacy against a number of HSPG-dependent  
161 viruses. The postulated mechanism of virus binding to HSPGs is reversible in nature. To render  
162 it irreversible we chose to replace MES with MUS as this ligand has a long hydrophobic  
163 backbone terminating with a sulfonic acid, allowing its terminal group to move with some  
164 freedom. Consequently, NPs coated with MUS are ideal for multivalent binding, in this case the

165 binding of multiple sulfonic acids to the HSPG-interacting motifs on the virus surface. Gold NPs  
166 coated with MUS ligands were selected, as they are the simplest non-toxic particles that can be  
167 synthesized with these ligands. Other NPs selected in the present study are the particles coated  
168 with a 2:1 mixture of MUS and 1-octanethiol (OT), as they are the most biocompatible, soluble,  
169 and resistant to protein non-specific adsorption version of MUS-coated gold particles that we  
170 have studied.<sup>33-36</sup> All used NPs are summarized in Table 1, and all synthetic methods and  
171 characterizations are presented in the supplementary information (SI) (Methods Section and  
172 Supplementary Figures 1 to 7).

173

#### 174 **Viral Inhibition**

175 Each virus was pre-incubated with different doses of gold NPs for 1 h at 37°C and 5%  
176 CO<sub>2</sub>; then the mixture was added to the cell culture (see Methods Section in SI for virus-specific  
177 protocol details, initial viral load, and cell types), and infectivity was tested 24-72 h post  
178 infection. For the GFP expressing viruses (LV-VSV-G, AD-5 and HPV-16) the infectivity was  
179 quantified by flow cytometry, while plaque assays were used for wild-type viruses. Table 2  
180 summarizes the results. It is noteworthy that the MUS functionalized NPs i) are indeed non-toxic  
181 at these concentrations showing favourable selectivity indexes, ii) are able to inhibit infection  
182 selectively for HSPG dependent viruses (i.e. no inhibition is observed for AD5), and that iii) all  
183 EC<sub>50</sub> are in the nanomolar range (see Methods Section in SI for calculations of moles of NP). It  
184 is important to underline that the monomeric sulfonated ligand (MUS molecule) was not  
185 effective in inhibiting LV-VSV-G (Supplementary Figure 8). One possible explanation for the  
186 lack of inhibition for the MUS molecule could be interactions between various chemical groups  
187 on the surface of viruses with the thiols at the end of the ligands. We believe that this explanation

188 is not the correct one as, no inhibitory activity of sodium undec-10-enesulfonate (pre-MUS), a  
189 molecule equivalent to MUS but lacking the thiol end-group, was detected against all the viruses  
190 tested.

191

192

193 To further test that the NPs affect infectivity by mimicking the attachment receptor for  
194 HSPG-binding viruses, we performed a series of control experiments. gold NPs coated with 11-  
195 mercaptoundecylphosphoric acid (MUP) ligands (Supplementary Figure 5) were synthesized,  
196 thus creating NPs of similar size, ligand- and charge-density to the MUS-NPs but replacing the  
197 sulfonate with phosphonate groups. In contrast to the MUS-NPs, the MUP-NPs showed no  
198 inhibitory activity when mixed with pseudo-lentivirus (LV-VSV-G), highlighting the importance  
199 of the sulfonic acid group for the activity of the particles. Finally, no inhibitory activity of 15 nm  
200 in diameter citrate-coated gold NPs was detected. In Supplementary Discussion 1 we detail  
201 experiments aimed at establishing that the particles actually do target the HSPG seeking VAL.

202

## 203 **Virucidal Results**

204 As explained above, other sulfonated materials <sup>16-19</sup> have also shown similar inhibitory  
205 effects as shown for MUS-NPs in Table 2, but these effects have been proven <sup>10,11</sup> or are  
206 assumed <sup>33</sup> to be virustatic and hence due to reversible attachment alone. To test whether a  
207 different inhibition mechanism was in place for our particles, we first verified the ability of our  
208 NPs to inhibit viral attachment, as is known for heparin (Supplementary Figure 9). Then, we  
209 verified the ability of MUS:OT-NPs, MES-NPs, and heparin of inhibiting viral infection. The

210 results are shown in the blue curves in Figure 1B and summarized in Table 1. In all cases we  
211 observed EC<sub>50</sub> in similar ranges. The inhibition assays were completed by standard toxicity tests.  
212 The orange curves in Figure 1B illustrate the results of cell viability studies. In all three cases no  
213 toxic effect was observed even at the highest concentrations. We then tested them for irreversible  
214 inhibitory activity through virucidal assays. These assays consist of an incubation of the virus  
215 and drugs at a concentration corresponding to the EC<sub>90</sub> for a given amount of time and the  
216 subsequent evaluation of the residual infectivity of the virus through serial dilutions of the  
217 inoculum. It is known <sup>19</sup> that if the effect is solely virustatic, the viral infectivity is fully  
218 recovered upon dilution, as we show here for heparin and MES gold NPs against HSV-2 (Figure  
219 1B). As expected in both cases we found these particles to have inhibitory activity in the  
220 nanomolar range,<sup>16</sup> but virucidal tests showed recovery of the viral infectivity indicating a simple  
221 virustatic inhibitory mechanism. If irreversible changes are induced in the virus particle, the  
222 infectivity is never regained at all dilutions tested, even though the dilution leads to a final  
223 concentration lower than the active dose<sup>22</sup>. MUS:OT-NPs also showed nanomolar inhibition of  
224 HSV-2 infectivity but, in contrast to heparin and MES-NPs, no infectivity was regained upon  
225 dilution (Figure 1B), confirming an irreversible effect (virucidal). In agreement with our  
226 hypothesis, all HSPG-binding viruses showed irreversible loss of infectivity when incubated with  
227 MUS:OT-NPs, although to differing extents (Figure 1C).

228

229 The HSV-2 virucidal tests were performed also at different time points, as shown in  
230 Figure 1D. While the virustatic effect is immediate, as shown by dose response curve at time 0 h  
231 in Supplementary Figure 10, the virucidal activity develops over time, with the effect being  
232 almost complete after 30 min. Indeed, when viruses and MUS:OT-NPs were mixed and

233 immediately added to cells, the inhibitory potency is reduced as compared to the pre-incubation  
234 experiment, confirming the time-dependent virucidal effect (Supplementary Figure 10).

235

### 236 **NPs-induction of Irreversible Changes in the Virus Particles**

237 To elucidate the fate of the viruses after NPs binding we performed a series of  
238 transmission electron microscopy (TEM) studies on HSV-2 exposed to MUS:OT-NPs and MES-  
239 NPs. Dry uranyl acetate negatively stained TEM were complemented by cryo-TEM studies (see  
240 Supplementary Discussion 2 for the choice of imaging and its validity). Figure 2 shows negative  
241 staining TEM (A) and cryo-TEM (B and C) images of viruses with and without NPs. It is  
242 possible to see different types of NP-virus association, categorized as follows: (1) virus with no  
243 NPs associated, (2) virus with some NPs associated (with particles being mostly isolated), (3)  
244 virus with NPs associated with at least one local cluster of NPs, and (4) deformed viruses mostly  
245 covered with NPs. We believe that stage (2) indicates that NPs have associated with the HSPG  
246 VALs, as time progresses the VAL attracts more particles leading to stage (3) forming NPs  
247 clusters; stage (4) is when the particles are associated with a broken virus or break the virus.  
248 Control experiments with particles that had no sulfonic acids show mostly stage (1) and in some  
249 rare cases stage (2) that we attribute to stochastic interactions. (Supplementary Figures 11).

250 The quantification of Cryo-TEM images illustrated in Figure 2 (D) shows that,  
251 immediately after mixing HSV-2 with MES NPs (0.2 mg/ml, approximate incubation time of 30  
252 sec), 75% of the viruses do not show any association with the NPs (stage 1), while 25% are  
253 associated with the particles (stage 2 and 3). For stage (2) and (3) we observed association  
254 primary at a single point. After 90 min of incubation at 37°C, 5% CO<sub>2</sub>, we find that the fractions  
255 of stage (1) versus NP-associated stages remain practically unchanged. The only noticeable

256 difference we found is that the fraction of viruses that was previously only associated to isolated  
257 particles (stage 2) now shows predominantly clusters (stage 3), with a 5% showing stage (4)  
258 deformed viruses fully coated with NPs. Our interpretation of this data is that in MES-NPs we  
259 observe an overall sporadic sizeable interaction with the VAL leading to a progression from  
260 stage (2) to stage (3), while the fraction in stage (4) provides us with a baseline to determine the  
261 fraction in our samples of deformed viruses that have lost their capsid and get coated with NPs.  
262 At the same concentration as MES-NPs, the effect of MUS:OT-NPs is markedly different. In  
263 this case, all viruses immediately associate with particles, showing 50% of stage (2) and 20% of  
264 stage (3), and already 30% of the viruses are deformed and fully coated (stage 4). After 90 min  
265 images show an evolution of the interaction, as only 13% of the viruses remain in stage (2) and  
266 the other 87% are deformed and fully coated (stage 4).

267 In our interpretation stage (2) and (3) are the imaging of a virustatic effect as they show  
268 NPs attached to viruses, while stage (4) is related to the virucidal effect, as it images viruses fully  
269 covered with NPs that most probably have lost their structural integrity. When comparing the  
270 images for MES-NPs and viruses with those for MUS:OT-NPs and viruses it is noticeable that  
271 the immediate association suggests stronger interaction with MUS:OT-NPs as images lack stage  
272 (1). While comparison of the images obtained at 90 min indicate that MUS:OT-NPs induce  
273 damage to a fraction of the viruses that is significantly higher to what observed for MES-NPs  
274 (87% vs. 5%, respectively). Moreover, image analysis leads to the conclusion that the virucidal  
275 action of the MUS:OT-NPs is progressive with time, as established also with virucidal assays  
276 (Fig. 1D), as the fraction of viruses imaged in stage (2) and (3) progressively evolves into stage  
277 (4). A similar progression can be observed with HPV-16 (Supplementary Figure 12). See

278 Supplementary Discussion 3 for gel studies to show that changes on the viruses happen on a  
279 whole population.

280

### 281 **Mechanistic Understanding *via* Simulations**

282 In order to understand how MUS-type NPs can induce irreversible changes upon  
283 interaction with HSPG-VAL, we performed atomistic molecular dynamics (MD) simulations of  
284 different NPs interacting with the capsid of HPV-16 (Figure 3). The simulations were performed  
285 in physiological solutions, where NPs were placed close to the solvent-exposed HSPG binding  
286 sites (amino acid residues K278, K356, K361, K54 and K59)<sup>37,38</sup> at the surface of HPV-16  
287 capsid L1 proteins.

288 The simulation results for MUS:OT-NP (2.4 nm core and two types of ligands, MUS and  
289 OT, 50 ligands of each kind) in Figure 3A (Movie M1) demonstrate that selective multivalent  
290 binding<sup>39</sup> develops between negative sulfonate groups of MUS:OT-NP and positive HSPG-  
291 binding lysine residues of L1 capsid protein complexes from the HPV-16 capsid. Within 50-80  
292 ns, 5-6 local charge interactions form on average between NP terminal sulfonate groups and L1  
293 HSPG-binding sites (Supplementary Figures 13 and 14), which are supported by a similar  
294 number of non-local coupling contacts between nonpolar alkyl chains of NP ligands and L1  
295 proteins. Each of the 5-6 sulfonate groups binds to positively charged amine groups of lysine  
296 residues with a relatively large Gibbs free energy of  $-6 \text{ kcal/mol}^{40}$ , totaling in  $\Delta G_{\text{bind}} \sim -34$   
297 kcal/mol, while the non-polar ligand chains acquire on average a non-local total binding energy  
298 of  $-21 \text{ kcal/mol}$  (see Methods in SI for details).

299



300 This multivalent binding can induce large stresses and deformations of the L1 complexes.  
301 Given the local nature of binding of the sulfonated groups, we can use this binding to estimate  
302 the effective force with which the NPs act on the L1 complexes. By considering the increase of  
303 binding energy,  $\Delta G_{\text{bind}}$ , during the NP motion,  $\Delta x$ , on the capsid surface, we can get an effective  
304 force that drives the NP binding and capsid deformation process forward,  $F \sim -\Delta G_{\text{bind}}/\Delta x$   
305 (Figure 3 C). By combining the above Coulombic energy change of  $\Delta G_{\text{bind}} \sim -28$  kcal/mol  
306 (considering at the beginning of simulations NP interacting with a Lysine) with a distance of  
307 10.4 Å over which the MUS:OT-NP moves (Supplementary Figure 15), while acquiring this  
308 binding energy, we obtain an effective forces of  $F \sim 189$  pN. This force can deform the L1  
309 complexes and even disturb a relative position of one L1 pentamer with respect to a neighboring  
310 L1 pentamer (Figure 3C, Supplementary Figure 16 and Movie M2). This disruption of viral  
311 capsids by NPs with a multivalent Coulombic binding is analogous to the pore formation in  
312 neural membranes by  $\text{Ca}^{2+}$  ions<sup>41</sup>. See Supplementary Discussion 4 for the effect of length on  
313 the sulfonated ligand.

314

### 315 **Ex vivo activity**

316 In order to develop an effective antiviral strategy the active substances have to act mainly  
317 after infection. We verified whether MUS:OT-NPs were effective also after virus infection of  
318 cells. Cells were infected with wild-type HSV-2 (multiplicity of infection, MOI 0.01 pfu/cell) for  
319 2 h at 37°C. After removal of the viral inoculum, different doses of MUS:OT-NPs were added to  
320 the cell monolayers immediately or after 2, 4 or 24h. Cells and supernatants were harvested  
321 when the untreated wells exhibited a cytopathic effect of the whole monolayer. The cell free  
322 supernatants were then titrated. We determined that MUS:OT-NPs had an  $\text{EC}_{50}$  of 4.4 µg/mL,

323 with complete inhibition at 400 µg/mL and 3 logs reduction at 80 µg/mL (Supplementary Figure  
324 17) and a relevant inhibition of infection in all the tested time points. Thus the NPs can either  
325 prevent infection or block an ongoing infectious process depending on whether they inactivate  
326 the virus inoculum or the viral progeny.

327 To further verify the increased activity of our NPs in a model similar to an *in vivo*  
328 infection, we performed antiviral assays in EpiVaginal tissues. These are composed of human-  
329 derived ectocervical epithelial cells grown on a collagen-coated membrane to form a  
330 multilayered and highly differentiated tissue that is similar to the vaginal mucosa. We used HSV-  
331 2 as a challenge due to its specific tropism for the genital mucosa. HSV-2 ( $10^5$  pfu) was pre-  
332 incubated with the NPs at 500 nM and then added on tissues for 2 h at 37°C. The tissues were  
333 washed apically every day and the viral titer was evaluated by titration. The results show a  
334 significantly better profile of inhibition of the MUS:OT-NPs compared to the MES-NPs (Figure  
335 4A). Moreover we performed experiments in EpiVaginal tissues also pre-treating the tissues for  
336 18 h and subsequently infecting with HSV-2 or infecting the tissue with HSV-2 and treating 24  
337 hpi. In both experiments we could observe a significant inhibition with MUS:OT-NPs (Figure 4  
338 B and C) confirming the preventive and therapeutic activity of MUS:OT-NPs observed in cell  
339 lines. Moreover the nanoparticles proved to be non toxic in MTT and LDH assays conducted on  
340 EpiVaginal tissues (Supplementary Figure 18) demonstrating their biocompatibility with a  
341 human mucosa.

342

### 343 **In vivo activity**

344 To provide the proof of concept that MUS:OT-NPs could exert inhibitory activity also *in*  
345 *vivo*, we tested them in Balb/c mice infected with RSV<sup>43</sup>. Three groups of 5 BALB/c mice were

346 treated at day 0 with (i) 50  $\mu$ l of PBS, (ii) 50  $\mu$ l of PBS, or (iii) MUS:OT-NPs in PBS (50  $\mu$ l at  
347 200  $\mu$ g/ml) in the latter two cases this was followed, 10 minutes later, by inoculation with RSV-  
348 Luc ( $10^4$  pfu). 3 days post-infection the luciferase expression in the lungs was analysed as a  
349 measure of the extent of infection. As shown in Figure 4D and 4E, untreated mice show a clear  
350 pulmonary dissemination of RSV infection. By contrast, the luciferase signal from the lungs of  
351 MUS:OT-NPs treated group was found to be statistically identical to the noise level set by the  
352 signal of uninfected mice treated solely with a PBS solution, indicating that MUS:OT-NPs  
353 treatment prevented the pulmonary dissemination of the infection.

354 Moreover, to investigate the biodistribution of MUS:OT-NPs, organ homogenates were  
355 subjected to inductively coupled mass spectrometry (ICP-MS) where it was possible to detect  
356 gold presence only in lung homogenates while there was no detectable signal from spleen, liver  
357 and brain (Supplementary Figure 19). Of note, the localization on the MUS:OT-NPs is consistent  
358 with their antiviral activity in the lungs as shown in Figure 4D and 4E.

359

## 360 **Conclusions**

361 We believe that the approach presented here has a chance to produce medically relevant  
362 virucidal drugs to fight viral infections. See Supplementary Discussion 5 for the extension of this  
363 work to biodegradable nanoparticles. The results found so far show outstanding virucidal activity  
364 over HSV-2 and LS-VSV-G, while the activity versus HPV and RSV, although remarkable  
365 should be improved. In any case, it should be stressed that the strategy proposed is intrinsically  
366 broad-spectrum, allowing the potential prevention and treatment of multiple viral infections with  
367 a single drug, a great advantage mostly in virology where rapid and at times unexpected  
368 infections occur. For example, West Nile, Yellow Fever, and Dengue are growing threats. All

369 these viruses belong to the Flaviridae family, and are HSPG-binding viruses. Preliminary results  
370 with gold NPs show nanomolar virucidal efficacy over Dengue 2 (see Figure 1C and  
371 Supplementary Figure 20). Similarly, the Filoviridae family contains several human pathogens  
372 causing haemorrhagic fevers, including Ebola virus, for which drugs are urgently needed. All  
373 bind HSPGs as attachment receptors, and are potentially susceptible to the antiviral NPs  
374 presented in this study. Overall, what presented here is a first step towards the development of  
375 treatments (whether prophylactic or therapeutic will be determined by further in-depth in-vivo  
376 experimentations) for many worldwide threatening viral infections.

377

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497

498 **Author contributions:**

499 V.C. was responsible for all activities involving HSV2, HPV, RSV under the supervision of D.L.  
500 and EpiVaginal experiments under the supervision of C.T. and L.K. P.A. and M.D. were  
501 responsible for all testing with VSV-LV-G under the direction of S.K.. P.J.S. was responsible for  
502 NP and ligand synthesis. M.M. was responsible for all cryo-TEM. S.T.J. was responsible for Iron  
503 oxide NP synthesis. M.G. R.L. were responsible for the in vivo experiments, R.W.M. and J.F.E.  
504 engineered the RSV-Luc used for in vivo experiments. M.V. was responsible for stained TEM  
505 imaging of the viruses. J.H. and J.W. conducted all testing with DENV-2. S.S. and Y.H. were  
506 responsible for molecular dynamics simulations under the direction of P.K. and L.V. E.R.J.  
507 synthesised MUP-NPs. A.B. synthesised MES NPs. B.S. synthesised EG2OH-NPs. C.M. and  
508 P.A. conducted the gel electrophoresis. M.D. was responsible for HSV-1 and 2 and dose  
509 response experiments. F.S. and S.K. first conceived the experiments, F.S. and D.L. developed the  
510 interpretation of the experiments. F.S., D.L., V.C., and S.T.J. wrote the paper.

511

512 **Competing Financial Interests**

513 The authors have no competing Financial Interest.

514

515 **Data Availability Statement**

516 Raw data of experiments are available at <https://figshare.com/s/19ed37fbbe0261a00254>

517

518

519 **Methods:**

520 Detailed procedures are provided in the Methods section in the Supplementary Information

521

522 Nanoparticles synthesis

523 MUS:OT and all MUS nanoparticles were synthesised using a slightly modified procedure  
524 reported by Verma et al.<sup>45</sup> all MES Au nanoparticles were synthesised following the synthetic  
525 procedure reported by Baram-Pinto et al.<sup>15</sup>

526

527 Viral inhibition

528 Viruses were pre-incubated with nanoparticles for 1 h and then added on cells. Viral infection  
529 was evaluated through plaque assay or FACS.

530

531 Virucidal assays

532 Viruses ( $10^4$ - $10^6$  pfu) and 100 to 1000  $\mu\text{g/ml}$  of MUS:OT-NPs were incubated at different time  
533 points at  $37^\circ\text{C}$  and the virucidal effect was investigated with serial dilutions of the mixtures.  
534 Viral titers were calculated at dilutions at which the NPs were not effective.

535

536 TEM and Cryo TEM

537 HSV-2 and HPV-PsV ( $10^5$  pfu) were incubated with or without  $100 \mu\text{g/ml}$  Au-NPs and were  
538 adsorbed on carbon- and Formvar-coated grids and negatively stained with 0.5% uranyl acetate  
539 and observed with CM 10 electron microscope. For cryo TEM, viruses and NPs were flash-  
540 frozen in their native hydrated state on carbon coated grids and imaged at  $-175^\circ\text{C}$  in a FEI Tecnai  
541 F20 Cryo 200kV TEM.

542

543 Ex vivo analysis

544 Epivaginal tissues were purchased from Mattek and cultured as indicated by the manufacturer.  
545 The tissues were infected with HSV-2 ( $10^4$ - $10^5$  pfu) and treated with different NPs (500-1500  
546 nM) and titrations of supernatants were conducted on Vero cells to evaluate viral replication.

547

548 In vivo analysis

549 Balb/c mice were treated with  $200 \mu\text{g/ml}$  of MUS:OT-NPs and then infected with RSV-Luc as  
550 previously described<sup>43</sup>. Luminescence was measured using the IVIS 200 imaging system  
551 (Xenogen Corp.).

552

553 Statistics

554 All results are presented as the mean values and sem from three independent experiments. The  
555  $\text{EC}_{50}$  values for inhibition curves were calculated by regression analysis with GraphPad Prism.  
556 The selectivity indexes SI were calculated dividing the  $\text{CC}_{50}$  for the  $\text{EC}_{50}$ . A unpaired t-test

557 analysis was performed for virucidal assays and in vivo assays. t values and degrees of freedom  
558 (df) are indicated in figure legends.

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## FIGURE LEGENDS

567 **Figure 1. Virucidal activity of MUS:OT-NPs.** A) Cartoon of the virucidal activity of  
568 MUS:OT-NPs compared to MES-NPs B) From top to bottom Heparin, MES-NPs and MUS:OT-  
569 NPs viral infectivity curves and virucidal assays. The percentages of infection were calculated  
570 comparing the number of plaques in treated and untreated wells. C) Virucidal activity of MUS  
571 coated NPs against HPV-16, RSV, LV-VSV-G (indicated as LV), and DENV-2 viruses. D)  
572 MUS:OT-NPs inhibition of viral infectivity against HSV-2 versus time (minutes). Results are the  
573 mean and sem of 3 independent experiments performed in triplicate. \*\*\*  $p < 0.001$  (two-tailed) in  
574 unpaired t test analysis. HSV-2  $t = 0.9788$   $df = 17$ , HPV  $t = 7.776$   $df = 16$ , RSV  $t = 44.32$   $df = 6$ , LV  
575  $t = 5.6$   $df = 2$ , DENV  $t = 38$ ,  $df = 4$ .

576 **Figure 2: HSV-2 and its association with MUS:OT-NPs.** The samples were imaged using dry  
577 negatively stained TEM (A) or unstained cryo-TEM (B,C). The scale bars are 100 nm. D)  
578 Percentage and distribution of NPs (MES or MUS:OT) associated with HSV-2 immediately and  
579 after 90 min were determined by analysing between 50 and 100 cryo-TEM images per condition.

580 **Figure 3: Molecular Dynamics Simulations.** A) Top view of a small sulfonated MUS:OT-NP  
581 (2.4 nm core) selectively binding to HPV capsid L1 protein pentamer, after 25 ns of simulations.  
582 Red and yellow spheres show negatively charged terminal sulfonate groups of the MUS-NP.  
583 Positively charged HSPG-binding residues of L1 (K278, K356, K361, K54 and K59) are shown  
584 in blue. Inset highlights the strong selective coupling between sulfonate groups and HSPG-  
585 binding residues (K356, K361, K54 and K59). B) Side view of the interactions of MUS, MES1  
586 (Figure S20 shows 5 nm MES2) and MUP NPs with a HPV L1 protein pentamer. Strong  
587 multivalent binding is developed within 15 ns simulation only for MUS NP. C) Schematic  
588 diagram illustrates how strong multi-site binding of MUS-type NPs to HSPG-binding residues  
589 can induce irreversible changes in the arrangement of L1 capsid proteins. Scale bars are 1 nm.



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**Figure 4: MUS:OT-NPs activity ex vivo and in vivo.** A) NPs activity against HSV-2 infected human cervicovaginal histocultures with pre-incubation of virus and NPs (500 nm) and addition on tissues. B) EpiVaginal tissues were treated with MUS:OT-NPs for 18 h and subsequently infected. C) EpiVaginal tissues were infected with HSV-2 and after 24 h MUS:OT-NPs were added on tissues. The percentages of infection were calculated comparing the viral titers in the treated tissues supernatant and in the untreated. Results are the mean and sem of 3 independent experiments performed in triplicate. D) Groups of 5 BALB/c mice were treated at day 0 with either 50  $\mu$ l of PBS or MUS:OT-NPs in PBS (50  $\mu$ l at 200  $\mu$ g/ml) and 10 minutes later where inoculated with RSV-Luc, with the exception of the mock group. Bioluminescence was measured at day 3 post-infection by intranasal injection of D-luciferin. Capture of photon emission was performed using the IVIS system. Luciferase activities were quantified for each mouse using Living Image software. Luciferase activity is expressed as photons per second (p/s). Results are the mean and sem of 3 independent experiments performed with 5 mice per group. \*\*\*  $p < 0.001$  (two-tailed) in unpaired t test analysis.  $t = 8.976$   $df = 8$  E) Ventral views of representative mice. The scale on the right indicates the average radiance: the sum of the photons per second from each pixel inside the region of interest/number of pixels (p/s/cm<sup>2</sup>/sr).