Broad-Spectrum Antiviral Peptides and Polymers

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Abstract

As the human cost of the pandemic caused by the SARS-CoV-2 is still being witnessed worldwide, the development of broad-spectrum antiviral agents against emerging and re-emerging viruses is seen as a necessity to hamper the spread of infections. Various targets during the viral life-cycle can be considered to inhibit viral infection, from viral attachment to viral fusion or replication. Macromolecules represent a particularly attractive class of therapeutics due to their multivalency and versatility. Although several antiviral macromolecules hold great promise in clinical applications, the emergence of resistance after prolonged exposure urges the need for improved solutions. In the present article, we review the recent advancement in the discovery of antiviral peptides and polymers with diverse structural features and antiviral mechanisms. Future perspectives, such as the development of virucidal peptides/polymers and their coatings against SARS-CoV-2 infection, standardization of antiviral testing protocols, and use of artificial intelligence or machine learning as a tool to accelerate the discovery of antiviral macromolecules, are discussed.

1. Introduction

Emerging and re-emerging viruses such as the Ebola virus, Zika virus, and more recently severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), have been at the origin of serious pandemics with high mortality rates worldwide, due to their genetic diversity, ability to replicate efficiently, and persistence within their hosts.[1-4] As vaccine development requires considerable time and is often ineffective against mutated viral strains, efficient broad-spectrum antiviral drugs are necessary to control and reduce infection rates of newly emerged viruses.[5]

Although a large morphological diversity was observed across the different viral species, the main components of viruses are retained and optimised to transfer nucleic acid from cell-to-cell: a virus particle (virion) is composed of genetic material, either single- or double-stranded RNA or DNA, protected by a protein coat (capsid) only (non-enveloped viruses) or a capsid surrounded in an outer envelope of proteins and lipids (enveloped viruses).[6] Multiple steps are involved in the propagation of viruses within host cells, starting with the entry process.[7] Firstly, viral attachment occurs via low-affinity interactions between viral envelope glycoproteins and attachment factors (e.g. glycosaminoglycans) at the surface of a host cell. Following this step, virions bind to specific cell receptors such as ACE2 for SARS-CoV-2.[8, 9] The high-affinity binding triggers viral penetration into the cytoplasm by receptor-mediated endocytosis or, for some enveloped viruses, by direct membrane fusion. Once viral entry is completed, genome replication and translation take place using the host cell machinery. Finally, new virions, which are assembled from the viral genome and proteins accumulated within the cell, are released extracellularly. The different stages of the virus life-cycle offer a range of targets for the design of antiviral drugs, yet the safety and efficacy of the treatment would be strongly affected by its mechanism of action.[10] The vast majority of currently approved antiviral drugs are limited to low molecular weight (generally below 700 $g.mol^{-1}$) compounds targeting viral enzymes such as viral RNA/DNA polymerase or protease, with high specificity (*e.g.* Acyclovir, Remdesivir, Saquinavir).[11] However, these drugs induce selective pressure on viruses during replication, causing mutations to occur within the viral genome.[12] In response to antiviral treatments, these mutations can produce resistant viral strains and render highly specific drugs ineffective.[13, 14] Conversely, multivalency was achieved with peptide- and polymer-based antivirals bearing diverse functionalities.[15, 16] Carefully designed macromolecules could thus potentially display a broad spectrum of antiviral activity, and lower the risk of emergence of resistant strains. In this article, we present an overview of peptides and polymers with intrinsic antiviral properties, and will not cover antiviral drug delivery systems as excellent reviews are presently available on this topic.[17, 18] We aim to provide an insight into the design optimisation of antiviral macromolecules, hence the review is organised according to the structural and/or functional features of the various antiviral peptides and polymers, which have been investigated so far (**Figure 1**). Finally, future research directions are proposed.

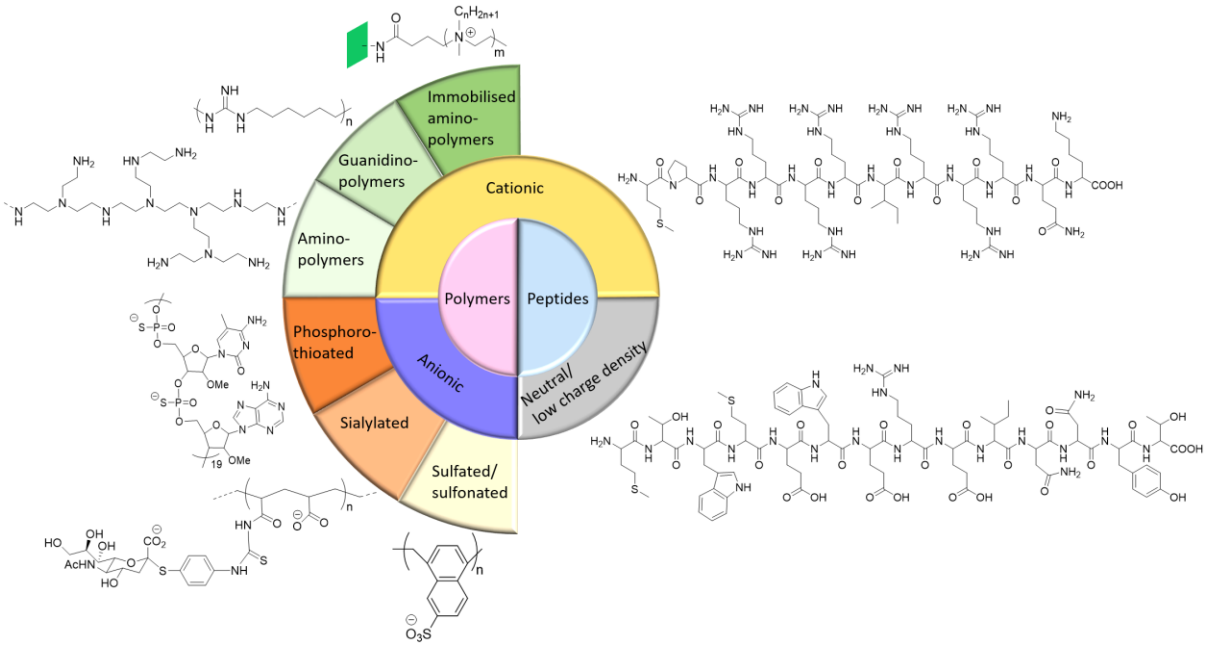


Figure 1. Diagram presenting the structural diversity of the antiviral macromolecules discussed in this review.

2. Antiviral peptides

While protein-based therapeutics are associated with high production costs, recent advances in the scale-up of peptide synthesis have rendered this class of oligomers particularly attractive as safe yet efficient broad-spectrum antivirals.[19, 20]

2.1. Cationic peptides

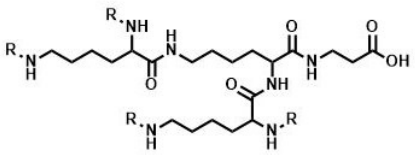
Heparan sulfate proteoglycans (HSPGs) are highly sulfated branched glycoproteins that are widely expressed at the surface of mammalian cells.[21] Interestingly, HSPGs can be recognised by viral proteins carrying positive charges and provide the initial contact between virions and host cells.[22, 23] A wide range of virions have been shown to bind to HSPGs via low-affinity interactions, thus facilitating subsequent viral attachment to high-affinity receptors prior to membrane fusion.[22, 24]

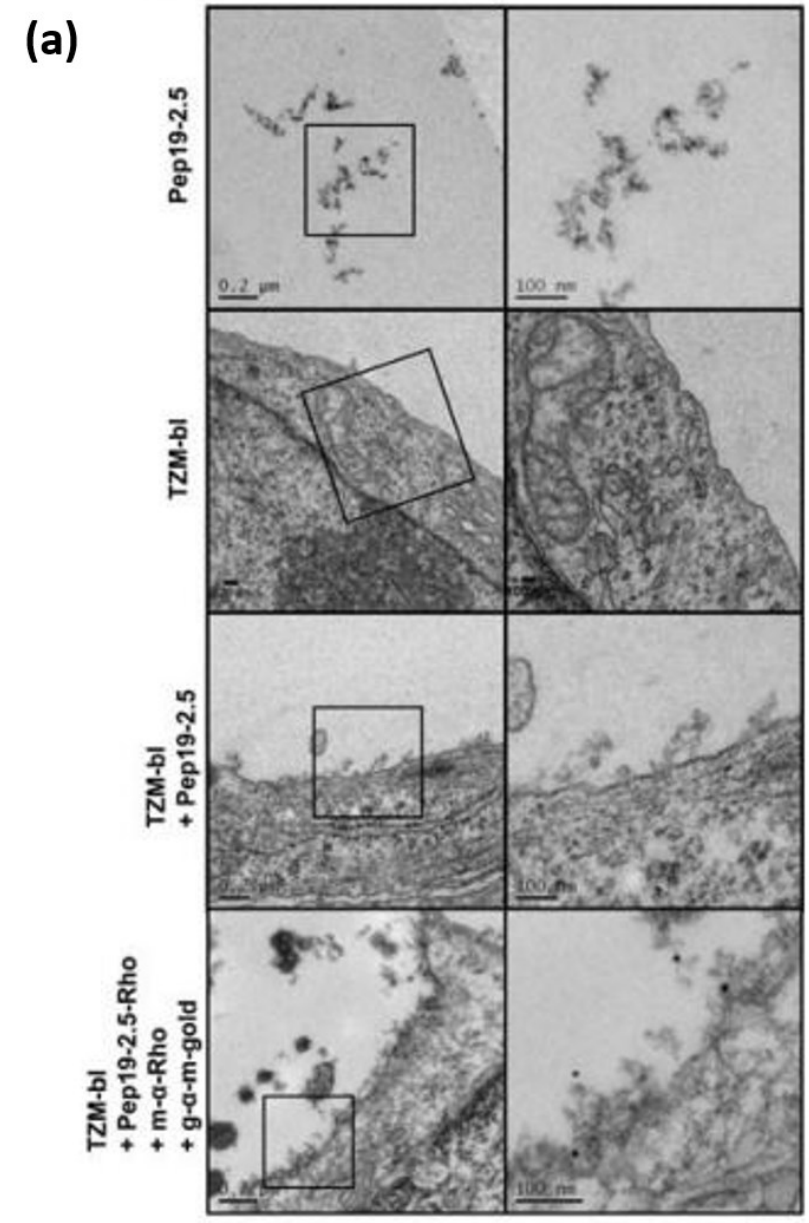
In the light of the role of HSPGs in viral entry, cationic peptides, which can interact with negatively charged HSPGs, have drawn increasing attention as potential inhibitors of viral attachment. Indeed, twenty-amino acid-long peptides containing lysine and arginine residues, Pep19-2.5 and Pep19-4, successfully inhibited viral infection against human immunodeficiency virus (HIV) type 1, herpes simplex virus (HSV) type 1 and 2, hepatitis B

virus (HBV), and hepatitis C virus (HCV) when the host cells were pre-treated with the peptides before infection (**Table 1**).[25] Binding assays demonstrated that the cationic peptides interacted with the HS moieties at the cell surface *via* electrostatic interactions. This finding was further confirmed by the peptide accumulation observed by TEM at the cell surface (**Figure 2A**). Therefore, Pep19-2.5 and Pep19-4 seem to block viral infection by binding to HSPGs at the cell surface, preventing the interactions of viral envelope proteins with cell receptors. However, Pep19-2.5 and Pep19-4 did not seem to interfere in the subsequent stages of viral infection. As the peptide sequence and the conformation of Pep19-2.5 and Pep19-4 varied significantly, the authors attributed the antiviral potency of both peptides solely to the presence of positive charges.

The effect of multivalency on the antiviral potency of cationic peptides was analysed using a library of linear, dimeric, and tetrameric peptides which were synthesized based on a branched peptidyl core functionalized with cationic peptide sequences.[26] As anticipated, the most potent peptides against human papillomavirus type 16 (HPV-16) pseudo-particles were the tetrameric cationic peptides, among which SB105 and its analog SB105-A10 exhibited particularly high activity (**Table 1**). This trend was correlated to the stronger affinity of the multivalent cationic peptides with the branched anionic structure of HSPGs, compared to their linear counterparts. In addition to multivalency, peptide sequence appears to play a crucial role in conferring potency to peptide-derivatized dendrimers. Studies screening a library of cationic tetrameric peptides against human cytomegalovirus (HCMV), HSV-1 and 2 infections demonstrated that cell pre-treatment with SB105 and SB105-A10 displayed the highest antiviral activity again.[27, 28] Their active concentration was relatively low as cell pre-treatment at 2 and 5 $\mu g.mL^{-1}$ for 1 hour reduced the replication of HSV-1 and 2, respectively, by 50 %.[28] The mechanism of action of both peptides was shown to be similar to that of Pep19-2.5 and Pep19-4: viral attachment was prevented by binding to HSPGs at the cell surface (**Figure 2B**). As SB105 and SB105-A10 did not compromise cell viability within their active range of concentrations, these cationic tetrameric peptides could be considered as potential candidates for the development of a topical microbiocide to prevent sexually-transmitted viral infections.[29]

Table 1. Sequence of the antiviral cationic peptides mentioned in this review

Peptide name	Peptide sequence
Pep19-2.5[25]	GCKKY RRFRW KFKGK FWFWG
Pep19-4[25]	GKKYR RFRWK FKFGK WFWFG
SB105 and SB105-A10[27, 28]	 R = ASLRVRIKKQ for SB105 ASLRVRIKK for SB105-A10
G2[30]	MPRRR RIRRR QK
p5[31]	GGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK Q
p5+14[32]	GGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK QAQKA QKAQA KQAKQ
p5R and $p5R_D$[33]	GGGYS RAQRA QARQA RQAQR AQRAQ ARQAR Q
RJ8[34]	ARRAA RRARR AARRA RRAAR R
Deca-$(Arg_D)_8$[35]	C_{10}–RRRRR RRR
T22[36]	RRWCY RKCY KGYCY RKCR



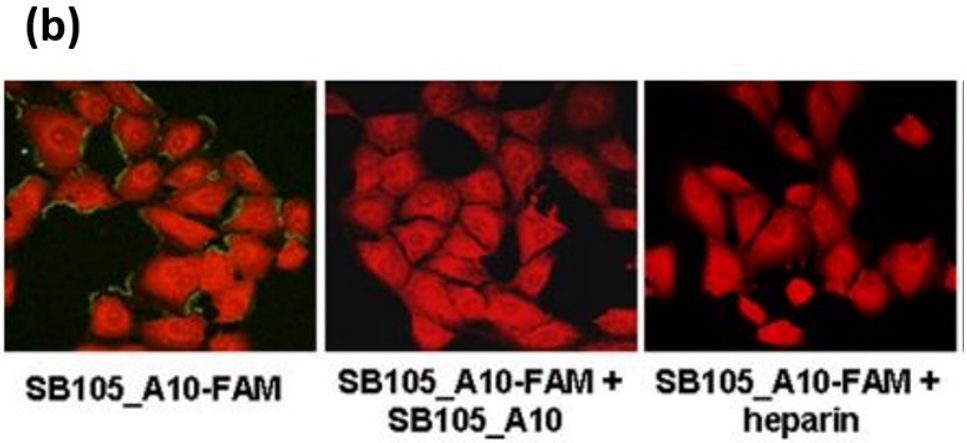


Figure 2. Cationic peptides prevent viral attachment by binding to the cell surface. a) Electron microscopy images of a Pep19-2.5 solution at 20 $\mu g.mL^{-1}$, TZM-bl cells, TZM-bl cells with Pep19-2.5 treatment at 20 $\mu g.mL^{-1}$ or rhodamine-conjugated Pep19-2.5 with immunogold-staining.[25] Adapted with permission. b) Confocal microscopy images of Vero cells (stained red) incubated in the presence of fluorescently-labeled SB105-A10 (SB105-A10-FAM, green) alone at 26 $\mu g.mL^{-1}$, in the presence of a 10-fold excess of unlabelled SB105-A10 or the presence of heparin (10 $\mu g.mL^{-1}$). [28] Adapted with permission.

Similar HSPG binding was reported for a 12-amino acid-long cationic peptide G2, containing a majority of arginine residues (**Table 1**).[30] Cell pre-treatment with G2 at 1000 $\mu g.mL^{-1}$ reduced the number of plaques in HSV-2 *in vitro* infection by 50 %. Unlike the previously presented cationic antiviral peptides, the authors demonstrated that G2 was able to prevent viral fusion, in addition to blocking viral attachment onto host cells. Unfortunately, the secondary structure of G2 peptide was not examined by the authors. An *in vivo* genital HSV-2 infection model with female mice demonstrated that the number of vaginal lesions decreased when G2 was administered prior to infection at a concentration of 2000 $\mu g.mL^{-1}$, compared to the control without pre-treatment. Further studies demonstrated the i*n vitro* efficacy of G2 against CMV and HSV-1 infections.[37] Despite these encouraging results, the applicability of G2 for the prevention of viral infections was quite limited due to its narrow therapeutic window.

The role of secondary structure in the antiviral activity of cationic peptides was highlighted in a study of seven different analogs of a cationic peptide of 31 residues with a repeating unit (KAQKAQA), p5, which was previously shown to bind to HSPGs.[31, 38] All seven analogs were designed to obtain an amphipathic helical structure with the lysine functionalities presented on one side of the helix. Among the screened peptides, only 3 were active against murine cytomegalovirus (MCMV) when used to pre-treat cells prior to infection, and p5+14, which contained an additional 14 amino acids compared to the sequence of p5, was the most potent candidate as it reduced MCMV infection by 90% at 500 $\mu g.mL^{-1}$, whereas p5 pre-treatment only reduced the infection by 53% at the same concentration (**Table 1**). Furthermore, the active concentration of p5+14 was lower than that of G2 peptide, with p5+14 cell pre-treatment reducing *in vitro* MCMV infection by 80 % at 10 μM, whereas no activity was observed by pre-treatment with G2 at this concentration. However, this result could also be attributed to the difference in the molecular weight of the peptides, with 10 μM corresponding to a concentration of 48 $\mu g.mL^{-1}$ v*s.* 17 $\mu g.mL^{-1}$ for p5+14 and G2, respectively. Subsequently, the broad-spectrum activity of p5+14 was successfully demonstrated against human CMV (HCMV), HSV-1 and 2.[31] Its inhibitory mechanism was attributed to the effective binding onto HSPGs at the cell surface, thus preventing viral attachment. This mode of action is in agreement with the observation that pre-treatment of MCMV with p5+14 did not induce any inactivation. In parallel, a set of p5 counter-parts were explored, p5R and $p5R_D$, which were obtained by replacing all lysines

with arginines in the p5 sequence, in L and D form, respectively (**Table 1**).[33] Cell pre-treatment with 700 μg.mL^{-1} of p5R and p5R$_D$ reduced HCMV infection by over 80 % in a range of different host cells. Despite both peptides presenting a helical secondary structure, p5R$_D$ was shown to have prolonged stability in serum as D-form peptides are resistant to protease degradation.[39] However, only a limited effect was observed against *in vivo* MCMV infection with pre-infection administration of p5R$_D$. This decrease in antiviral activity was explained by the rather fast HSPG turnover rate (half-life of around 4 hours).[40]

Naturally occurring antimicrobial peptides (AMPs), which bear cationic residues and adopt an amphipathic α-helical conformation in aqueous solution, also exhibit antiviral properties.[41, 42] Jenssen *et al.* screened ten cationic α-helical peptides of 21 amino acids against herpes simplex virus,[33] based on the sequence of lactoferricin, a naturally occurring AMP.[34, 43] As expected, the cationic peptides prevented viral attachment by binding to HSPGs and peptide affinity with HSPGs increased with cationic content. Hence, a charged residue of 43 mol% and above was necessary to obtain lactoferricin analogs which were active against HSV-1 and 2 infections.[34] The most potent peptide, arginine-rich RJ8, inhibited HSV-1 and HSV-2 viral infection by 50 % after cell pre-treatment at 50 and 30 μg.mL^{-1}, respectively (**Table 1**). Nonetheless, the spatial positioning of the positive charges appeared to be of higher importance than the overall net charge to inhibit HSV-1 and 2 infections.[44]

Despite the necessity for high cationic content, hydrophobicity also appears to improve the antiviral efficacy of cationic peptides. Indeed, substitutions with hydrophobic residues increased the potency of the afore-mentioned lactoferricin analogs against HSV-1 and 2.[34] This result is supported by findings from screening α-helical oligo-D-arginines (with 6 to 10 residues) conjugated to a range of lipids of varying chain length.[35] Among the tested compounds, the octa-arginine conjugated to decanoic acid, Deca-(Arg$_D$)$_8$, was the most potent conjugate for post-infection (p.i.) treatment of duck HBV (DHBV) infection (**Table 1**). After a 5 day p.i. treatment at 3 μg.mL^{-1}, Deca-(Arg$_D$)$_8$ inhibited DHBV replication by over 85 % against 60 % with (Arg$_D$)$_8$ under the same conditions, whereas no activity was observed with decanoic acid itself. Unlike other cationic peptides, cell pre-treatment with Deca-(Arg$_D$)$_8$ did not prevent DHBV infection, thus demonstrating that this compound did not block viral attachment. Instead, the authors provided evidence that Deca-(Arg$_D$)$_8$ acted at a later stage of the viral life cycle and prevented the release of new virions by inducing the intracellular aggregation of preS/S envelope proteins before virion formation (**Figure 3**). Enhanced cell internalisation of the lipid conjugated cationic peptide compared to the peptide alone further supported this finding.[45] Based on this study, the introduction of hydrophobic groups seems not only to increase the potency of cationic peptides against viral infection but also appears to alter its mode of action.[46]

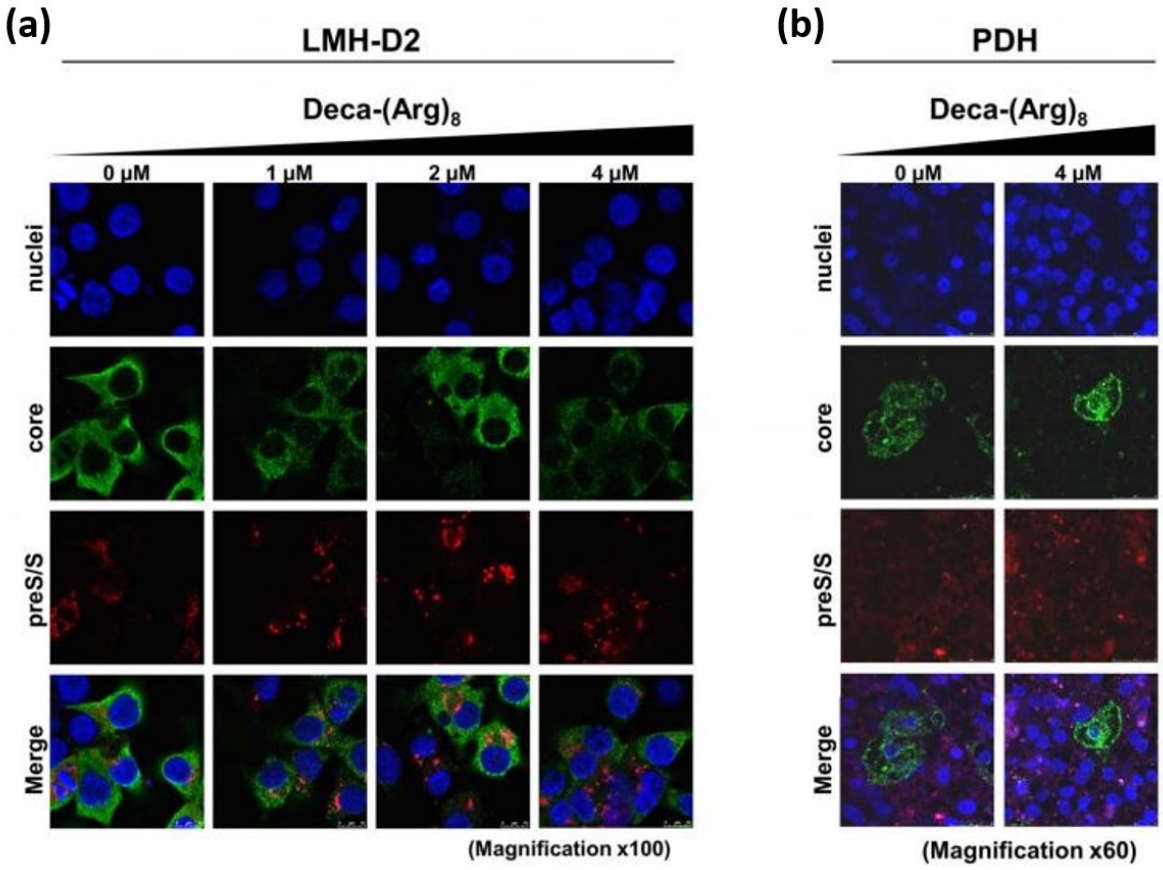


Figure 3. Lipid-conjugated oligo-arginine Deca$(Arg_D)_8$ induced the accumulation of large clusters of DHBV envelope proteins. Confocal microscopy images of DHBV-infected a) LMH-D2 and b) PDH cells treated daily with fluorescently-labeled Deca$(Arg_D)_8$.[35] Four days after treatment, the cells were fixed and stained for DHBV core proteins (2nd row) and DHBV preS/S proteins (3rd row). The fluorescence signal of DHBV-core (green), DHBV-preS/S (red) and cell nucleus (blue) are shown in the absence of treatment (1st column), and with increasing concentration of Deca$(Arg_D)_8$ (subsequent columns). Adapted with permission.

In addition to α-helical cationic antimicrobial peptides (AMPs), a wide range of naturally occurring AMPs presenting different conformations were screened against viral infections.[47, 48] Interestingly, an α-helical structure does not seem to be required for an AMP to inhibit viral infection. For instance, defensins, which are 29 to 42 amino acid-long cationic peptides with β-sheet domains linked by disulfide bonds, exhibited a broad spectrum of antiviral activity against enveloped viruses such as HIV-1, HSV-1 and 2.[49, 50] In the case of defensins, however, a variety of mechanisms of action were described depending on virus type and peptide structure, ranging from the inhibition of viral attachment to interference in viral replication.[51] Similar peptides were investigated to better understand the role of spatial configuration in antiviral efficacy, using analogs of tachyplesin and polyphemusin, which are two naturally occurring cationic AMPs comprising β-sheets.[36] The most potent candidate

against HIV-1 was identified as T22, an 18 amino acid-long arginine-rich peptide which is comprised of β-sheets linked by two disulfide bonds (50 % reduction was observed at 7×10^{-3} $\mu g.mL^{-1}$ when administered at the time of infection) (**Table 1**). Other than the number of arginine residues towards the N- and C-terminal regions, the ability of T22 in inhibiting HIV-1 infections was attributed to its disulfide rings. Indeed, the secondary structure of T22 was shown to be indispensable for its antiviral activity, as it enables the peptide to prevent viral attachment by specifically binding to CXCR4, a T-cell receptor exploited by HIV-1 for viral entry.[52]

Although cationic peptides present a broad spectrum of activity, high systemic doses are generally required to inhibit viral infections due to their non-specificity. Furthermore, they are relatively toxic towards mammalian cells, hence displaying a narrow therapeutic window. Therefore, cationic peptides could be considered as last resource prevention of viral infections. Despite the majority of cationic peptides being potent at preventing infection only when administered to host cells prior to infection, a handful of candidates were shown to inhibit viral replication post-infection (e.g. Deca-$(Arg_D)_8$ and β-defensins).

2.2. **Peptides as viral fusion inhibitors**

The majority of cationic peptides have been shown to block viral attachment by non-specific interactions with HSPGs at the surface of host cells. Viral fusion inhibitors are peptides that act at the next stage of viral entry, by disrupting membrane fusion between virions and host cells.[53] This approach, which is specific to enveloped viruses, is particularly relevant as most emerging or re-emerging infectious diseases originate from this category of viruses.[54] Briefly, the viral fusion process is initiated by non-covalent interactions between viral fusion proteins (fusogens) and cell surface receptors, triggering substantial conformational changes in viral fusion proteins (**Figure 4A**).[55-57] Viral fusion proteins are comprised of three C-terminal and one N-terminal region which can fold into coils when activated.[58] By designing viral fusion inhibitors mimicking the functional region at the C- or N-terminus (C- and N-peptides) of viral fusion proteins, the inhibitors could bind to the complementary domain and prevent the conformational transitions of the proteins upon viral attachment.[54, 59] This category of antiviral peptides has mainly been studied for the treatment of HIV-1. As HIV-1 viral fusion is correlated to the conformational changes of HIV-1 trans-membrane glycoprotein gp41, viral fusion inhibitors have been designed based on the C- and N-terminal heptad repeats (seven residue-motif comprised of hydrophobic, polar, and charged amino acids) of this protein (**Figure 4B**). Furthermore, this category of antiviral peptides displays a low charge density and generally presents both cationic and anionic residues (**Table 2**).

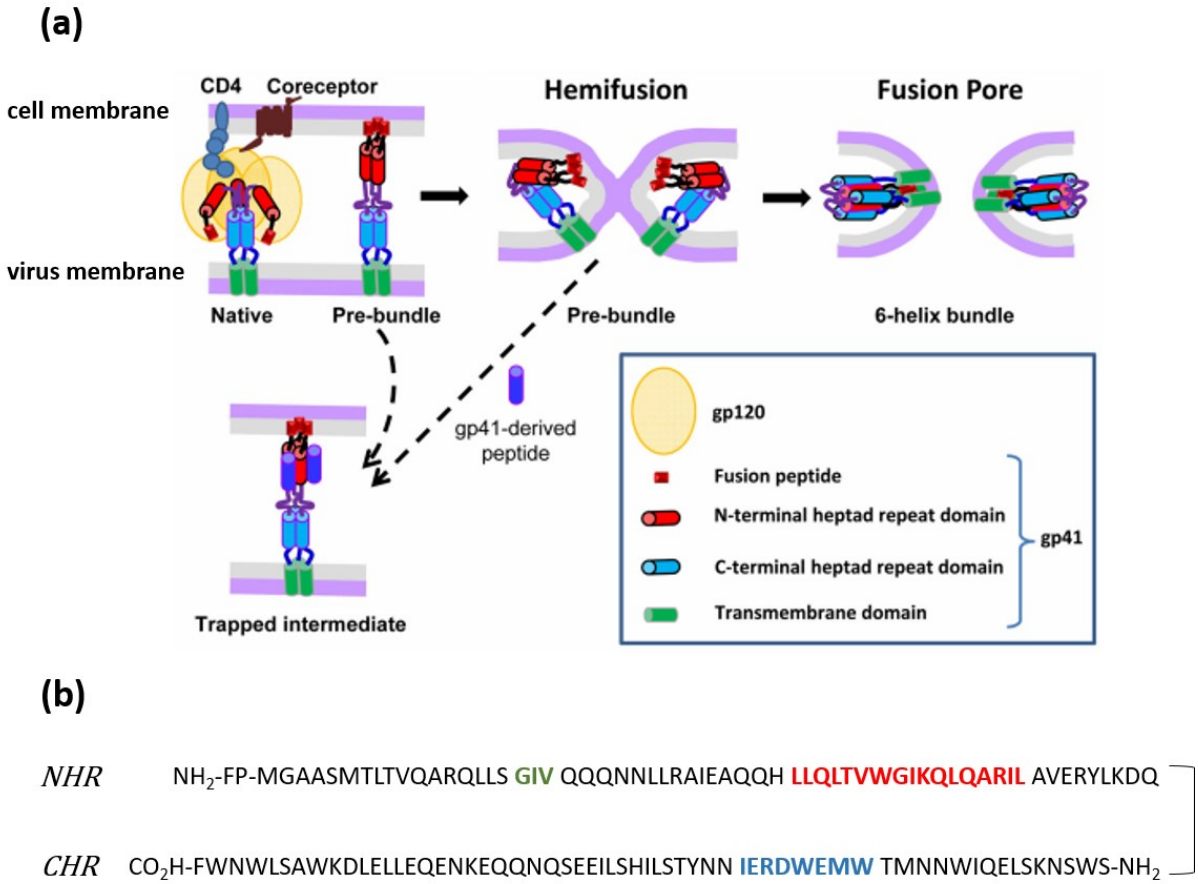


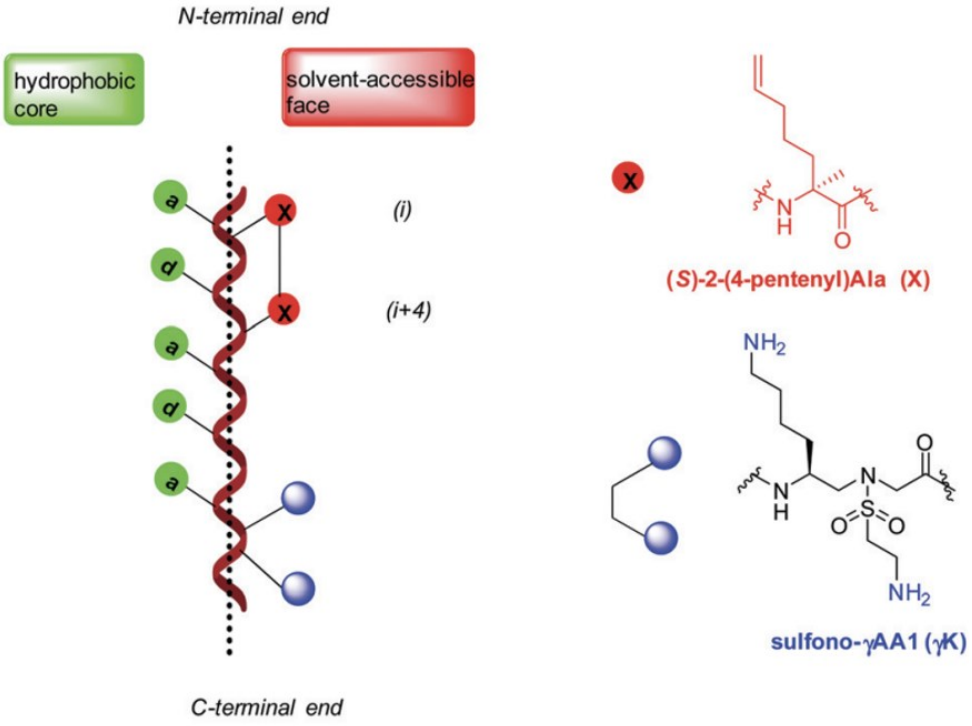
Figure 4. Interactions of viral fusion inhibitors with HIV-1 gp41. a) Key stages of HIV viral fusion and its inhibition with C-peptides. The viral envelope glycoprotein gp41 undergoes various conformational transitions during the fusion process before reaching the 6HB post-fusion.[60] Adapted with permission. b) Functional regions of HIV-1 gp41. The six-helix bundle (6HB) is driven by the interaction of the red domain in the CHR with the blue domain in the NHR (pocket-forming region). The green domain, which corresponds to the target of T20 and C34, is prone to mutations among the resistant variants.[61]

The first generation of viral fusion inhibitors generated for the treatment of HIV was designed based on the sequence of the C heptad repeat of gp41. T20 (also labeled Enfuviritide, Fuzeon, or DP-178) and C34 peptides, with 36 and 34 amino acids, respectively, demonstrated strong activity against HIV-1 when administered during or post-infection (**Table 2**).[62-64] Their ability to prevent viral fusion was attributed to their binding affinity to the N-terminal region of gp41, hence disrupting the function of the viral protein.[65] T20 was approved for clinical use in 2003 for the treatment of HIV, yet the emergence of T20-resistant HIV-1 variants urged the search for a new type of viral fusion inhibitor.[66] Due to the similarities between the sequence of C34 and that of T20 (22 amino acid overlap), C34 was inactive against T20 resistant variants of HIV-1 (**Figure 4B**).[64, 67] Additionally, the poor pharmacokinetic properties of C34 and T20 (half-life of around 1 and 4 hours *in vivo* for C34 and T20, respectively) entailed frequent administrations and high dosing requirements for the

treatment of HIV-1 with the C-peptides.[68, 69] Techniques to rigidify the structure of C-peptides were thus implemented to improve the stability of viral fusion inhibitors.

Stabilisation of the α-helicity of C-peptides was enabled by the introduction of intramolecular salt-bridges within the secondary structure. SC34EK, which is a C34 derivative obtained by substituting some of the residues on the solvent-accessible side of the α-helix with charged amino acids such as glutamate and lysine, was shown to have higher helical stability than its parent peptide, as well as an increased potency against HIV-1 (**Table 2**).[70] Similar results were reported with SC29EK, which was obtained from the remodeling of a shorter C-peptide C29 (29 amino-acids) in order to increase the stability and the activity against HIV-1.[71] More importantly, SC29EK inhibited the replication of wild-type and T20-resistant variants of HIV-1 (50 % inhibitory concentration IC_{50} of 18 $\mu g.mL^{-1}$), whereas C29 only displayed a weak to absent activity. Therefore, the addition of non-covalent interactions within a C-peptide seems to increase its affinity for the N-terminal region of gp41, hence preventing the conformational transitions which drive viral fusion. However, the electrostatically constrained peptides were prone to protease degradation *in vivo*, and their activity towards HIV-1 was decreased in the presence of serum, to a similar extent as T20.[72, 73] Bolarinwa *et al.* also looked into the introduction of additional charge by substituting one of the residues of an α-helical C-peptide of 24 residues by one unit of sulfo-γ amino acid to alter the physicochemical properties of the peptide (**Figure 5**).[74] Although the substitution did not seem to affect the secondary structure of the C-peptide and provided a slight improvement in the anti-HIV-1 activity compared to its parent peptide, the stability of the modified peptide towards proteolytic degradation decreased dramatically in the presence of sulfo-γ amino acid. On the contrary, hydrocarbon stapling provided significant enzymatic stability to the sulfo-γ modified C-peptide, which became less prone to hydrolysis after the addition of a covalent cross-linking, whilst maintaining its anti-HIV activity.

(a)



(b)

Peptide	Sequence	Modification
1	MTWEEWDKKIEEYTKKIEELIKKS	—
2	MTWXEWDXKIEEYTKKIEELIKKS	Hydrocarbon stapling
3	MTWEEWDKKIEEYTKKIEELIγKS	Sulfono-γ-AA1 (γK)
4	MTWXEWDXKIEEYTKKIEELIγKS	Both

Figure 5. a) Schematic illustration of the positioning of the hydrocarbon staple and sulfono-γ-AA1; b) List of peptides and modifications from reference **74**.[74] Adapted with permission.

As covalent bonds appeared to provide a better *in vivo* stability to C-peptides than salt-bridges, constrained α-helical hydrogen-bond surrogates (HBS) were investigated by replacing one H bond with a covalent link within a 14 amino-acid long C-peptide.[75] One of the synthesized α-helical HBS inhibited gp41-mediated cell fusion (50 % inhibition at 100 $\mu g.mL^{-1}$), whilst the original C-peptide did not display any activity, despite binding to gp41 with a similar affinity to the active HBS. Similar to cationic antiviral peptides, the use of D-amino acids for the synthesis of C-peptides was explored in combination with constraining techniques. Eckert and co-workers identified short D-form C-peptides (18 amino acids) cyclized *via* intramolecular disulfide bonds with high binding affinity to the hydrophobic pockets of gp41, which were formed during viral fusion, thus preventing further conformational transitions that lead to viral entry.[76] The IC_{50} of these C-peptides (e.g. D10-p1-2K) against HIV-1 infection was in the micro-molar range when administered at the time of infection, whereas C34 and T20 prevented viral entry from a nanomolar concentration

(**Table 2**). In a separate study, D-form multimers with short polyethylene glycol (PEG) linkers were designed as C-peptides in order to match the trimeric nature of gp41.[77] The potency of the trimeric peptide $(PIE7)_3$ against HXB2, a laboratory HIV-1 strain, was over 1000 fold higher than that of PEG-PIE7 (**Table 2**), the unimeric peptide modified with PEG (IC_{50} of 0.25 nM for $(PIE7)_3$ *vs.* 0.94 μM for PEG-PIE7). Despite having similar association rates to a gp41 N-trimer mimic, $(PIE7)_3$ had a much slower dissociation rate than its unimeric counter-part PIE7, which would explain the increased antiviral activity of the $(PIE7)_3$. Although D-from peptides have been associated with extended circulation time and resistance to enzymatic degradation, the introduction of flexible linkers could drastically affect the *in vivo* stability of the resulting multimeric D-peptides. Similarly, Sia *et al.* introduced chemical cross-linking as a constraining method in the structure of C14, a short C-peptide (14 residues) with weak inhibition of cell fusion (**Table 2**).[78] In this case, none of the peptides (cross-linked or unmodified) presented a helical structure when unbound to gp41. Nonetheless, the cross-linking appeared to stabilise the helical structure formed after binding of the modified C14 peptides to gp41, hence conferring high HIV-1 inhibitory efficacy (active concentration 15 times lower than that of C14). Therefore, a loosely constrained C-peptide might be more suitable for the enhancement of the efficacy of viral fusion inhibitors. Further work would be required to establish the enzymatic stability of these peptides.

Table 2. Sequence of the viral fusion inhibitors mentioned in this review.

Peptide name	Peptide sequence
T20[69]	YTSLI HSLIE ESQNQ QEKNE QELLE LDKWA SLWNW F
C34[63]	WMEWD REINN YTSLI HSLIE ESQNQ QEKNE QELL
SC34EK[70]	WZEWD RKIEE YTKKI EELIK KSQEQ QEKNE KELK
C29[71]	WMEWD REINN YTSLI HSLIE ESQNQ QEKN
SC29EK[71]	WEEWD KKIEE YTKKI EELIK KSEEQ QKKN
D10-p1-2K[76]	Ac-KKGAC EARHR EWAWL CAA
PIE7[77]	KGACDYPEWQWLCAA
$(PIE7)_3$[77]	PEG–$(KGACDYPEWQWLCAA)_3$
C14[78]	MTWME WDREI NNYT
α/β peptide[79]	β^3T-TWE-β^3A-WD-β^3R-AIA-β^3E-YA-β^3A-RIE-β^3A-LI-β^3R-AAQ-β^3E-QQ-β^3E-KNE-β^3A-AL-β^3R-EL
WT[80]	EWDRE INNYT SLIHS LIEES QNQQE KNEQE LLELD

	KWASL W
LP-40[69]	YTSLI HSLIE ESQNQ QEKNE QELLE LDK-C_{16}

Finally, unnatural oligomer backbones with similar spatial conformation to natural peptides, unnatural foldamers, have been explored for their improved metabolic stability.[81] Stephens and co-workers have demonstrated the strong affinity of α-helical β^3-peptides to gp41 as well as their ability to inhibit the replication of HIV-1 despite their relatively short sequence (10 amino acids).[82, 83] In parallel, the superior stability of unnatural oligomer backbones was demonstrated with C-peptides by substituting some *α*-amino acid residues by their corresponding β^3 analogs in the sequence of a C-peptide (**Table 2**).[79] Although the half-life of the modified peptides in the presence of proteolytic enzymes extended by 20-fold, their binding affinity to gp41 was reduced compared to their parent peptide. Again, rigidification of the structure using β^3 residues with 5-membered cyclic side chains allowed to obtain *α*/β peptides acting as viral fusion inhibitors towards HIV-1, with a similar mode of action as *α*-C-peptides. Unnatural foldamers present attractive biological properties as viral fusion inhibitors, yet their clinical development is hampered by their challenging synthetic process and the lack of knowledge on their potential immunogenicity.[84]

Chemical conjugation is a common strategy for the improvement of the pharmacokinetics of peptides and proteins. PEGylation, which has been used for FDA-approved drugs, not only increases the water-solubility of viral fusion inhibitors, but more importantly, slows down their *in vivo* degradation.[85, 86] By conjugating a 40 kDa branched PEG to C34 peptide (PEG40k-C34), the *in vivo* half-life reached 10 hours after subcutaneous injection in rats, which was significantly longer than that of unmodified C34 ($t_{1/2}$=0.6 hour). Although PEG40k-C34 was more stable than C34 *in vivo*, its IC_{50} against HIV-1 was notably higher than that of C34 (18.5 nM and 1.7 nM for PEG-40k-C34 and C34, respectively). A similar loss of antiviral activity was reported with the conjugation of 2kDa PEG on WT, a 41 amino acid-long T20 analog (33 residue overlap, Table 2).[80] Interestingly, depending on the PEG conjugation site, the reduction in IC_{50} value against HIV-1, could vary from 29 to 3 folds compared to that of the parent peptide. PEGylation towards the non-interacting central region of the peptide minimized the reduction in antiviral potency, whilst extending the trypsin degradation half-life by over 3 fold, relative to the unmodified peptide.

As lipopeptides are known to have an extended *in vivo* half-life compared to peptides without lipid moieties, the effect of hydrophobic modifications on the antiviral activity of viral inhibitors was examined by substituting the tryptophan-rich motif of T20 with a C16 fatty acid to obtain LP-40.[69] LP-40 not only had a higher *in vitro* potency towards wild-type HIV-1 compared to T20, but also inhibited viral fusion with T20-resistant variants, due to an increased membrane affinity. Given that viral fusion and budding of HIV are highly mediated

by cholesterol, chemical modification of viral fusion inhibitors with a cholesterol moiety successfully improved the efficacy of C-peptides against HIV infections.[87-89] For instance, Ingallinella *et al.* screened C34 peptide and its cholesterol-conjugated analog (C34- Chol) against a range of HIV primary isolates, and reported a 25 to 100-fold lower IC_{50} for C34-Chol, relative to its parent peptide.[90] HIV-lipid bilayer models confirmed that enhancement in the antiviral activity of cholesterol-conjugated peptides correlated with an increase in membrane affinity.[91, 92] Moreover, an extended *in vivo* half-life was observed with cholesterol-derivatized viral inhibitors, rendering the modified peptides more suitable for the treatment of HIV infections. For example, in a comparative subcutaneous pharmacokinetic study in mice, no C34 peptide was observed in plasma 6 hours after administering a 3.5 mg/kg dose subcutaneously, whilst 130 nM of C34- Chol was still detectable in plasma 24 hours post-injection.[90]

Although this category of antiviral peptides is specifically designed according to the viral fusion proteins of the targeted virus, a recent study of a viral fusion inhibitor, RVFV-6 peptide, developed against Rift Valley fever virus (RVFV) suggested that the peptide could also prevent viral fusion of Ebola virus and vesicular stomatitis virus.[93] Therefore, viral fusion proteins appear to present similarities across the different types of viruses, which could be exploited in the development of broad-spectrum viral fusion inhibitors.[54]

3. **Antiviral polymers**

Unique versatility and multivalency are the major attributes of polymeric materials as broad-spectrum antivirals.[94] Additionally, their repeated monomer units enable them to achieve high binding affinity to biological receptors.[95] With remarkable improvements in polymerization techniques, the composition of polymers can be optimised to obtain highly selective antivirals.

3.1. **Anionic polymers**

3.1.1. **Sulfated polysaccharides and glycopolymers**

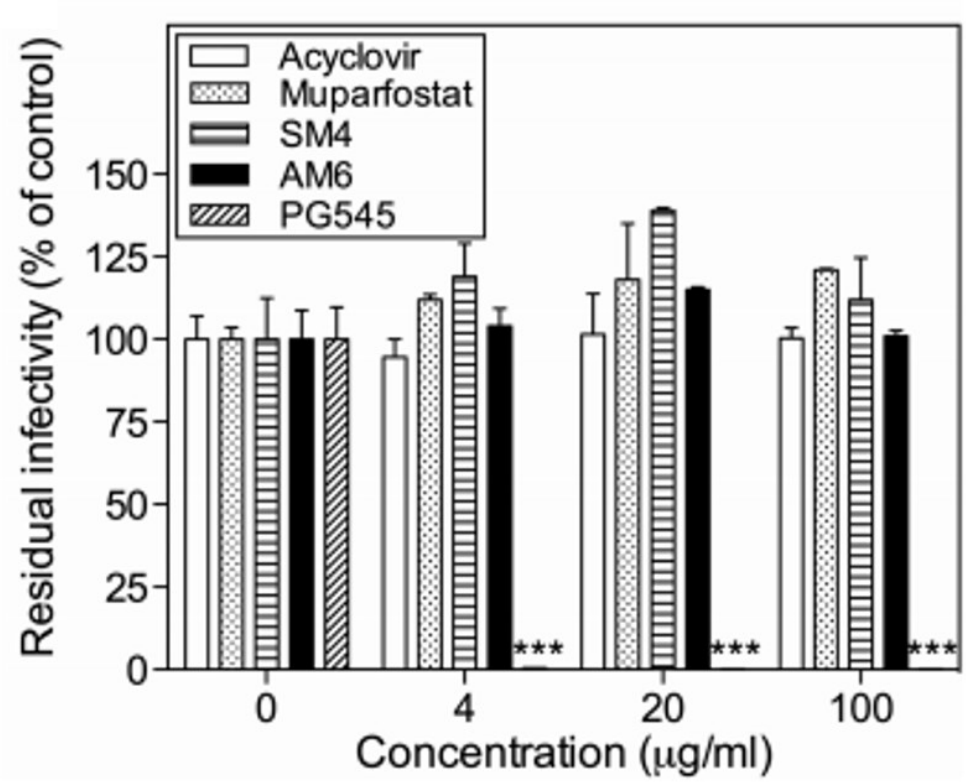
Heparan sulfate proteoglycans are negatively charged branched polysaccharides present at the surface of host cells which are widely used by viruses as binding receptors.[22] Sulfated polysaccharides (SPs) have been used as mimics of heparan sulfates and have been shown to shield the cationic sites of glycoproteins presented on the viral envelope, prior to cell attachment.[96] By interacting with viruses, sulfated polysaccharides prevent viral attachment onto host cells, in a non-specific manner.

SPs derived from marine algae or animal sources, such as heparin,[97] carrageenan[98], or dextran sulfate, have been reported as antivirals (**Table 3**).[96, 99, 100] This class of antiviral polymers featured a broad spectrum of *in vitro* activity against enveloped viruses such as HSV-1 and 2, HIV, respiratory syncytial virus (RSV) and dengue virus (DENV).[99] Variations in viral envelope glycoproteins can affect the susceptibility of the virus to SPs, which is in agreement with the mechanism of action associated with the polymers.[100] Due to the diversity of the SPs which have been screened for antiviral properties, the determination of a structure-activity relationship has been challenging to establish. However, for each particular class of SPs, it was reported that the antiviral activity increased with the degree of sulfation.[100] This trend can be correlated to the enhancement of electrostatic interactions with viral proteins by a higher density of negative charges along the polymer backbone. Across the various types of SPs, the lowest degree of sulfation required for a SP to display an antiviral activity was reported to be 20 mol %.[96] Furthermore, the molecular weight (MW) of the polymers seemed to be an essential parameter in determining the infection inhibition pathway. Indeed, high molecular weight SPs inhibited viral attachment, whereas low molecular weight polymers were shown to reduce the intercellular spread of virions.[96]

Muparfostat (PI-88), a mixture of highly sulfated mannose containing oligosaccharides (M_n=2.4 kDa, ranging from 1.4 to 3.1 kDa) with the major components being penta and tetrasaccharides, but also comprising di, tri and hexasaccharides, was considered as a promising candidate for antiviral treatment (**Table 3**). Muparfostat, which was obtained from a modification of a natural polymer extracted from the yeast *Pichia holstii*, presented a lower potency against HSV compared to a 15 kDa heparin, however, it inhibited the intercellular spread of HSV to a greater extent than the high MW heparin.[101] This result is consistent with the effect of molecular weight mentioned previously. The impact of hydrophobicity on antiviral activity was also investigated by modifying Muparfostat with various hydrophobic functionalities such as an alkyl group, aromatic ring or cholestanyl.[102] The virucidal activity against HSV significantly increased with the addition of a hydrophobic component to the structure. Among the tested compounds, Muparfostat modified with a cholestanyl group was shown to not only block viral attachment but also irreversibly inactivate viral particles. Muparfostat was also shown to be effective against dengue and flaviviral encephalitis in a mouse model.[103] Although, the safety of the compound was established after successful phase I and II clinical trials as adjuvant therapy for patients with hepatitis-related liver cancer, phase III clinical trials were prematurely terminated due to unsatisfactory results obtained from the interim analysis.[104, 105] Similar improvements in antiviral activity were reported with PG545 (2.38 kDa), which was obtained by conjugating cholestanyl to the sulfated tetrasaccharide derived from Muparfostat (**Table 3**). For instance, the antiviral activity of PG545 (IC_{50}=2.2 $\mu g.mL^{-1}$) against RSV was 5-fold that of Muparfostat (IC_{50}=9.9 $\mu g.mL^{-1}$).[106] Furthermore, PG545 was shown to be virucidal and block viral entry. The conjugated tetrasaccharide only displayed a slightly higher potency (IC_{50}=0.8 $\mu g.mL^{-1}$) for the treatment of HSV-2 compared to Muparfostat (IC_{50}=1.5 $\mu g.mL^{-1}$)

as shown in **Figure 6A**. Interestingly, a potent virucidal effect was attributed to PG545 against HSV-2 by disruption of the lipid bilayer of viral particles, whereas the sulfated tetrasaccharide control compound (and Muparfostat) without any hydrophobic functionality, did not affect the integrity of the viral envelope despite its antiviral activity (**Figure 6B**).[107] Interestingly, no or weak viral resistance was observed when HSV-2 or RSV was in prolonged contact with PG545,[102] whereas mutations of envelope proteins involved in viral attachment were observed with Muparfostat-treated virions.[108]

(a)



(b)

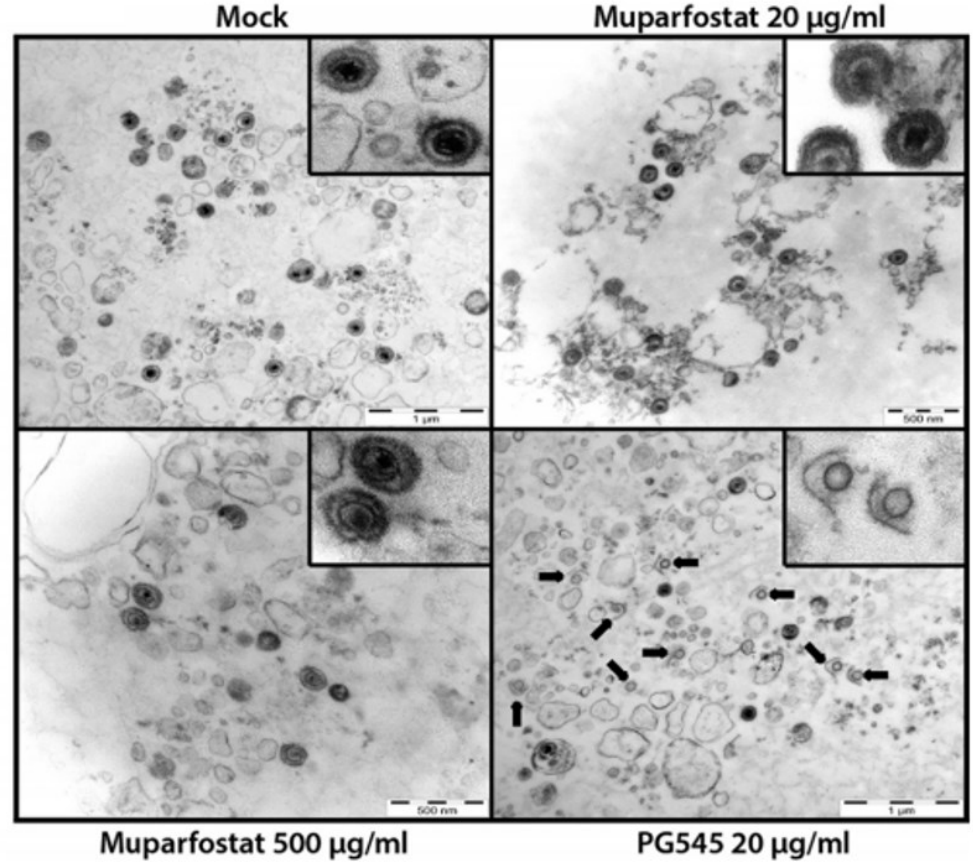
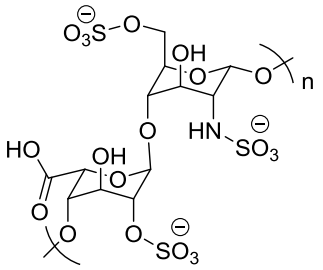
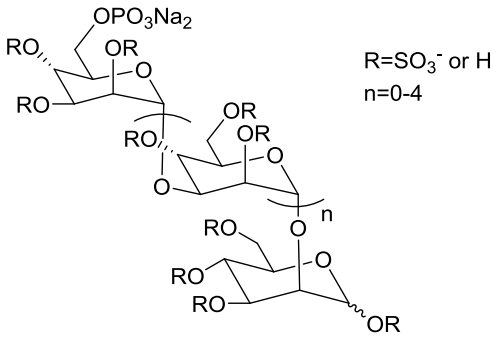
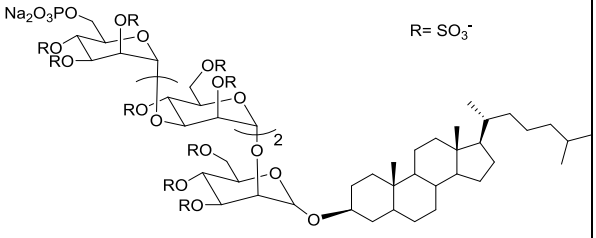
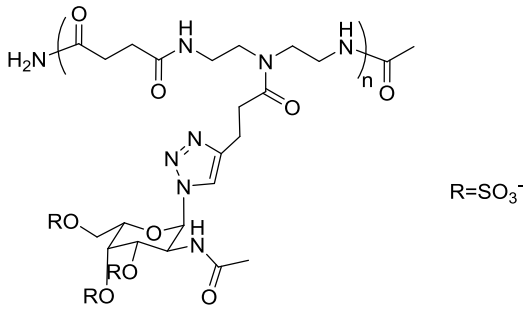
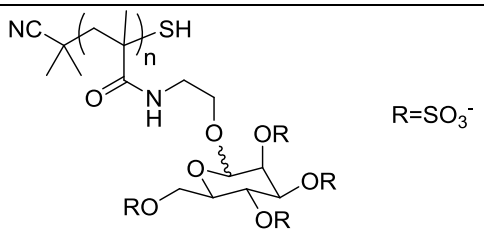
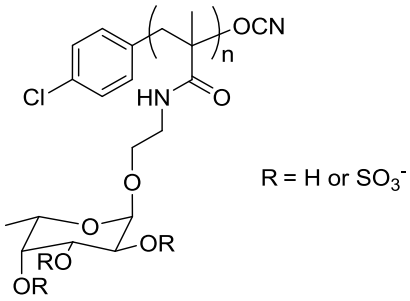
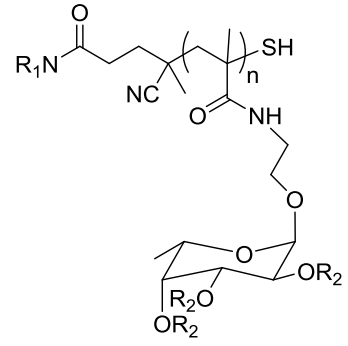


Figure 6. PG545 exhibited a virucidal activity against HSV-2. a) Effect of anti-HSV-2 compounds on the infectivity of HSV-2 after a 15 min incubation at various concentrations. PG545 permanently inactivated HSV-2 from a concentration of 4 μg.mL^{-1}, whereas the other anti-HSV-2 compounds tested such as Muparfostat were not virucidal (the triple asterisks indicating a significant difference of $P < 0.001$).[107] b) Effect of mock, Muparfostat and PG545 treatment on the integrity of HSV-2 viral membrane after a 30 min incubation. Electron microscopy images of viral preparation demonstrate the presence of disrupted viral particles (highlighted with black arrows) for the treatment with PG545, whilst no membrane activity was observed with Muparfostat treatment.[107] Adapted with permission.

Table 3. Chemical structure of antiviral sulfated polysaccharides.

Name	Structure	Reference
Heparin M_n from 5 to 8 kDa		Ghosh *et al.*[96] Ito *et al.*[97] Witvrouw *et al.*[100]
Muparfostat (PI-88) M_n=2.4 kDa		Nyberg *et al.*[101] Ekblad *et al.*[102] Lee *et al.*[10] Ricciuti *et al.*[104] Chen *et al.*[105]
PG545 cholestanoyl conjugated tetrasaccharide M_n=2.38 kDa		Lundin *et al.*[106] Said *et al.*[107] Ekblad *et al.*[102] Adamiak *et al.*[108]

Glycooligomers M_n from 1.6 kDa to 8.2 kDa		Soria-Martinez *et al.*[109]
RAFT glycopolymers M_n from 22 to 52 kDa		Soria-Martinez *et al.*[109]
M_n=30 kDa		Tengdelius *et al.*[110]
RAFT glycopolymers M_n from 2 to 4 kDa		Tengdelius *et al.*[111]

One of the main issues undermining the use of naturally occurring polymers in antiviral treatments is the high batch-to-batch variability of the biological properties, which is correlated to inconsistencies in the chemical properties of the polymers.[112] Therefore, synthetic sulfated glycopolymers were explored as an alternative to naturally sourced HS mimics. Soria-Martinez and co-workers compared the antiviral activity of highly sulfated glycooligomers (1.6 to 8.2 kDa) obtained by solid-phase synthesis and glycopolymers synthesized by RAFT (**Table 3**).[109] The molecular weight of the glycooligomers was well-

defined and ranged from 1.6 kDa to 8.2 kDa, whereas the polymers had a higher molar mass (from 22 to 52 kDa) and their dispersity varied substantially ($Đ$=2.0 and 1.08, respectively). Both glycooligomers and glycopolymers inhibited HPV-16 and HSV-1 infections when the viral particles were pre-treated with the compounds for an hour before infection, albeit with a superior antiviral potency for glycopolymers. Similar to heparin, the glycopolymers were also able to reduce HPV-16 infection *in vivo*, by inhibiting viral attachment onto host cells. Interestingly, influenza A was susceptible to the glycopolymers, whilst no activity was observed for the glycooligomers or heparin (mixture ranging from 6 to 30 kDa). Influenza A is known to bind to the sialylated glycans of host cells instead of HSs, hence no activity was expected for any of the sulfated materials. This result seems to support that the binding affinity of sulfated glycopolymers does not only rely on electrostatic interactions, but its carbohydrate moieties are also essential in the binding with viral particles.

In a separate study, sulfated glycopolymers were synthesized by post-polymerization sulfation of a polymethacrylamide with pendant fucoside moieties generated by cyanoxyl-mediated free radical polymerization (**Table 3**).[110] Compared to polysaccharides which are short and well-defined, the polymer had a high molecular weight (30 kDa) and a broad dispersity ($Đ$=2.15). The sulfated glycopolymer inhibited HSV-1 infection when the polymer was added at the time of infection, whereas the control glycopolymer (without sulfate groups) did not exhibit any antiviral activity. As neither post-infection treatment nor pre-treatment of the cells with the sulfated glycopolymer had any notable effect on HSV-1 infection, the fucoidan mimic seems to prevent viral attachment by binding to viral particles.

Based on the finding that modification of sulfated polysaccharides with hydrophobic moieties could enhance their antiviral properties, a library of sulfated glycopolymers with varying degrees of hydrophobicity was screened by Tengdelius and co-workers (**Table 3**).[111] RAFT polymerization allowed the introduction of hydrophobic moieties at the terminal end of sulfated fucoside-functionalized homopolymers by using chain transfer agents (CTAs) bearing octadecyl, dioctadecyl or chloesteryl moieties. The resulting polymers ranging from 2 kDa (DP 25) to 4 kDa (DP 50) with narrow $Đ$ values (between 1.13 and 1.20) formed aggregates of around 30 to 60 nm when dissolved in water due to their amphiphilic nature. The lipophilic glycopolymers were potent against HSV-1 *in vitro* when added at the time of infection and post-infection, but did not display any virucidal activity. The molecular weight of the polymers seemed to influence the antiviral activity, as the shorter polymers (DP 25) were more effective than the DP 50 polymers. This result seemed to contradict that of the above study from Soria-Martinez *et al*., which demonstrated that short glycooligomers were less potent than their polymer counterpart (**Table 3**). The difference in the structure-activity relationships between the two studies could be explained by the self-assembling properties of the lipophilic glycopolymers in this case, as the critical aggregation concentration (CAC) is lower than the treatment concentration. Additionally, the lack of any virucidal activity of the lipophilic glycopolymers against HSV-1 could be caused by the hydrophobic moieties being hidden from the viral surface in an aggregate conformation. Unfortunately, this study did not include control glycopolymers without a lipophilic end group, which would have provided

further insights into the effect of hydrophobicity on the antiviral activity of sulfated glycopolymers.

3.1.2. **Sulfated/Sulfonated non-glycosylated polymers**

As sulfated glycopolymers suffer from a short plasma half-life, poor bioavailability, and laborious synthetic route, alterations to their chemical structures were required to improve their suitability as antiviral agents.[113] Recently, greater flexibility in the design of HS mimics has been observed with the investigation of sulfated and sulfonated polymers without carbohydrate moieties.

One of the early examples of non-glycosylated sulfonated polymers used as antiviral agents is PRO2000, a commercially available 5 kDa naphthalene sulfonate polymer. Promising *in vitro* results were reported using PRO2000 against HIV-1 infections, and inhibition of viral attachment was observed in the presence of the polymer (**Table 4**).[114, 115] Clinical trials were carried out to evaluate the safety and the performance of PRO 2000 as a vaginal gel to prevent the transmission of HIV-1.[116] Despite being considered as safe during phase I and II, PRO2000 failed phase III clinical trials due to the lack of protection against HIV-1.[117] Therefore, a more complex architecture was designed as an alternative to PRO2000, a 16 kDa dendrimer functionalized with sodium 1-(carboxymethoxy) naphthalene 3,6-disulfonate labeled as SPL7013 (**Figure 7A**). SPL7013 inhibited HIV-1, HSV-1, and HSV-2 infections *in vitro* and in animal models, even when administered post-infection.[118, 119] There is evidence that the dendrimer inhibits both viral entry and replication, thus acting at different stages of infection. Based on these encouraging results, SPL7013 was formulated into a vaginal gel (3 % wt) named VivaGel, which was under examination in a clinical trial for the prevention of HIV-1 and HSV infections (**Figures 7B and 7C**).[120, 121] VivaGel lubrified condoms are now commercially available in Japan, Australia and Canada.[122]

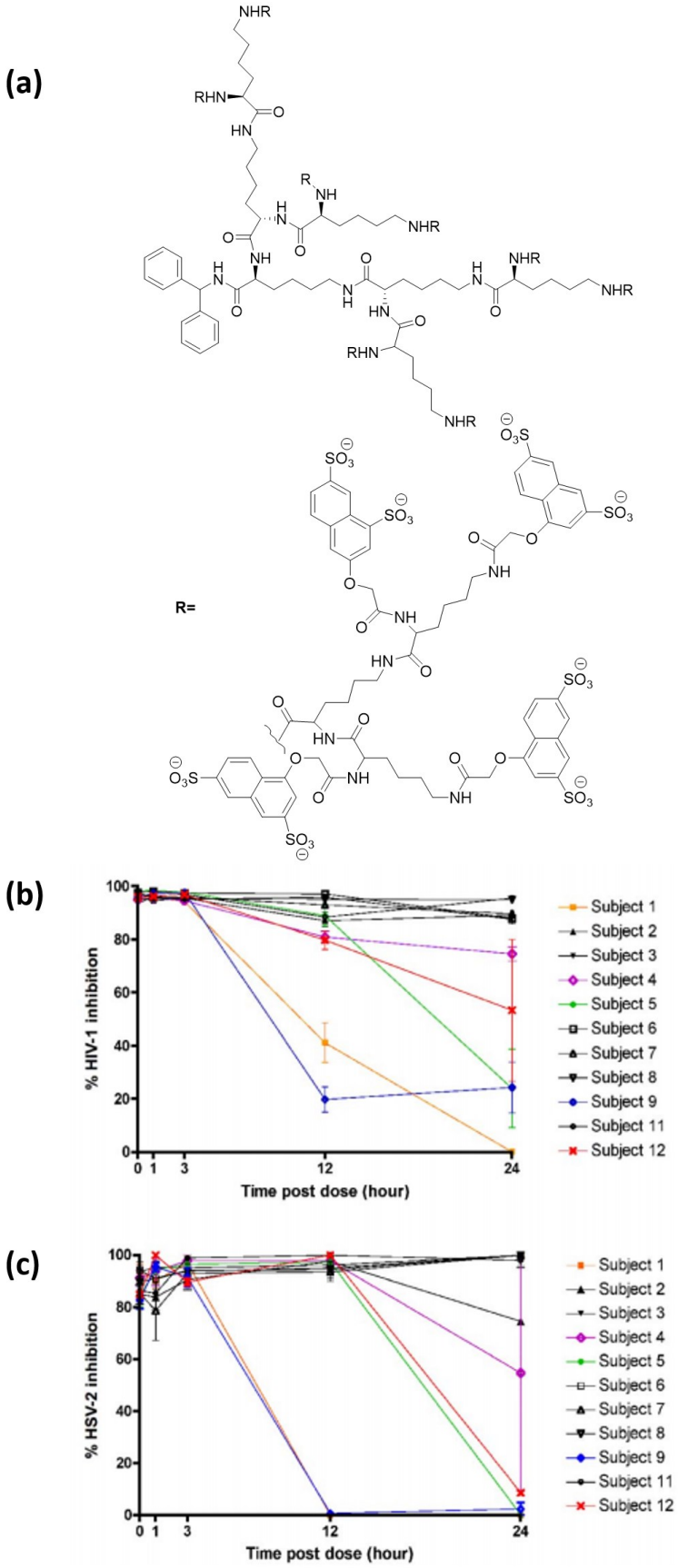
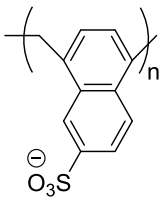


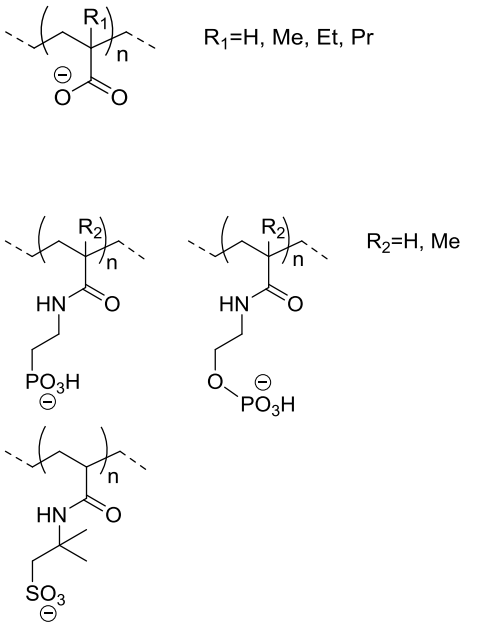
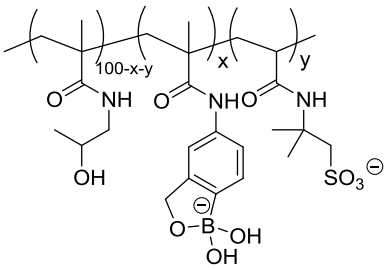
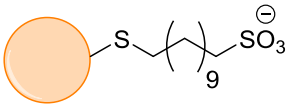
Figure 7. SPL7013-based vaginal gel (Vivagel) retains an inhibitory activity against HIV-1 and HSV-2 *after* vaginal administration. a) Chemical structure of SPL7013. Cervicovaginal fluid samples of healthy women who received 5 doses of Vivagel were collected and the

antiviral activity of the samples was assessed towards b) HIV-1 and (C) HSV-2 in a cell culture assay.[121] In 6 out of 11 women, over 90 % of the initial antiviral activity was retained 24 hours post-dose. Adapted with permission.

In order to establish a structure-activity relationship for antiviral anionic polymers, Schandock and co-workers screened a library of anionic homopolymers bearing different types of charge (carboxylate, sulfonate, and phosphate/phosphonate) and degrees of hydrophobicity (**Table 4**).[123] The antiviral activity of the polymers synthesized using a RAFT process, was tested against a range of viruses. For each type of charge, the authors demonstrated that hydrophobicity was an essential feature in antiviral activity, as anionic charge alone was not sufficient to obtain potent efficacy. Although it was found that sulfonated polymers were more active against HIV-1 and HSV than their carboxylated, phosphated, or phosphonated counterparts, the trend was not as clear against pseudo particles of H5N1 influenza, Ebola, or SARS. However, a large variation in the number average molar mass of the polymers (M_n ranging from 1.8 to 84.7 kDa) was noted, thus affecting the values of active concentrations reported in $mg.L^{-1}$ and rendering any structure-activity relationship less significant.

Table 4. Chemical structure of antiviral sulfated and sulfonated polymers without carbohydrate group.

Name and molecular weight/diameter	Structure	Literature
PRO2000 M_n=5kDa		Huskens *et al.*[114] Keller *et al.*[115, 117] Pirrone *et al.*[116]
SPL7013 (VivaGel) M_n=16 kDa	See Figure 7A	Rupp *et al.*[118] Dezzutti *et al.*[119] McGowan *et al.*[120] Price *et al.*[121]

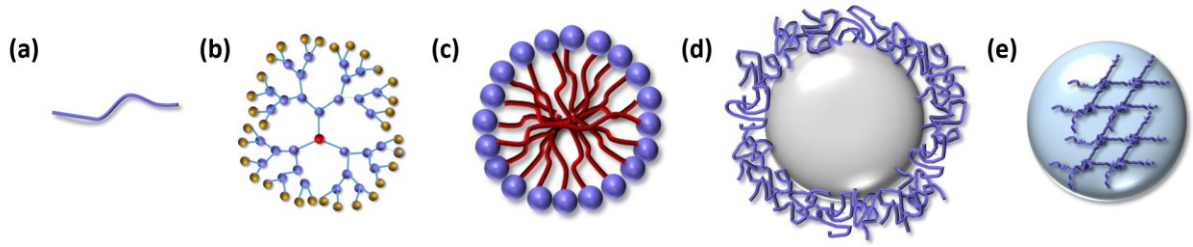
Carboxylate/phosphonate/sulfonate RAFT polymers M_n from 1.8 to 84.7 kDa		Schandock *et al.*[123]
Polyacrylamides containing benzoboroxole moieties obtained by free radical polymerization M_n=100 kDa		Mahalingam *et al.*[124]
MUS-functionalized gold NPs ≈ 2.5 nm		Cagno *et al.*[5, 125]

As sulfonated polymers without carbohydrate moieties solely rely on electrostatic interactions for binding to viral particles, other types of non-covalent interactions were introduced within the polymers to enhance their affinity with the virus surface. Synthetic mimics of lectins were designed using copolymers of benzoboroxole acrylamide and *N*-(2-Hydroxypropyl) methacrylamide (HPMA) to target glycoproteins present on the HIV envelope (**Table 4**).[124] Free radical polymerization was utilised to obtain polyacrylamides of high molar mass (100 kDa) with different benzoboroxole content. The antiviral activity of the copolymers against HIV-1 was assessed by adding the polymers at the time of infection. Entry inhibition against HIV-1 increased with benzoboroxole content, which correlated with an increase in ligand density. Furthermore, the incorporation of 10 mol% of sulfonate moieties obtained by copolymerization of 2-acrylamido-2-methylpropane sulfonic acid (AMPS) with HPMA and the benzoboroxole monomer, improved the antiviral activity of the

benzoboroxole copolymers. This observation could be explained by the introduction of electrostatic interactions supplementing the binding affinity of the benzoboroxole polymers towards the viral envelope.

Strategies to improve the binding affinity of the polymers to virus surfaces were investigated using nanoparticles (NPs). Cagno and co-workers synthesized gold NPs which surface was functionalized with long and flexible sulfonated ligands mimicking HS (**Table 4**).[125] The surface-functionalized NPs (≈ 2.5 nm in diameter) were potent *in vitro* against HSV-2 and pseudo particles of HPV-16 and lentivirus when the viral particles were pre-treated before infection and when the polymers were administered at the time of infection. The NPs were able to distort viral particles upon attachment, thus rendering the binding irreversible and preventing infection. The length of the linkers appeared to be crucial for the efficacy of the polymers: the NPs functionalized with 3-mercaptoethylsulfonate were only virustatic, whereas the NPs with 11-mercapto-1-undecanesulfonate (MUS) were virucidal. Interestingly, additional studies demonstrated that the MUS-functionalized NPs were virucidal against influenza A (H1N1), though its mechanism for viral entry was independent of HSPGs and was instead enabled by the binding of viral particles to the sialic acid groups presented at the surface of host cells.[5] This activity was partly attributed to the affinity of influenza hemagglutinin protein to sulfate or sulfonate moieties, but also to the long alkyl spacer of MUS being an essential attribute for the inactivation of viral particles, as heparin was only weakly virustatic against H1N1. As promising *in vivo* efficacy was reported with MUS-functionalized NPs for the treatment of RSV-infected mice, further analyses on a wider range of viral strains were recommended.[5]

In addition to unimolecular polymers, dendrimers, self-assembling polymers, and NPs, nanogels have also been investigated as potential antiviral agents (**Scheme 1**). For example, dendritic polyglycerol sulfate was used to generate nanogels (≈ 200 nm) with different flexibilities.[126] They were shown to reduce viral activity *in vitro* when introduced during HSV-2 infection. However, pre-treating the host cells with the nanogels did not inhibit viral activity. The authors observed higher inhibition of viral entry with increased nanogel flexibility, which could be explained by a better shielding of viral particles from cellular surfaces.



Scheme 1. The various polymeric structures used as antiviral agents a) unimolecular linear polymer, b) dendrimer, c) micelle, d) nanoparticle with inorganic core and organic shell, and e) nanogel.

Sulfated and sulfonated polymers are suitable antiviral agents against viruses that rely on the presence of HSPGs at the cell surface for viral attachment. Although the majority of viruses follow this mechanism, some viruses bind to host cells via different receptors. An additional concern arising from the administration of sulfated or sulfonated polymers is the significant risk of bleeding which has been reported in previous clinical trials.[127] This adverse effect has been associated with the anticoagulant properties of these polymers.

3.1.3. **Sialylated polymers**

The surface glycoprotein hemagglutinin of influenza viruses is characterized by its strong binding affinity to the sialylated glycans present at the surface of host cells.[128] Sialylated glycans are short, branched glycans featuring a few sulfonate moieties and a sialic acid group at its terminal end. As their properties considerably differ from those of HSs which are highly sulfonated, long and unbranched structures, sulfated/sulfonated polymers generally present limited efficacy towards the treatment of influenza viruses. Therefore, a new class of antiviral agents emerged in the treatment of influenza viruses are based on polymers bearing sialic acid moieties. These polymers inhibit viral attachment by exploiting the strong binding affinity between sialic acid motifs and hemagglutinin. Furthermore, the conjugation of sialic acids to a polymer backbone was shown to improve the stability of sialic acid motifs as the monomeric form is prone to enzymatic degradation.[129] The studies detailed in this section focus on the post-infection treatment of influenza viruses.

A simple one-pot synthesis of sialic acid functionalized polymers was reported by Umemura *et al*.[130] Linear chitosan was conjugated with α2,6-sialyllactosamine (6'SLN), a trisaccharide recognised by all non-egg-adapted human influenza viruses (**Figure 8A**).[131] The degree of substitution (DS) of the glycopolymers was 32 and 38 %, and the molecular weight was 140 and 155 kDa, respectively. Both glycopolymers inhibited H1N1 infection, but the one with the higher DS was more potent (IC_{50}=0.2 $mg.mL^{-1}$ *vs.* 0.5 $mg.mL^{-1}$). Notably, the IC_{50} of 6'SLN alone was 40 to 20 times lower (in molar concentration) than that of the glycopolymers. A similar system was explored by conjugating 6'SLN to a high molecular weight copolymer incorporating a NHS-functionalized acrylate and *N*-2-hydroxyethylacrylamide (MW 1500 kDa).[132] *In vivo* tests revealed that aerosol treatment with 6'SLN functionalized polymer inhibited H1N1 infection in mice (**Figure 8B**). However, virus variants resistant to the polymer seemed to emerge after repeated contact with the polymer.

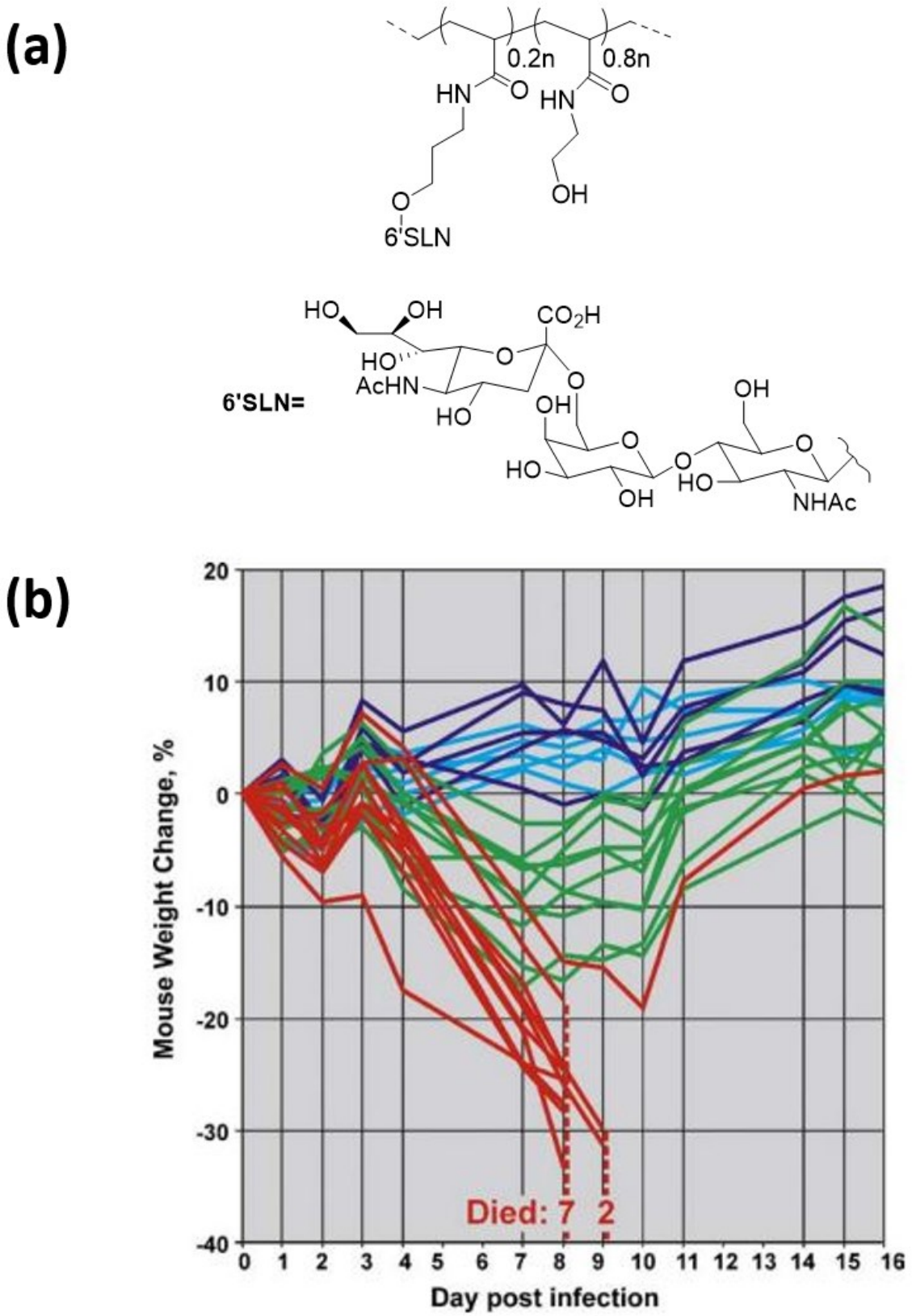
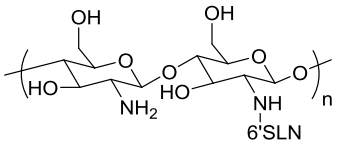
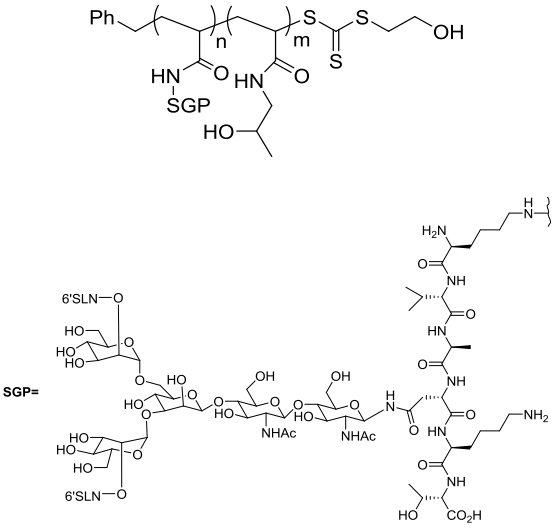
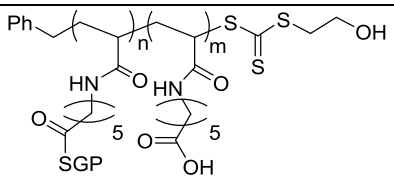
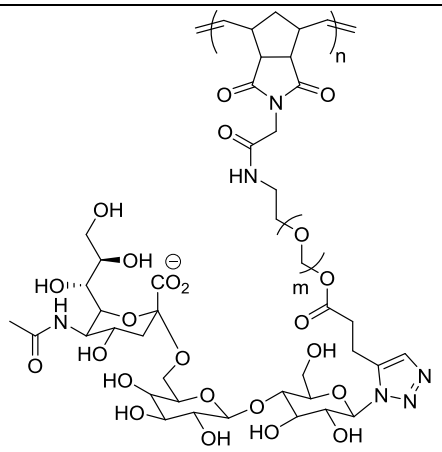
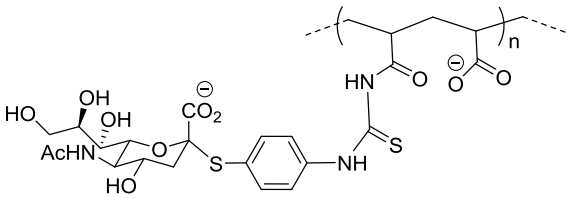
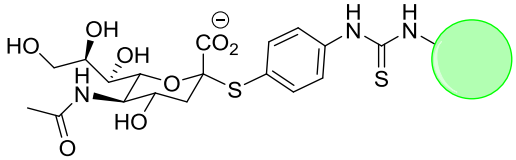
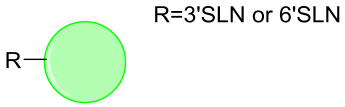


Figure 8. Aerosol treatment with 6'SLN-functionalized polymers successfully inhibited H1N1 infection in mice. a) Chemical structure of the 6'SLN functionalised polymers. b) Weight variations and survival of non-infected mice treated with placebo in dark blue (5 mice), non-infected mice treated with 6'SLN-functionalized polymer in light blue (5 mice),

H1N1-infected mice treated with placebo in red (10 mice) and the green curves correspond to the infected mice treated with 6'SLN-functionalized polymer (10 mice).[132] Steep drop in curve indicates the death of the mouse. Treatment of the mice was administered between day 2 and day 5 post-infection by 10 min exposure to the aerosol every 2 hours, with a 10 hour night break. Adapted with permission.

Table 5. Chemical structure of antiviral sialylated polymers.

Name and molecular weight/diameter	Structure	Literature
6'SLN-conjugated linear chitosan M_n from 140 to 155 kDa		Umemura *et al.*[130]
6'SLN-conjugated linear polyacrylamide M_n = 1500 kDa	See Figure 8A	Gambaryan *et al.*[132]
SGP-conjugated RAFT polyacrylamides M_n from 12 to 60 kDa		Tanaka *et al.*[133]
SGP-conjugated RAFT polyacrylamides M_n from 60 to 80 kDa		Tsuji *et al.*[134]

Sialylated poly(norbornene imide) M_n from 28 to 100 kDa		Tang *et al.*[135]
Sialic acid conjugated linear polyacrylamide M_n from 28 to 68 kDa		Reuter *et al.*[136]
Sialic acid conjugated-PAMAM dendrimer M_n from 9 to 48 kDa	 G2, G4 or G5 PAMAM dendrimer	Reuter *et al.*[136] Landers *et al.*[137]
3'SLN or 6'SLN conjugated-G1 PAMAM dendrimer M_n from 4 to 7 kDa	 R=3'SLN or 6'SLN G1 PAMAM dendrimer	Gunther *et al.*[138]

The effect of polymer length and sialic acid content was investigated by Tanaka and co-workers based on a library of sialylated linear polyacrylamides with different DPs (45 and 145) and degrees of substitution (5 and 10 mol%) by conjugating a sialylglycopeptide (SGP) onto a NHS functionalized polyacrylate after polymerization (**Table 5**).[133] The polymer with the higher DP and higher DS was shown to have a stronger binding affinity with human

influenza A virus strains. The same research group optimised the synthesis of sialic acid-functionalized polymers by using a sulfo-NHS acrylamide monomer to enable the post-polymerization modification to be conducted in water. The number average molar mass of the sialylated polymers ranged from 60 to 80 kDa, with a DP of 150 and different degrees of substitution (5, 8, and 24 mol%).[134] They were able to demonstrate that the synthesized polymers had a higher binding affinity to the hemagglutinin of the human influenza A virus than the free sialylglycopeptide. This observation was attributed to the mutlivalency of the saccharide motif on the polymer backbone.[139]

More complex architectures have been explored with sialylated polymers in order to improve their binding affinity towards viral particles. The antiviral activity of brush polymers against influenza viruses was investigated using ring-opening metathesis polymerization (ROMP) to incorporate a sialic acid terminated PEG norbornonyl macromonomer (**Table 5**).[135] The study examined the effect of DP (25, 50, and 100), length of side chains, and density of sialic acid motifs on the biological properties of the brush polymers. Inhibition of H1N1 infection was improved with increasing DP, side-chain length and density of sialic acid motifs. Their results indicated that the distribution of sialic acid motifs along the polymer backbone had to match the spatial configuration of the glycoprotein hemagglutinin at the viral surface.

Dendrimers are monodispersed surface-functionalized nano-objects, which have been extensively employed in targeted drug delivery due to their enhanced three-dimensional multivalency compared to their linear counterparts.[140, 141] More recently, the unique properties of dendrimers have also been proven to be beneficial in antiviral applications. Inhibition of viral infection in the presence of sialylated linear and dendritic polymers was compared by Reuter and co-workers.[136] Linear polyacrylamides and dendritic polymers generated from partial or full hydrolysis of poly(2-ethyl-2-oxazoline) were conjugated to an isothiocyanate-functionalized sialic acid via the primary and secondary amines of the polymers (**Table 5**). Linear and dendritic sialylated polymers were up to 200-fold more effective at preventing influenza A H2N2 agglutination of chicken erythrocytes than the monomeric form of sialic acid. The dendrimers also inhibited influenza infection in mammalian cells, whereas the activity of the linear counterparts was not evaluated due to their high toxicity. These results demonstrated that modifying the architecture of sialylated polymers significantly improved their selectivity. The synthesis of another type of sialylated dendrimer (43 kDa) was reported by Landers *et al.* from a G4 polyamidoamine (PAMAM) dendrimer, which was functionalized with sialic acid via an aromatic thiourea linker (**Table 5**).[137] Interestingly, the *in vitro* antiviral activity of the dendrimer seemed to be strain-specific: whilst inhibition of H3N2 and H1N1 infections was reported, no effect was observed against H2N2. Results from the murine influenza pneumonitis model suggested that the dendrimer also inhibited H3N2 infections *in vivo*. Likewise, sialylated dendrimers were obtained by conjugating a PAMAM dendrimer to cyclic carbamate derivatized 3′-sialyllactose (3'SLN) or 6′-sialyllactose (6'SLN) as shown in **Table 5**.[138] The dendrimer conjugated with 6'SLN only demonstrated activity against human influenza A virus strains,

whilst the 3'SLN modified dendrimer was able to inhibit avian influenza strains despite its reduced activity towards human influenza strains *in vitro*.

3.1.4. **Nucleic acid polymers**

Nucleotide and nucleoside analogs have been verified as potent antiviral agents since the late 1970s due to their ability to inhibit viral DNA replication.[142] However, viruses were shown to efficiently adapt to their antiviral mechanism, with the emergence of resistant variants upon prolonged contact.[143] In parallel, the synthesis of oligonucleotides has been extensively investigated as a tool for selective gene regulation, but the structural modification was necessary to improve their poor *in vivo* half-life.[144] The phosphorothioation of the phosphodiester linkages increased the hydrophobicity of the backbone, whilst preventing nuclease attack.[145, 146] Being both anionic and ampiphatic, single-stranded phosphorothioated oligonucleotides, which are commonly referred to as nucleic acid polymers (NAPs), emerged as potential candidates in antiviral therapy (**Figure 9A**). Importantly, the hydrophobicity of the local environment was shown to induce a transition between a charged and uncharged state of the phophorothioate linkage depending on the location of the free electron from the sulfur atom.[147] This transition renders NAPs highly adaptable as they can match their hydrophobicity to that of their environment.

NAPs presented a broad spectrum of activity *in vitro* (e.g. HIV-1, herpesviruses, cytomegalovirus, HBV, and HCV) when introduced at the time of infection.[148] Systematic studies support that the potency of NAPs is independent of their sequence (i.e. type of base), whereas their amphipathicity and their length (between 20 and 40 units) appeared to be essential characteristics. The potency of NAPs against a wide range of viruses (e.g. HSV, CMV, and HBV) was maintained *in vivo*.[148-150] Their mechanism of action was shown to be quite similar to that of sulfated polysaccharides: NAPs interact with the fusion glycoprotein gp41 at the surface of virions, hence preventing viral attachment onto host cells.[151] However, when treating HBV infections, NAPs were shown to exhibit a post-entry effect in addition to the inhibition of viral entry.[152] By preventing virus budding, NAPs inhibited the secretion of HBsAg subviral particles from infected hepatocytes.[153] Results from clinical trials are in agreement with this proposed mechanism as levels of serum HBsAg and HBV DNA decreased in patients with chronic HBV infection after treatment with NAPs.[154]

Treatment tolerability of NAPs can be affected by DNA or RNA-sensing receptors which trigger an undesirable immune response upon recognition of single-stranded nucleotides.[155, 156] Structural modifications at the 2' ribose position of NAPs have been incorporated to prevent pro-inflammatory effects from occurring upon administration of the polymers without altering their antiviral properties. Such adjustments (5'methylcytosine and 2' O-methylribose) were performed to obtain the optimised structure of REP2139 (**Figure 9A**), which consists of an alternating sequence of 2 different bases repeated 20 times. This

NAP is currently undergoing phase II of clinical development for chronic HBV infections in a combination treatment with tenofovir disoproxil fumarate (TDF) and pegylated interferon (pegIFN).[147, 157, 158] After 48 weeks of randomised trial, the treatment resulted in a similar level of tolerability to the treatment with TDF and pegIFN alone. It is worth noting that in patients treated with REP2139, the levels of HBsAg declined at a faster rate, whilst the rate of HBsAg seroconversion increased compared to those solely treated with TDF and pegIFN (**Figure 9B**).[158]

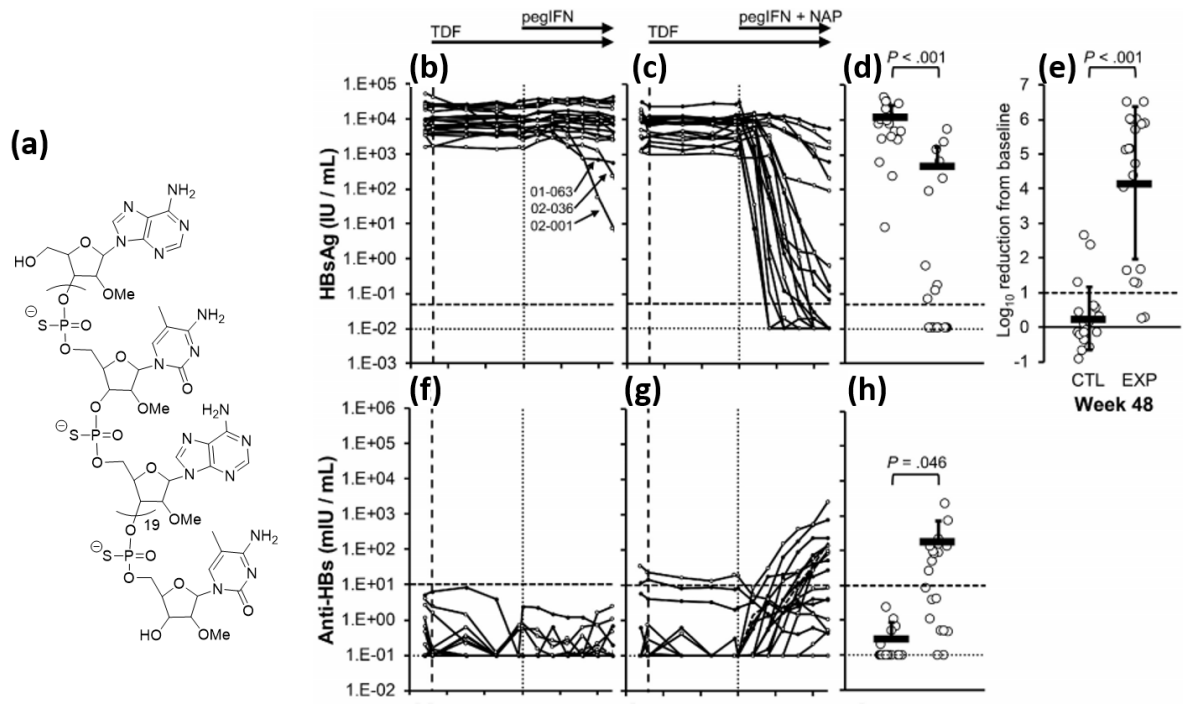


Figure 9. NAP REP2139 improves HBsAg reduction and seroconversion in patients with chronic HBV infection. a) Chemical structure of NAP REP2139 Variations in serum HBsAg and anti-HBs levels in the control group treated with TDF and pegIFN (graphs b and f) and the experimental group treated with REP2139 combined with TDF and pegIFN (graphs c and g) over the course of a 48 week-randomized trial.[158] Differences in initial and final HBsAg (graphs d and e for absolute and $\log_{10}$ reductions, respectively) and anti-HBs levels (graph h) were analysed statistically. Adapted with permission.

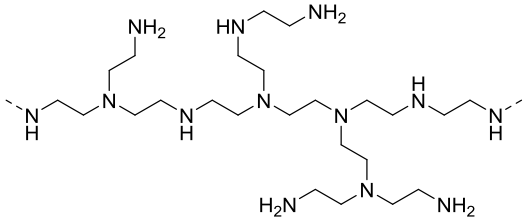
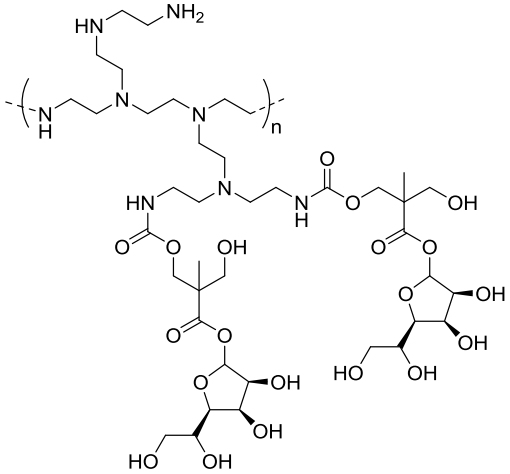
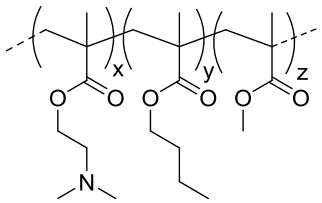
Although these results are promising for the treatment of HBV, further studies are necessary to elucidate the adaptability of viruses to NAPs which would determine the long-term relevance of this class of antiviral agents.[159] Furthermore, the scale-up of NAPs might be hampered by their tedious synthetic process.[160]

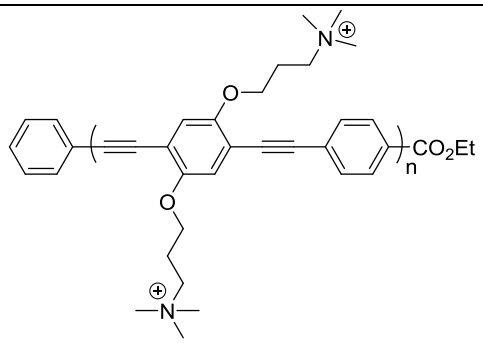
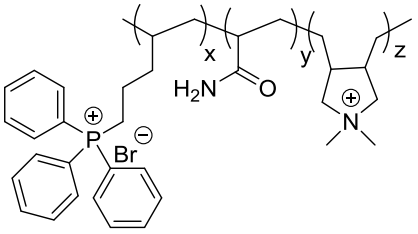
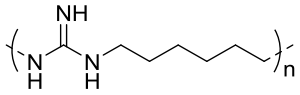
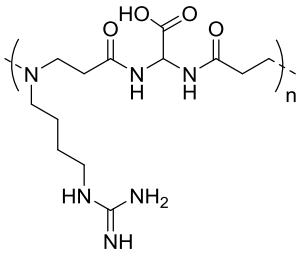
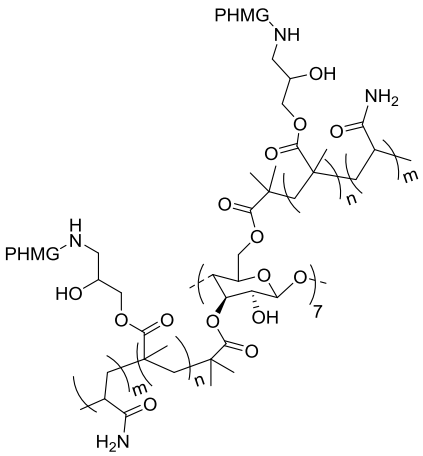
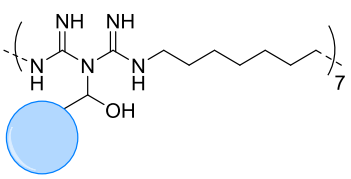
3.2. **Cationic polymers**

3.2.1. **Amine-functionalized polymers**

Given that cationic peptides were shown to prevent viral attachment onto host cells, positively charged polymers could also display similar properties. Although polyethylenimine (PEI) has been more commonly utilised as a non-viral delivery vector for nucleic acids, both linear and branched PEI were reported in recent years to display a broad spectrum of antibacterial properties.[161, 162]

Table 6. Chemical structure of cationic antiviral polymers.

Name and molecular weight/diameter	Structure	Literature
Branched PEI M_n = 75 kDa		Wang *et al.*[163]
Mannose-functionalized branched PEI M_n from 20 to 60 kDa		Ichiyama *et al.*[164]
Eudragit E100 M_n = 47 kDa		Alasino *et al.*[165, 166]

Cationic poly(phenylene ethynylene) M_n from 0.5 to 1.4 kDa		Wang *et al.*[167] Martin *et al.*[168]
Quaternary phosphonium tripolymer M_n = 30 kDa		Xue *et al.*[169]
PHMG M_n = 1.4 kDa		Lysytsya *et al.*[170]
AGMA1 M_n = 10 kDa		Donalisio *et al.*[171, 172]
Guanidinium-functionalized ATRP star polymer M_n = 19 kDa		Pan *et al.*[173]
PHMB-functionalized NPs 150 to 250 nm		Bromberg *et al.*[174]

The antiviral effect of 25 kDa linear PEI against non-enveloped HPV-16 and enveloped HCMV was investigated *in vitro*.[175] When the cells were pre-treated with PEI prior to infection or when PEI was administered during infection, a critical reduction in viral infection was observed. Binding assays supported that PEI acted on host cells by blocking HSPGs, the primary binding receptors of HPV and HCMV, thus effectively prevented viral attachment. A comparison of the *in vitro* antiviral activity of linear and branched PEI was established by Wang and co-workers using a 40 kDa linear PEI and a 75 kDa branched PEI against porcine reproductive and respiratory syndrome virus (PRRSV) infection on MARC-145 (**Table 6**).[163] The number of infected cells was drastically reduced when 40 kDa linear PEI was administered as a cell pre-treatment or during infection, whereas only a slight antiviral effect was observed in the presence of the branched PEI. As there was no antiviral activity detected with PEI treatment post-infection, the mechanism of action of the polymer seemed to depend on preventing viral attachment but was not fully elucidated. Unexpectedly, the authors reported an enhancement in the number of PRRSV-infected cells after cell pre-treatment with 40 kDa linear PEI, compared to the untreated control, when porcine alveolar macrophages (PAM) cells were used as host cells instead of monkey kidney derived cells MARC-145. An increase in PRRSV infection was also observed when the polymer treatment was administered post-infection to PAM cells. 40 kDa linear PEI was only able to reduce PRRSV infection in PAM cells when added during infection. The discrepancy in treatment outcome across cell lines could be caused by differences in the susceptibility of the cells to PRRSV or in the mechanism of viral attachment and internalisation. The adverse effect of PEI on viral infection observed in this study is in agreement with an investigation from Owada *et al.* reporting that 70 kDa linear PEI promoted HIV-1 *in vitro* infection using human T-cells (MT-4 and MOLT-4).[176] Although the polymer inhibited viral infection when pre-incubated with viral particles prior to infection, it accelerated viral infection when administered during or after infection. The work suggested that PEI stimulated membrane fusion between viral particles and host cells by increasing membrane fluidity.

An additional concern which arises from the use of PEI for systemic administration is its relative cytotoxicity.[177] Ichiyama and co-workers were able to reduce the toxicity of the cationic polymer by conjugating mannose-substituted cyclic carbonates to 10 kDa branched PEI (**Table 6**).[164] The selectivity of the polymers against dengue virus (DENV-2) substantially increased after mannose-functionalization, though the antiviral activity of the polymer decreased. The polymer inhibited infection *in vitro* when administered during or early after infection by preventing viral attachment onto host cells. Molecular docking computations of the mannose-modified PEIs suggested that hydrogen bonding with viral surface proteins took place in addition to electrostatic interactions with cell surface receptors (**Figure 10**). Interestingly, the active concentration of the polymer against DENV-2 remained constant after prolonged exposure, which was attributed to the non-specific supramolecular interactions with both the viral surface and the host cell surface.

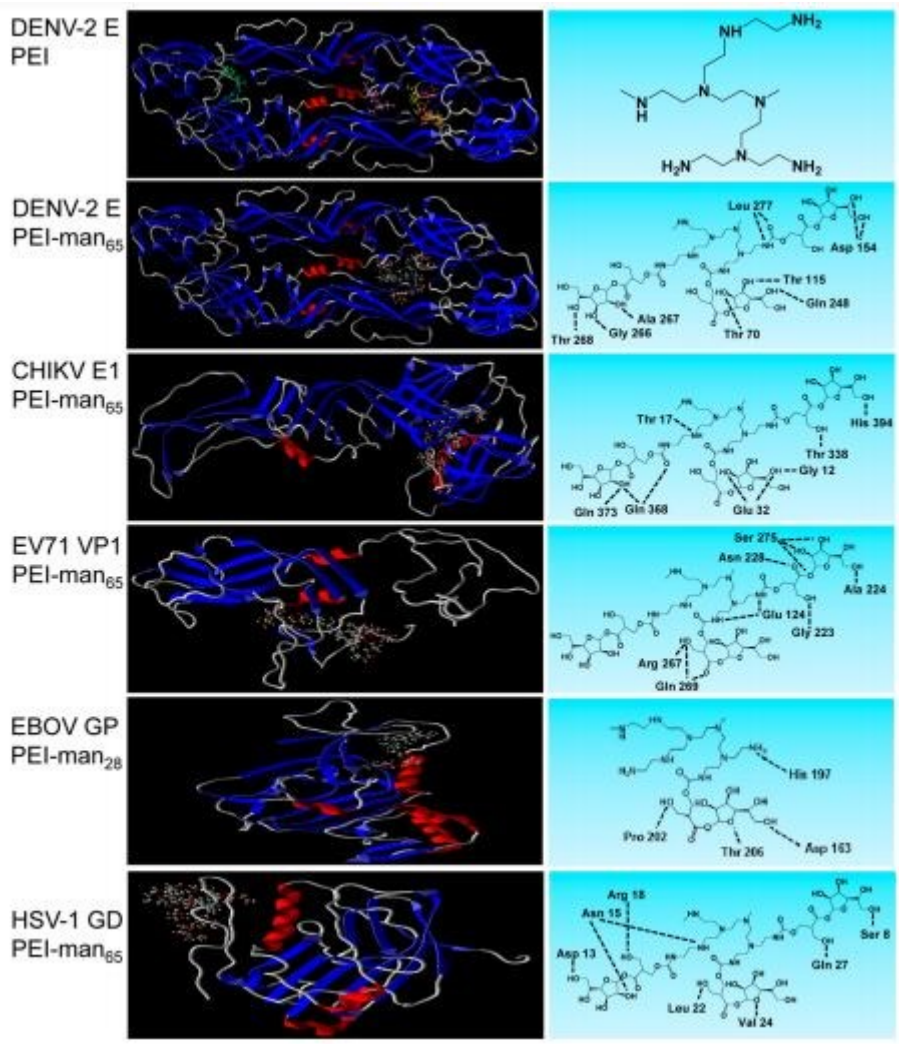


Figure 10. Modeling of interactions of polymers with virus proteins using a blind docking computation study. On the left column, binding model of one unit of polymer with the corresponding virus protein (examples with dengue virus DENV-2, Chikungunya virus CHIKV, Enterovirus EV71, Ebola virus EBOV, and HSV-1). On the right column, predictions of hydrogen binding sites between a unit of polymer and viral protein residues using the cavity detection algorithm of Molegro Virtual Docker.[164] Adapted with permission.

Rather than systemic administration, PEI could be more suitable as a topical virucidal drug, as suggested by studies from Hayashi *et al*.[178, 179] The authors demonstrated that intravaginal administration of 3.6 kDa branched PEI reduced viral replication in HSV-1 and HSV-2 infected mice.[178] When applied an hour post-infection, the polymer treatment prevented the formation of genital herpes, which have been associated with an enhancement in HIV transmission.[180] However, the virucidal effect of the linear PEI significantly decreased when applied 24 hours post-infection. Furthermore, conjugation of the linear PEI to liposomes improved the efficacy of the treatment in vivo, whilst reducing its

cytotoxicity.[179] No viral transmission study has been carried out using PEI, probably due to safety concerns.

In parallel to PEI, other ammonium-functionalized polymers were explored for their ability to inhibit viral infections. Eudragit E100 is a copolymer of methyl methacrylate, *N*,*N* dimethylaminoethylmethacrylate, and butyl methacrylate in a 1:2:1 composition with Mn ≈ 47 kDa, widely employed as an oral drug excipient (**Table 6**).[181] Recent studies looked into its antiviral properties, which were attributed to its amphiphatic nature.[165] Indeed, when HSV-2 was pre-treated with E100 for 30 min prior to infection, the infectivity rate significantly dropped. Similar effects were observed against other enveloped viruses such as vesicular stomatitis virus (VSV), bovine viral diarrhea virus (BVDV) or Measles virus (MV).[166] However, E100 was shown to exhibit some membrane-destabilizing properties against mammalian cells at similar concentrations to that inhibiting viral infections, rendering it unsuitable as an antiviral agent.[165]

Lately, highly bactericidal properties of cationic conjugated polymers have been reported after exposure to UV/visible light via the generation of reactive oxygen species (ROS).[182] These findings inspired researchers to study the antiviral activity of cationic conjugated materials. Wang *et al.* provided insights into the properties of cationic oligomeric conjugated polyelectrolytes (OPEs) based on synthetic poly(phenylene ethynylene) (PPE) against MS2 and T4 bacteriophages (**Table 6**).[167] The materials demonstrated high potency against bacteriophages, which was enhanced under UV/visible light irradiation. Molecular dynamics simulations suggested that the OPEs strongly bound to the viral capsid via Van der Waals and electrostatic interactions, thus causing capsid distortion.[168]

The antiviral efficacy of cationic polymers towards non-enveloped viruses was also investigated with 30 kDa cationic copolymer containing quaternary ammonium and phosphonium groups synthesized by free radical polymerization. In addition to its antibacterial activity against *E. coli*, the copolymer was demonstrated to be active against non-enveloped adenovirus.[169] As adenoviruses lack a lipid bilayer, inhibition of viral attachment to cell receptors is, in this case, thought to take place through electrostatic interactions between the phosphonium polymer and the adenovirus capsid.

3.2.2. **Guanidine-functionalized polymers**

Previous studies investigated the use of guanidine-functionalized low molecular weight compounds as antiviral agents.[183, 184] The results suggested that the guanidine moiety could inhibit entry of both enveloped and non-enveloped viruses into host cells. Based on these findings, macromolecules presenting guanidinium functionalities have been investigated to enhance viral infection inhibition.

In addition to a well-documented broad spectrum of antibacterial activity, polyhexamethyl guanidine (PHMG, M_n=1400 g.mol^{-1}) has been explored as an antiviral agent against enveloped viruses such as Equine herpesvirus type 1, Rhinotracheitis infectious bovine, and Equine infectious anemia virus (**Table 6**).[170] The study demonstrated that for all three viruses, pre-treatment of bovine tracheal cells or chicken embryo fibroblasts with PHMG before infection successfully increased cell viability. The cationic polymer might protect the cells from viral particles by binding to the phospholipids at the surface of the cell membrane.

The antiviral mechanism of guanidinium polymers was investigated using a polyamidoamine with guanidinium pendant groups (10 kDa) prepared by Michael-type polyaddition of (4-aminobutyl)guanidine to 2,2-bis(acrylamido)acetic acid (**Table 6**).[171] Treatment with the guanidinium polymer (AGMA1) at the time of infection reduced the infectivity of HCMV, HPV-16, HSV-1 and 2. Importantly, the active concentration of the polymer was over 100-fold lower than its CC_{50} (50% cytotoxic concentration). Complementary *in vitro* assays were performed by administering the polymer treatment at different points of infection using HSV-1 and 2 to understand the inhibition mechanism.[172] Strong reduction in viral infection was observed following pre-treatment of host cells with the polymer prior to infection, whereas pre-treatment of viral particles with AGMA1 did not induce any inactivation and viral internalisation was not inhibited when the polymer was administered post-infection. These findings suggested that the guanidinium polyamidoamine reduced cell susceptibility to viral attachment by binding to the HSPGs at the surface of the cell membrane. Based on the promising results obtained *in vitro*, AGMA1 was used as a topical microbiocide against genital HSV infection in an animal model. Not only was the polymer safe to use, but it also provided protection from HSV infection even when applied 30 minutes before exposure to high viral loads.

Other polymer structures were studied in order to improve the binding affinity to cells. Pan *et al.* reported the synthesis of a star polymer generated from the copolymerization of a PHMG macromonomer and acrylamide by ATRP using cyclodextrin as a macroinitiator (**Table 6**).[173] The star polymer (19 kDa) was active against non-enveloped adenovirus when added at the time of infection. A similar mechanism of action to that of cationic linear polymers was proposed: the star polymer was interacted with HSPGs present at the surface of host cells, thus preventing viral attachment. Larger cationic structures were designed by Bromberg and co-workers using cationic NPs (150-250 nm) with a magnetite core and a silica shell, surface-functionalized with PEI or poly(hexamethylene biguanide) (PHMB).[174] Following pre-treatment of viral particles, NPs reduced the infectivity of both enveloped and non-enveloped viruses such as HSV-1, bacteriophage MS2, infectious pancreatic necrosis virus (IPNV), and viral hemorrhagic septicemia virus (VHSV).

3.2.3. **Amine-functionalized surfaces**

Due to the elevated toxicity associated with the systemic administration of ammonium or guanidinium-functionalized polymers, their application towards antimicrobial coatings has attracted increasing interest, although most studies were solely focused on the antibacterial activity of cationic surfaces. Recently, the virucidal activity of amphiphilic ammonium-functionalized coatings was reported by Klibanov and co-workers.[185] These surfaces were obtained on glass slides after solvent evaporation of a solution of branched *N*,*N*-dodecyl,methyl-PEI (DMBPEI), which was synthesized by quaternization of 750 kDa branched PEI (**Figure 11A**). The surface exhibited virucidal activity against wild-type strains of influenza viruses H1N1 and H3N2, as well as drug-resistant H3N2 and H4N1 strains.[186] Interestingly, polymer solution did not reduce viral titer. Therefore, the leaching of polymer did not contribute to the potency of the coating. Mechanistic studies revealed that DMBPEI-coated surfaces compromise the integrity of viral particles, which release their genomic material into solution after adhesion to the surface (**Figures 11 B** and **11C**).[187] Although DMBPEI is highly toxic when in solution, DMBPEI coated surfaces were shown to be biocompatible and suitable as coatings for metallic implants *in vivo*.[188]

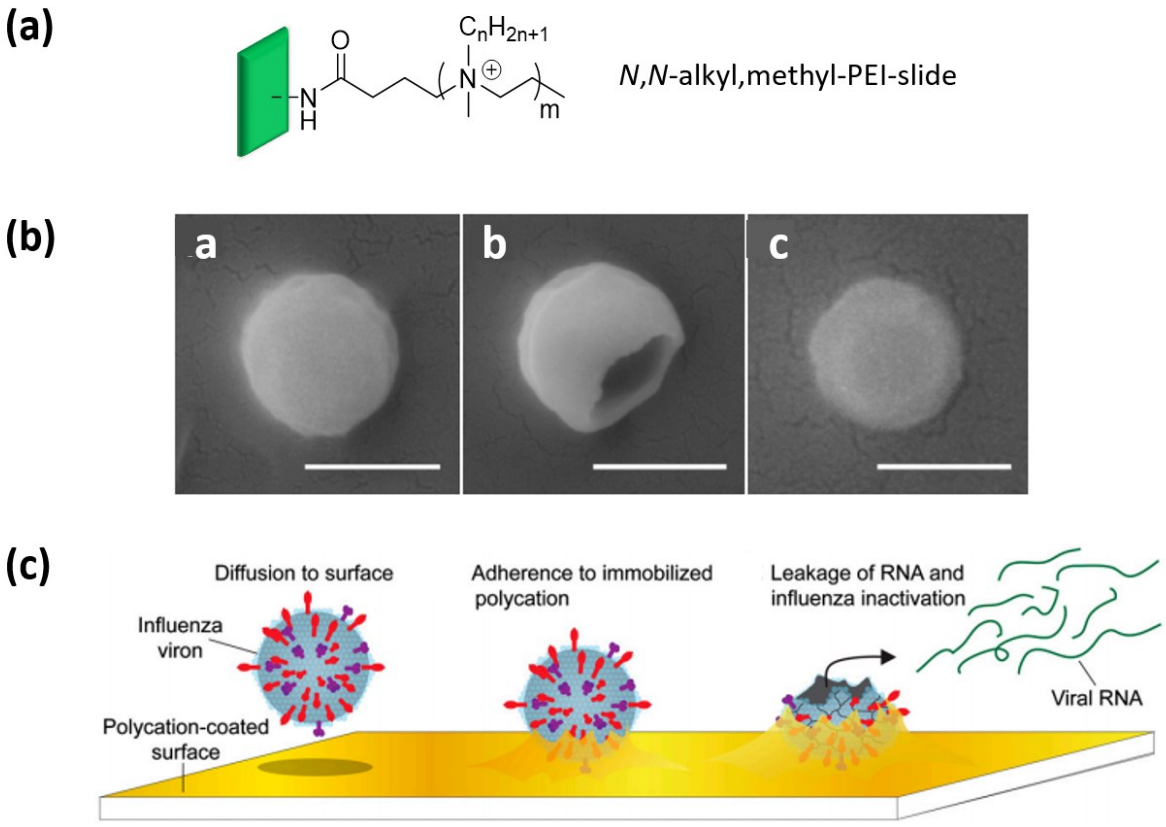


Figure 11. DMBPEI-functionalized surfaces display a potent antiviral activity. a) Schematic representation of a DMBPEI-functionalized surface. b) Scanning electron microscopy images of WSN influenza strain after exposure to plain (a) and DMBPEI-functionalized silicon wafers (b and c).[187] The majority of virions exposed to the cationic surfaces presented structural damage as shown in the photo (b), and only a few did not appear to have any damage as shown in the image (c) (Scale bars, 100 nm). c) Schematic representation of the mechanism of action of DMBPEI-functionalized surfaces against influenza viruses.[187] Adapted with permission.

Coating of surfaces by solvent evaporation is restricted by the geometry of the surface to coat, hence other techniques have been employed to improve the uniformity and the stability of cationic amphiphilic virucidal coatings. A layer-by-layer approach was utilised by Wong and co-workers to assemble linear *N*,*N*-dodecyl,methyl-PEI (DMLPEI from 217 kDa LPEI) and polyacrylic acid (50 kDa) on silicon substrates.[189] This facile and versatile technique enabled greater control and reproducibility over the coatings than the solvent evaporation process. The resulting surfaces were highly potent against H1N1 and a range of bacteria, whilst being non-toxic to mammalian cells. Similar to DMBPEI coatings, the virucidal activity was attributed to the surface and not to the leaching of the polymers. The potency of the coatings increased with the number of layers, which was explained by higher surface coverage. Another example of improved surface functionalization is the immobilisation of 217 kDa linear PEI onto bromine-functionalized glass surfaces, followed by quaternization of amines.[190] The virucidal activity of the resulting surfaces was investigated against H1N1 and H3N2. The most potent surfaces, functionalized with *N*,*N*-hexyl,methyl-PEI and *N*,*N*-dodecyl,methyl-PEI, were able to reduce viral titer by over three logs. The authors suggested that the antiviral activity of the coatings increased with the density of quaternary ammoniums on the surface.

The virucidal performance of glass surfaces coated with didodecyldimethylammonium bromide (DDAB) functionalized silica NPs was also studied.[191] After exposing H1N1 suspension onto the cationic surface for 30 minutes, no remaining virus was found. Analysis of the washing solutions demonstrated that the antiviral activity was not attributed to the leaching of the cationic silica NPs but to the functionalized surface.

These examples of amphiphilic cationic coatings with polymers incorporating amines quaternized with long hydrophobic alkyl chains, demonstrated high biocompatibility and activity towards influenza viruses such as H1N1, H3N2, and H4N1. However, their potency towards viruses other than the influenza type is yet to be investigated to ensure their suitability as broad-spectrum antiviral coatings.

4. Conclusion and perspectives

A variety of peptide and polymer structures have been explored as antiviral agents with various functional mechanisms (**Figure 12**). The majority of the reviewed antiviral macromolecules prevented viral attachment onto host cells by targeting either the host cell (e.g. cationic peptides and polymers) or the virions (e.g. anionic polymers). Entry of enveloped viruses could also be interrupted by viral fusion inhibitors, which are peptides mimicking the functional sequence of viral fusion proteins. Finally, nucleic acid polymers were recently demonstrated to block the secretion of newly formed virions, in addition to preventing viral attachment.

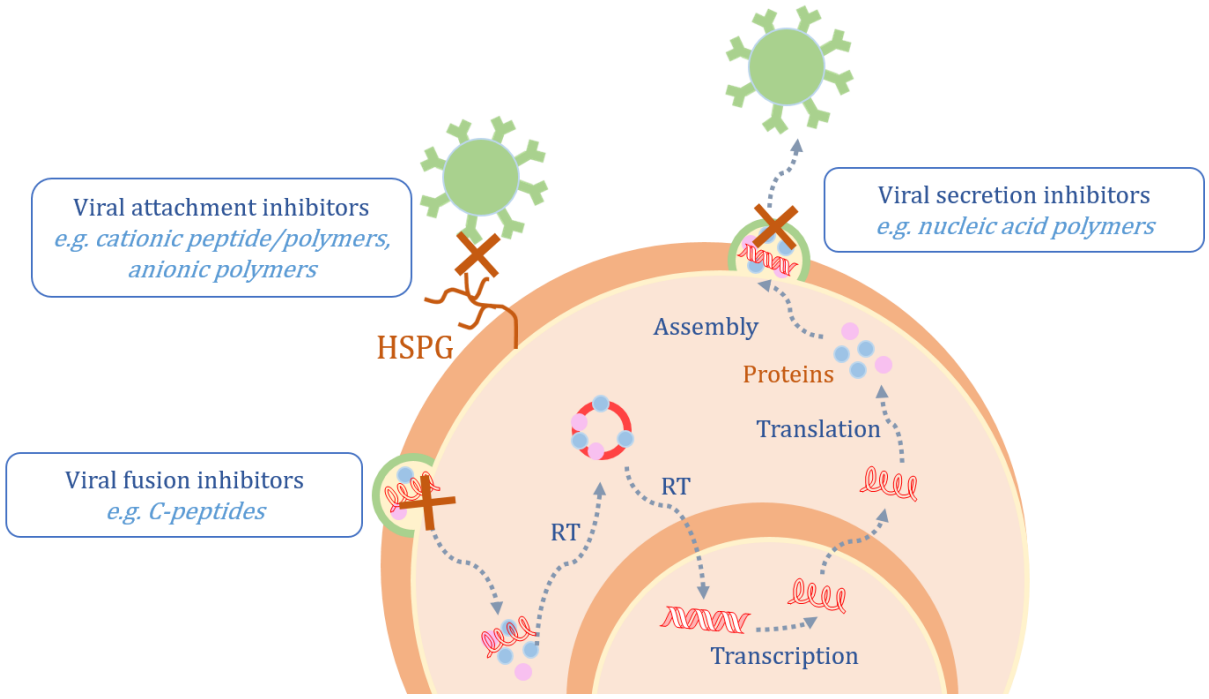


Figure 12. Main targets of the different types of peptide- and polymer-based antivirals, using the example of the life-cycle of HIV. RT stands for reverse transcription.

As recently experienced with the SARS-CoV-2 pandemic, reducing viral transmission is one of the key aspects in the management of the crisis.[192] Several studies demonstrated the ability of antiviral peptides to inhibit the binding of SARS-CoV-2 to the cell receptor ACE2 or to block the membrane fusion process.[193, 194] However, these peptides are highly specific to the type of virus. Therefore, broad-spectrum antivirals are urgently needed in the case of a future pandemic caused by a new virus. In this regard, the most promising materials appear to be anionic polymers due to their activity towards a range of viruses and biocompatibility which allows for a large therapeutic window. Interestingly, indications that heparin could

reduce the mortality of patients infected with SARS-CoV-2 have been reported.[195] Clinical trials are currently ongoing in order to determine the safety of heparin as a treatment against SARS-CoV-2, as well as its efficacy in reducing thrombosis and inflammation in infected patients.[196] Advances in amphiphilic cationic coatings also represent an encouraging avenue, as polymers incorporating amines quaternized with long alkyl chains were shown to be virucidal against various influenza virus strains. Additional studies are still required to determine the range of antiviral activity of the cationic polymer coatings, and in particular, their effect against coronaviruses has yet to be investigated. Despite the great progress in the clinical trial of nucleic acid polymers for post-infection treatments, their activity currently appears to be limited to HBV and HDV. Polymer-drug conjugates might be able to provide a means to further broaden the activity of antiviral polymers. Studies demonstrated that the pharmacokinetics of small antiviral agents was improved after being conjugated to an antiviral polymer, while their potency towards viruses was synergized with that of the polymer.[184, 197, 198]

However, comparisons between various studies are undermined by variations in the type of assay performed. First of all, *in vitro* conditions for infection assays varied significantly, even for the same virus strain: in some cases, the polymer/peptide treatment was administered at the time of infection, whereas in other studies, the viral particles (or the host cells) are pre-treated with the polymer/peptide prior to infection (with variations in the duration of the pre-treatment). As previously mentioned, the type of cell line used as host cells can further alter the antiviral efficacy of the screened compounds. Following infection, the type of downstream assays performed to evaluate the efficacy of the polymer/peptide treatment also varied from a plaque or a fluorescence assay to determine the number of infected cells, to the quantification of viral surface protein level or viral genetic material.[199] In the case of anti-influenza materials, a rapid and high-throughput screening method is available: the hemagglutinin inhibition (HI) assay evaluates the effect of the tested compounds on the interactions between the influenza virus and the hemagglutinin proteins present at the surface of red blood cells.[200]

The drawbacks of antiviral polymers as therapeutics, which hinder their clinical translation, include their potential toxicity, polydispersity in their molecular weight distribution and difficulty in determining their pharmacokinetics. Not only can their metabolites exhibit toxicity, but the accumulation of polymers with a non-degradable backbone also represents a hurdle to their application as antiviral agents.[201, 202] Concerns over adverse immunogenic reactions caused by the administration of polymers *in vivo*, as reported with polyethylene glycol, also need to be addressed in order to encourage the development of antiviral polymers.[203, 204] Cationic polymers or peptides exhibit higher *in vivo* toxicity compared to their anionic counterparts, as they can cause damage to mammalian cell membranes as well as aggregation of red blood cell.[205-207] Therefore, conjugation of cationic antiviral polymers or peptides to a targeting moiety and delivering them specifically to virus or infected cells may mitigate their toxicity. In addition, molecular weight distribution is another concern as molecular length may affect antiviral efficacy. Technical

advances in synthetic methods seem to have rapidly progressed in the last decade to help overcome this barrier.[208-210] The study of pharmacokinetics of polymers is challenging due to polydispersity in molecular weight. Isotopic labelling may provide a solution. Although peptides have narrow molecular weight distribution and can be readily quantified in the plasma using MALDI or LC-MS to study pharmacokinetics, they have shortcomings of high cost and short blood circulation time due to enzymatic degradation.[211, 212] These issues may be overcome by rigidifying the structure of peptides, as mentioned in section 2.2.[74, 75, 78]

Antiviral material research would progress rapidly with standardisation efforts among different laboratories as this would allow the use of machine learning to accelerate the development of broad-spectrum antiviral macromolecules against emerging or re-emerging viruses such as the SARS-CoV-2 virus. Artificial intelligence (AI) has recently been used to design novel antimicrobial agents by mining large volumes of data to predict new potential leads. [213-215] AI has also been employed to repurpose approved drugs for the treatment of SARS-CoV-2 infection.[216, 217] It is envisaged that AI will play an important role in new drug development for the prevention and treatment of SARS-CoV-2 infection or other emerging viral infections.

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Conflict of Interest

The authors declare no competing financial interests.

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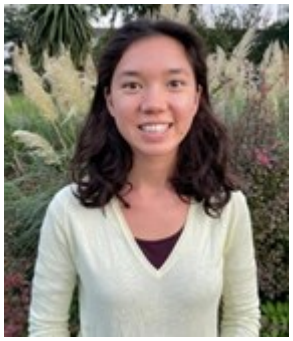

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Various targets of the viral life-cycle can be exploited to inhibit viral infection, from viral attachment to viral fusion or replication. This review discusses antiviral peptides and polymers with diverse structural features and functional mechanisms, analyzes limitations of polymers/peptides as therapeutics and provides future perspectives of accelerated peptide/polymer development, and ways to bring them from the lab to the clinic.