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Macromolecular (pro)drugs in antiviral research

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Macromolecular (pro)drugs are a sub-discipline of medicinal and polymer chemistries aiming to optimize the delivery of drugs to their site of action. In recent decades, this field of science has undergone a tremendous development, with the soundest achievements registered in the delivery of anticancer drugs. Surprisingly, the development of these tools for applications in antiviral treatment lags significantly behind – despite the fact that the first *in vivo* successes of polymers in fighting viruses were reported half a century ago. Furthermore, the unique scope and utility of polymers in antiviral research is that macromolecules themselves exhibit highly potent activity against diverse viruses. Herein, in an attempt to revive the research interest in this field, we aim to provide an overview of successes (and failures) of polymers as antiviral agents and macromolecular prodrugs. Specifically, we discuss inhibition of the entry of the virus into mammalian cells by polymers, give an overview of the synthetic schemes applied for the conjugation of drugs to carrier polymers, and also present guidance with regard to potential reporter systems which can be used for the characterization of novel drug delivery systems in virus-free cell cultures.

Introduction

Polymer therapeutics is an interdisciplinary field of study positioned at the interface between polymer science and medicinal chemistry. This discipline has matured since its advent into a well-established biomedical opportunity with numerous research reports appearing in press annually.[1–4] Polymer–protein conjugates,[5] polymer-conjugated drugs,[4] and block-copolymer micelles as drug carriers[6] are particularly successful, with multiple formulations having progressed to advanced clinical trials and the clinic. The power of polymers is associated with their tuneable, and typically high molecular weight which results in opportunities to change the pharmacokinetics of associated drugs, specifically to achieve prolonged circulation of the drug in the human body and/or enhanced accumulation of the drug within the desired tissues.

Polymer–protein conjugates is a highly successful field of biomedical research and industry, in which the increased molecular weight of the conjugates facilitates localization of the protein in the blood and extends the half-life in circulation to as much as 7–10 days. We note that this discipline has recently been reviewed in this journal,[5] and for the remainder of this contribution polymer–protein conjugates are omitted from consideration.

Polymer-conjugated drugs are essentially macromolecular prodrugs (MP), with the therapeutic benefit being dependent on each of the following: nature of the polymer carrier, average molar mass of the chains, drug loading, nature of the linkage between the drug and the polymer, and the presence of ligands to achieve active homing into the desired tissues.[1–4] Successes of polymer-associated drug delivery systems are particularly well-documented for anticancer research, in which the abnormal physiology of the cancerous tissue facilitates accumulation of the payload within the tumour through the enhanced permeation and retention (EPR) effect. This field of research is well reviewed and readers are referred to earlier contributions for detailed discussion on this subject. In contrast, the successes and failures of macromolecular (pro)drugs in the context of antiviral research has received far less attention. This topical review aims to fill this gap.

Viruses need cellular machinery and metabolism for their own replication. To infect the cell, the virus has to attach to specific receptors on the cellular surface of the host and penetrate through the cellular membrane. Subsequently, viral genomic nucleic acid is released from the viral capsid, replicated within the cell, and serves as a template for the synthesis of viral proteins. Newly synthesized viral components undergo self-assembly and mature virions can be released from the host cell.[7] When developing antiviral agents, there are various stages in which viral inhibition can be implemented; (i) shielding of viral particles outside the cell, inhibition of viral adsorption and viral entry, (ii) inhibition of viral replication, and (iii) inhibition of viral release or targeting newly produced virus particles both inside and outside the cell.

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The essential ingredients for a successful antiviral macromolecular (pro)drug (MP) are the same as those outlined above for the counterparts in anticancer treatment.[4] However, the significant difference lies in that polymers themselves – not only the conjugated drug – have been documented to be powerful tools against the progression of viral diseases.[8–10] Macromolecules are capable of preventing viral entry into mammalian cells, and the first section of this review is dedicated to the discussion of this phenomenon, its mechanism, and documented successes. The second part of the review discusses MPs and the intracellular delivery of antiviral drugs using polymers, specifically in the context of therapies to prevent progression of the human immunodeficiency virus (HIV). Next, we review our own results and those from other groups on the delivery of drugs against the hepatitis C virus (HCV). We also include a discussion related to the methods available to evaluate the success of newly synthesized MPs. This review aims to highlight the power of polymers and MPs as tools in antiviral research and stimulate further research in the field.

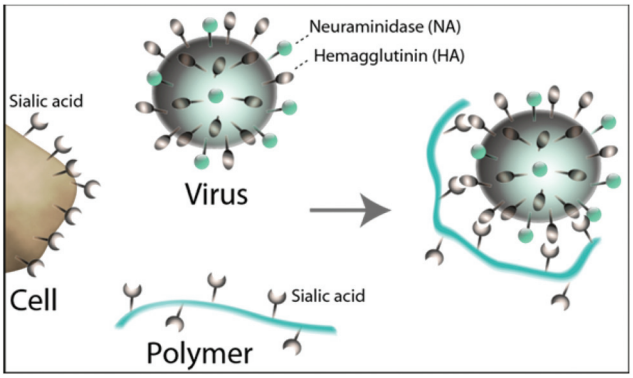

Fig. 1 Schematic illustration of the inhibition of influenza virus cell entry by means of polymer therapeutics. The virus particle presents two glycoproteins involved in the cell entry, neuraminidase (NA) and hemagglutinin (HA), either one having affinity for sialic acids on the cell surface as well as within mucosal membranes. Polymer therapeutics presenting sialic acids can inhibit viral entry through competitive binding to the virus, blocking its access to the host.

Inhibition of viral entry by polymers

Influenza

Influenza is an airborne respiratory virus which targets the epithelial cells of the respiratory tract.[11,12] The virus first has to penetrate through a layer of mucus, a hydrogel-like substance comprising polysaccharide chains rich in sialic acids (SA, variants of *N*-acetylneuraminic acid). Viral particles are equipped with copies of neuraminidase (NA), a protein with an affinity for sialic acid and an ability to degrade the polysaccharide chains. When the virus reaches the cell surface, another viral protein, hemagglutinin (HA), binds sialic acid-containing receptors on the cell surface (Fig. 1) leading to viral internalization.

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Anton Smith, Mille Kryger and Benjamin Wohl are working towards their PhD under the supervision of Dr Zelikin at Aarhus University.

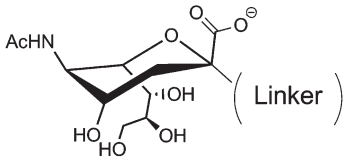
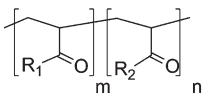
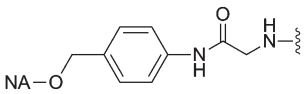
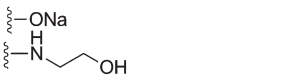
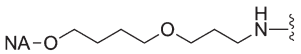
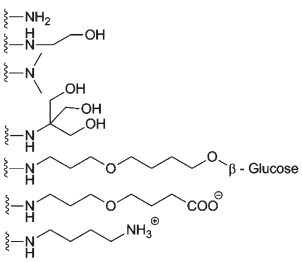
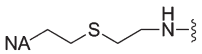
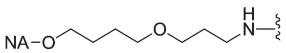
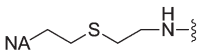
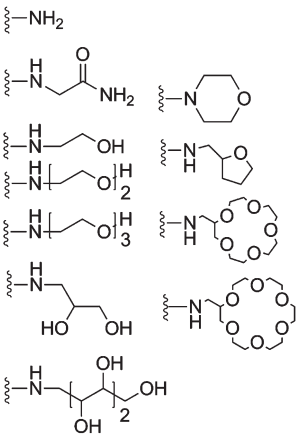
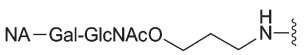
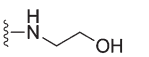
Dr Pau Ruiz-Sanchis earned his PhD in Organic Chemistry (2011) under the supervision of Prof. M. Álvarez and Prof. F. Albericio at the Institute for Research in Biomedicine Barcelona (Spain) and joined Dr Zelikin's laboratory as a postdoctoral fellow in 2012.

Kaja Zuwala is pursuing her academic career under the co-supervision of Dr Zelikin and Dr Tolstrup.

Dr Martin Tolstrup received an MSc degree in Molecular Biology and subsequently completed his PhD in basic retrovirology with Professor Finn Skou Pedersen at Aarhus University. He then moved to a Research Fellow position at the Department of Infectious Diseases, Aarhus University Hospital, during which he did a training period with Professor Sharon Lewin in Melbourne. In 2013, Martin was appointed as an Associated Professor at Aarhus University.

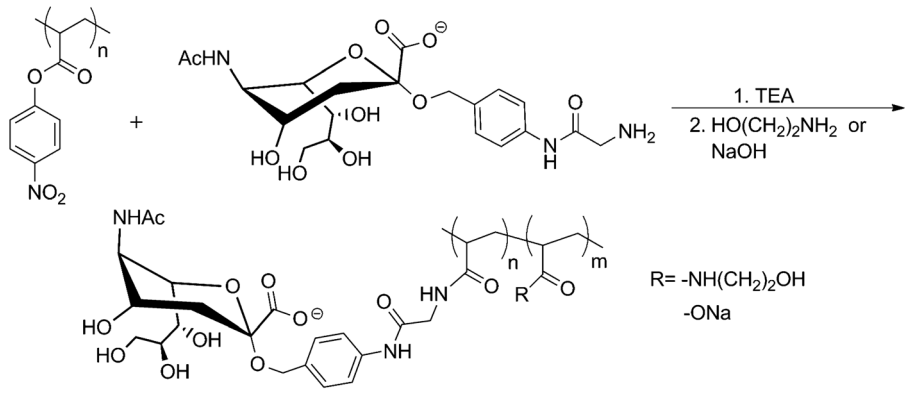
Dr Alexander N. Zelikin received a PhD in polymer chemistry from Moscow State University (Russia) in 2003 (advisor: Prof. V. A. Izumrudov). He held research positions at MIT with Prof. R. Langer, at Cornell University with Prof. D. Putnam, and with Prof. F. Caruso at the University of Melbourne (with an Australian Postdoctoral Fellowship). In 2009, Alex joined Aarhus University and now holds a position as an Associate Professor in Medicinal Chemistry. He is a recipient of the Sapere Aude Starting grant from the Danish Council for Independent Research, Technology and Production Sciences, Denmark and the European Research Council Consolidator grant. His research interests cover diverse aspects of biomedical engineering, specifically polymer chemistry and bioconjugation, delivery of antiviral drugs, hydrogel biomaterials, enzyme-prodrug therapy, and site-specific drug delivery.

Table 1 Sialic acid-containing polymers

N-Acetylneuraminic acid (NA)		
		
Linker to NA (R_1)	Carrier polymer pendent group R_2	Ref.
		10
		26
		27,31
		28
		25
		24

Together, the isotype of HA and NA on the viral envelope designates the viral name H#N# (*e.g.* H1N1, the virus responsible for one of the recent pandemics). Following intracellular replication of the viral nucleic acid and proteins, NA facilitates virion release from the host cell.[13] While all of the steps in the viral life cycle are subject to therapeutic intervention, arguably the most successful drugs are transition state analogues of sialic acid inhibiting NA, *e.g.* Tamiflu.[14]

One of the strategies developed by the viruses to achieve a high affinity for the target is that of multivalency: while a single interaction of *e.g.* NA and sialic acid is weak, a high number of NA copies on the viral particle and multiplicity of contacts with sialic acid within the mucus leads to an overall high affinity of the virus for the target. Similarly, polymer chains can be designed to contain multiple copies of a nominated ligand leading to very high overall affinities for the target. Taking advantage of this opportunity, polymers have been developed to be potent inhibitors of cell entry of the influenza virus.[15] Specifically, polymer therapeutics have been synthesized to mimic the interaction between the virus and sialic acid within mucus and/or receptors on the host cells. Inhibition occurs through competitive interactions between sialic acid-carrying polymers and the viral HA/NA, thus shielding the virus. Example of successful polymer designs are listed in Table 1 [10,16–31] and discussed in detail below.



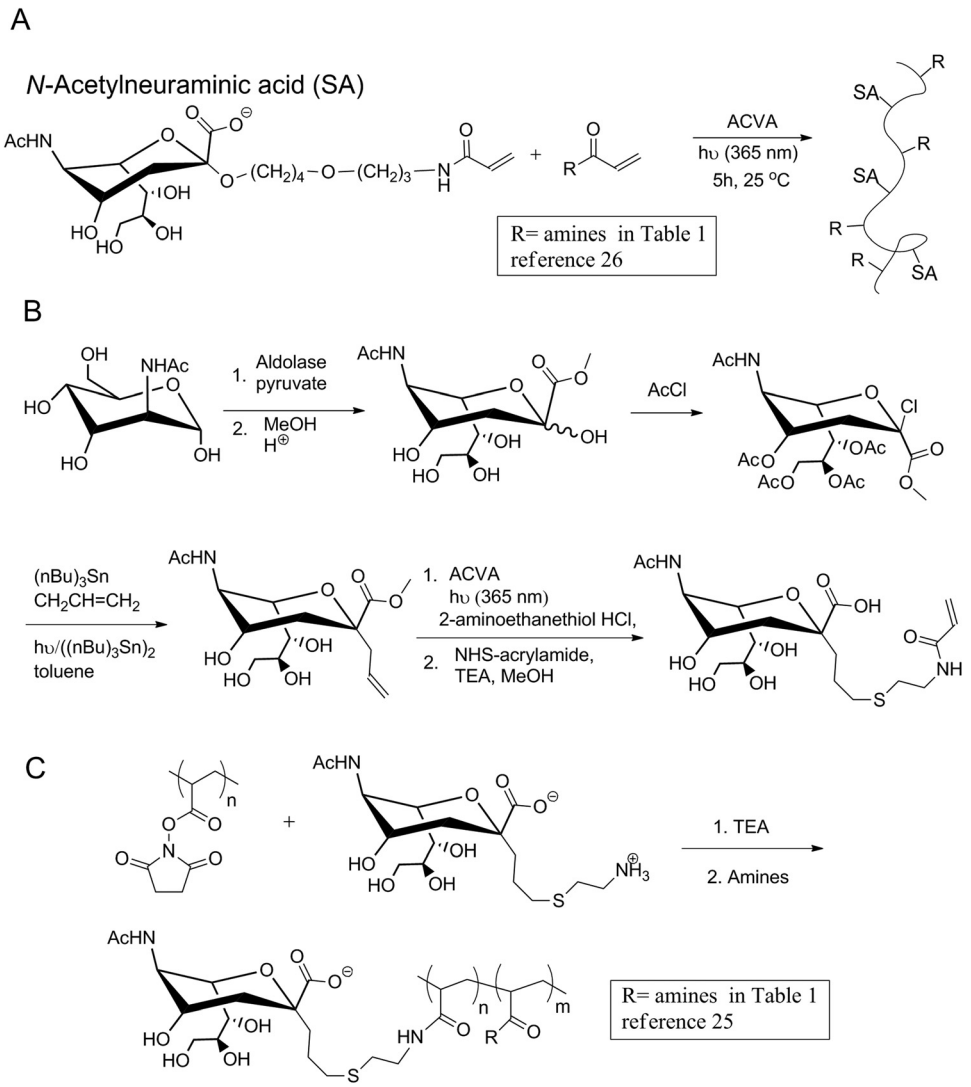
Scheme 1 Schematic illustration of the synthesis of polymers containing the sialic acid functionality through a polymer-analogous reaction. Poly-(4-nitrophenylacrylate) was successfully used as a reactive polymer-precursor towards the synthesis of amides *via* conjugation of the amines with polymer-bound activated ester groups. Adapted from ref. 10.

To the best of our knowledge, a series of publications which marks the advent of synthetic SA-containing polymers as tools in biomedicine dates back to late 1980s when Roy *et al.* developed "pseudo-polysaccharides" towards their use in immunology research.[29] The polymers were obtained *via* free radical polymerization and the use of acrylamide derivatives of SA. Shortly after, in 1990, Matrosovich *et al.* synthesized a series of polymers containing varying amounts of sialosides through a polymer-analogous reaction using poly(4-nitrophenylacrylate) and a glycine-4-amidobenzyl derivative of sialic acid (Scheme 1).[10] Water-soluble polymers were obtained through hydrolysis of the unreacted nitrophenyl esters into acrylic acid or 2-hydroxyethylamide using sodium hydroxide or ethanolamine, respectively. The average molar mass of the hydrolyzed poly(4-nitrophenylacrylate) was reported to be ~100 kDa. The antiviral activity of the polymers was 1000-fold higher than that of the monovalent sialosides, thus providing an early example of the utility of polymer therapeutics in antiviral research and also revealing a high potency of polymer-based drugs. With regard to the structure–function correlation, the 10 mol% SA-loaded polymer was the most effective as an antiviral agent with both 5% and 20–30% being less effective, indicating a "sweet spot" for sugar loading.[10]

Independently but simultaneously, the Whitesides group developed the synthesis of polymers with structures similar to that discussed above through the copolymerization of acrylamides containing SA (Scheme 2A) and various other functionalities (positive and negative charge, glucose, methyl- and hydroxymethyl-derivatives, *etc.* see Table 1).[26–28] The synthesized polymers were analyzed with regard to their ability to inhibit virus-induced hemagglutination, *i.e.* aggregation of erythrocytes, the latter having a membrane rich in SA. Lead formulations exhibited a 10^4–10^5-fold increase in activity as compared to the monovalent methyl α-sialoside.[26] The initial report from this group made use of an acrylamide with SA conjugated to the polymer through a glycosidic linker. The functionality of this polymer was readily negated upon treatment with neuraminidase, *i.e.* removal of SA from the polymer structure.[26] This potential shortcoming was overcome through the development of SA-containing monomers without glycosidic linkages, similar to the monomers initially used by Roy *et al.*[29] Such exhibited similar effectiveness in inhibiting virus-induced hemagglutination and were non-cleavable by NA (Scheme 2B).[27,30]

In the next iteration, the Whitesides lab used poly(NHS-acrylate) (Scheme 2C) and obtained SA-containing polymers through a polymer-analogous reaction using an amine-containing derivative of sialic acid and a panel of amines with diverse structures (size, charge, hydrophobicity, *etc.*).[25] The polymers had a weight average molar mass of ~146 kDa and a polydispersity of ~2. Increasing the SA content to ~20% increased the potency of the polymers with regard to inhibiting virus-induced hemagglutination. However, further increase in the SA content was not beneficial. Introducing charge to the polymer, be it positive or negative, resulted in a decrease of the potency of the polymer in the hemagglutination assay. Further to this, the introduction of hydrophilic side chains more bulky than acrylamide led to a significant drop in the potency of the polymers in their capacity to prevent hemagglutination caused by the virus. In contrast, hydrophobic side chains increased the potency of the polymers but also significantly limited their solubility.[25] Interestingly, the potency of these polymers in the hemagglutination assay was enhanced when the assay was performed in the presence of "classic" transition state inhibitors of NA, although this effect became significant at concentrations ~1000-fold higher than the inhibition constant of the transition state inhibitor taken individually.[31] Taken together, these studies present a comprehensive investigation of SA-containing polymers as inhibitors of interaction between the virus and human erythrocytes.

An elegant visual illustration of the polymer binding to the virus was obtained with the use of a biotin-containing antiviral polymer, streptavidin protein functionalized with gold nanoparticles, and transmission electron microscopy as an imaging technique.[32] The biotin-labeled polymer binds to both the virus and gold particles, through SA ligands and the biotin–streptavidin interaction, respectively. TEM visualization revealed gold nanoparticles clustering around the viral



Scheme 2 (A) Schematic illustration of the synthesis of polymers with sialic acid functionality through direct co-polymerization of comonomers, one of which is an acrylamide derivative of SA. (B) Synthetic pathway leading to an acrylamide derivative of SA lacking scissile glycosidic bonds. (C) Illustration of the synthesis of SA-containing polymers *via* polymer-analogous reaction using polymeric NHS-activated esters and aliphatic amines – derivatives of SA. Adapted from ref. 25–28 and 30.

particles (Fig. 2) thus providing a picture to substantiate the above discussed research findings.

Thus, sialic acid-containing polymers are highly effective as inhibitors of cell entry of the flu virus. Polymers can either be made through copolymerization of functionalized monomers or post-polymerization modifications. Polymeric sialosides protected mice from an otherwise lethal infection by a murine adapted human influenza virus, showing the applicability in an *in vivo* setting[24] and further revealing the power of polymer therapeutics as tools against the influenza virus. We highlight that in the majority of studies, polymers were not properly characterized per average molar mass or dispersity (typical reported range of polymer molar masses being between 100–450 kDa[10,32] and as high as 1500 kDa[24]). A report from the Whitesides lab indicates that increasing the concentration of the initiator and the use of a chain transfer reagent, both favoring the synthesis of polymer chains with lower average molar masses, resulted in polymers with lower potency in inhibiting hemagglutination.[28] Thus, one would expect that the average polymer molar mass strongly affects its capacity to inhibit viral cell entry, but, to the best of our knowledge, no report to date addresses the structure–function correlation in this context. For macromolecular carriers of anticancer drugs, polymer chain length defines the blood residence time, effectiveness of tumor accumulation through the EPR effect, and is of tremendous importance for the possibility of renal elimination.[1,2] Admittedly, polymers acting against the flu virus are not expected to enter the bloodstream, and none of the above listed factors are important for anti-influenza polymers. However, proper characterization of the polymers is highly important from the standpoint of reproducibility of results and to avoid batch-to-batch variation of the antiviral activity of

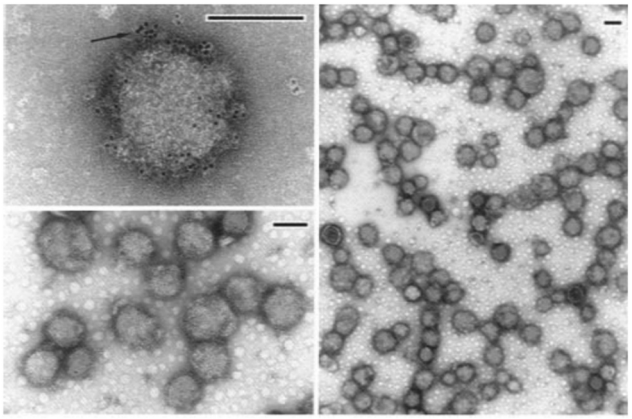


Fig. 2 Transmission electron microscopy (TEM) images illustrating association of the SA-containing, biotin-functionalized polymer chains with the viral particles. Sialic acid moieties effectively anchor the polymer on the surface of the virus. Biotin is responsible for association with streptavidin on the surface of the gold nanoparticles. The latter are clearly visible in TEM, and together the presented images visualize adsorption of the polymer chains onto the virus, a phenomenon responsible for the prevention of viral cell entry. Scale bars: 100 nm. Reprinted with permission from ref. 32. Copyright 1996 American Chemical Society.

polymer samples. The studies described in this section preceded the development of controlled radical polymerization techniques (*e.g.* atom transfer radical polymerization, ATRP,[33] reversible addition–fragmentation chain transfer, RAFT[34,35]). Plausible developments in the field therefore include the synthesis of polymers with controlled composition *and* chain length leading to a detailed structure–function analysis of polymer therapeutics acting against the influenza virus.

HIV and other viruses

In this section, we discuss the progress in designing polymer therapeutics with inherent activity against HIV and other viruses manifesting itself through non-specific inhibition of viral cell entry. In the late 1960s, De Somer *et al.* observed that negatively charged polymers inhibit adsorption and replication of the vesicular stomatitis virus (VSV), Sindbis and the vaccinia virus (VV).[9,36] Then and throughout subsequent decades, it was shown that this effect is observed for a vast range of structurally dissimilar negatively charged polymers, revealing that the mechanism behind inhibition of viral infectivity is non-specific and related to the polymer charge (Table 2). The proposed mechanism of activity of the polymers was that of electrostatic adsorption of the polymers onto the viral particle. In the original reports from De Somer, the most pronounced effect was achieved using poly(methacrylic acid) (PMAA).[9,36] Virus adsorption was measured by the disappearance of viral particles from the cell medium, and it was shown that 80–90% of the particles were recovered in the presence of PMAA.[9,36] Polyacetal carboxylic acid (chlorite-oxidized oxypolysaccharide, **18**) was shown to have antiviral activity against VV in mice.[37] Various glycosaminoglycans such as heparin, heparan sulfate, chondroitin sulphate, dermatan sulphate and keratan sulphate[38] exhibited efficient binding to the viral envelope resulting in shielding of the viral particles.[39,40] Hepatitis C and B viruses (HCV and HBV, respectively) can be isolated from patient sera through electrostatic interactions between positively charged amino acids in the E2 envelope protein and negatively charged polymers such as heparin.[41] Basu *et al.*[39] showed that sulfated heparin (**20**) inhibits HCV cell entry through binding to the E2 envelope protein, and a study by Barth *et al.*[42] revealed that O-sulfated heparin exhibits an enhanced effectiveness as an antiviral polymer. Chondroitin sulphate (**21**) was shown to inhibit viral entry, through binding to the E envelope antigen of dengue virus.[43]

In a similar fashion, naturally occurring and synthetic negatively charged polymers were shown to inhibit the adsorption of HIV onto the membrane of mammalian cells (Table 3).[44–46] Historically, the anti-HIV activity of polyanions was hypothesized to occur through the inhibition of the viral reverse transcriptase, but was subsequently shown to be extracellular and to do with the inhibition of the viral cell entry.[8] The mechanism of HIV cell entry has been well studied since its isolation in 1983.[13,47–50] The first step in this process involves interaction of the viral glycoproteins (gp120) with CD4 receptors on the immune cells, CD4+ T cells. The conformational change of the viral protein, association of components of the cellular membrane, and recruitment of co-receptors result in virus cell entry.[51] As with the polymers designed against influenza, competitive interaction of the polymers with the viral particles can

Table 2 Polymers with inherent antiviral effect

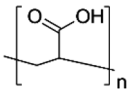
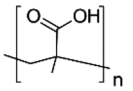
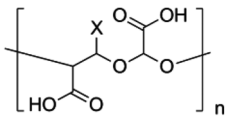
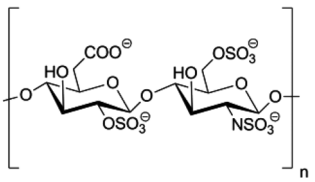
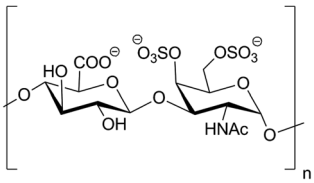
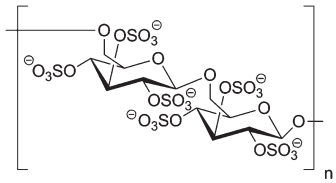
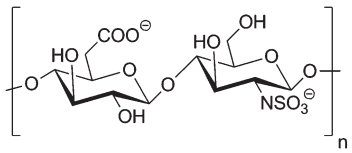
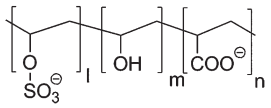
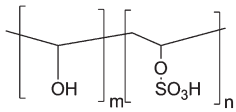
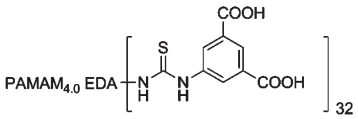
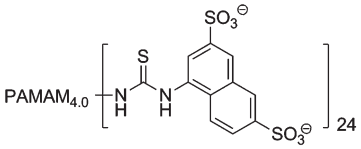
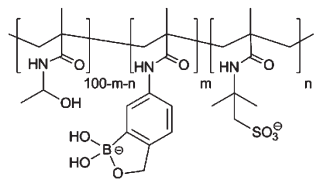
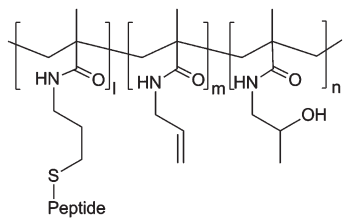
Polymer		Virus	Ref.
	16	VV	9,36
	17	VSV, Sindbis and VV	9,36
 X = Chlorite-oxidized oxyamyloses	18	VV	37
	19	HCV	39
	20	Dengue virus	43

Table 3 Negatively charged polymers with anti-HIV effect

Polymer		Ref.
	8	46
	9	53
	10	54
	11	54
	12	55
	13	55
	14	59
	15	60

prevent association of the latter with the T cells. Anionic polymers bind the positively charged amino acids in the V3 loop of HIV gp120, thereby preventing viral binding to the CD4 receptor and in doing so, inhibit the viral cell entry (Fig. 3).[52,53]

Inhibition of viral adsorption by synthetic polymers was first shown for HIV using the examples of poly(acrylic acid-*co*-vinyl alcohol sulphate) (PAVAS, **10**) and poly(vinyl alcohol sulphate) (PVAS, **11**).[54] The two polymers were prepared through post-sulfation of poly(vinyl alcohol) and poly(vinyl alcohol)-*co*-poly(acrylic acid) respectively, and were shown to inhibit HIV

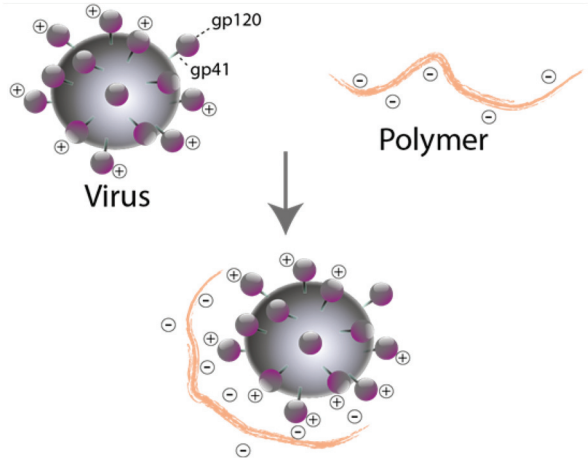
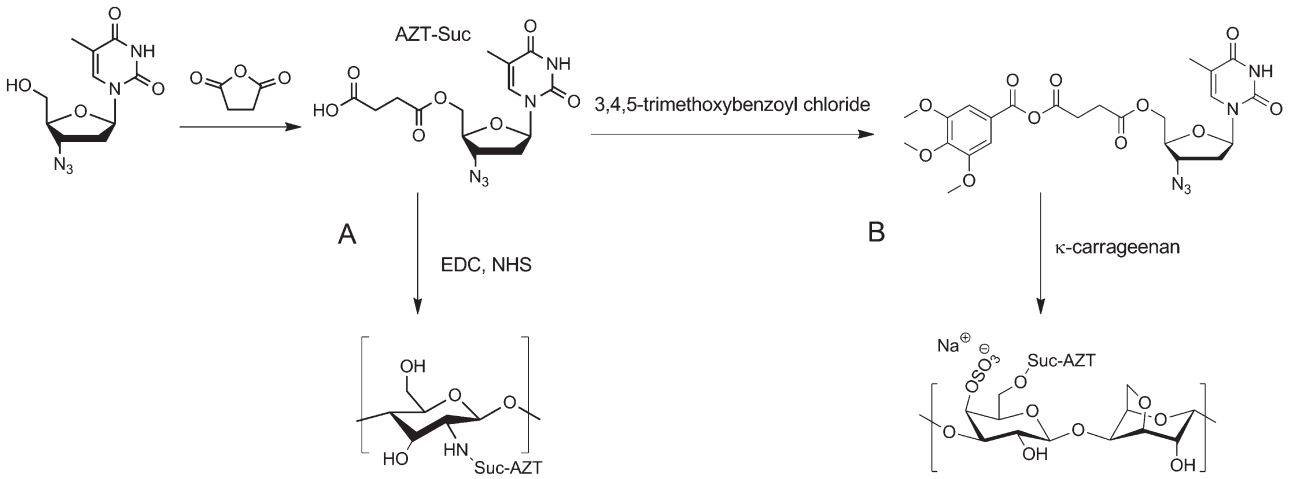


Fig. 3 Schematic illustration of the mechanism by which negatively charged polymers prevent infectivity of HIV. The viral particles of HIV contain a glycoprotein gp120 which possesses positively charged amino acids. This glycoprotein is important in binding to the CD4 receptor on the host cell, thereby facilitating entry. Polymer therapeutics containing negative charges bind to positively charged residues on the gp120 envelope protein and block its normal function in HIV entry.

entry using a T cell line. Additionally, and in contrast to azidothymidine (AZT), these polymers showed suppression of HIV-induced giant cell formation (*i.e.* cell fusion) in a T cell leukemia model cell line. In comparison to dextran sulfate, a 25-fold better inhibition of giant cell formation was observed and this was attributed to the higher average molar mass of the synthetic polymers (M_n 10 000 and 20 000) compared to dextran sulfate (M_n 5000). With regard to cellular adhesion, the study described above determined that sulfated polymers were increasingly effective with increasing molecular weight and degree of sulfation. These findings are in good agreement with the hypothesized electrostatic interactions driving the inhibition: increased sulfation and increased chain length both enhance the polyanionic character of the polymers. For an in-depth overview of the development of polyanionic inhibitors of HIV, we refer the readers to an excellent recent review on the subject.[8]

Similarly to the linear polymer discussed above, carboxylated (**12**) or sulfonated (**13**) dendrimers have also shown anti-HIV activity, with the mechanism of action identical to that for the linear polymers.[55] A particularly promising dendrimer was equipped with both, naphthyl groups giving it a hydrophobic character and sulfonic acid groups giving it high surface charge.[56] This dendrimer inhibited viral reverse transcriptase at concentrations similar to those at which it inhibited viral entry (0.1 μM to 0.2 μM, dependent on HIV strain), revealing a dual mode of activity.[56] When testing against a simian variant of HIV in macaques, it was observed that administration of the dendrimer prevented viral infection in all of the 8 tested monkeys.[57]

The interest in macromolecular entry inhibitors and the high success rate demonstrated by these antiviral agents *in vitro* culminated in several clinical trials, the course of



Scheme 3 Synthetic pathway to AZT conjugates with chitosan and κ-carrageenan. In both cases, conjugation was achieved through a succinic linker, using carbodiimide-mediated coupling for chitosan and 3,4,5-trimethoxybenzoyl chloride-based activation of the carboxyl for κ-carrageenan. Adapted from ref. 62 (chitosan scheme) and 64 (carrageenan scheme).

which are reported in detail elsewhere.[8] In brief, all the agents failed the trials, both as systemic and topical agents. The only clinical study on dextran sulphate administered intravenously revealed an increase (not a decrease) in the HIV-p24 antigen and also demonstrated significant side effects such as thrombocytopenia and hair loss.[58] These studies were quickly terminated. Subsequent clinical studies were centred on formulating the polyanions as topical gels, but these too failed, providing no statistically significant protection from HIV acquisition as compared to the control groups. The origin of this dramatic discrepancy between laboratory and clinical findings remains to be fully explained. Recent findings present successful prophylaxis of HIV infection using both oral tablets (Truvada) as well as topical gels containing tenofovir, a nucleoside analogue. These results further detract the focus of research attention from the polyanionic non-specific inhibitors of HIV.

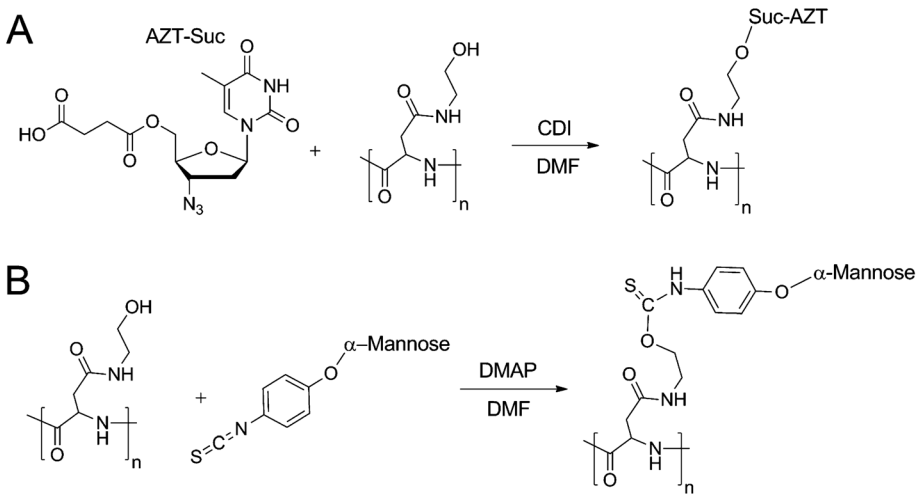
Macromolecular prodrugs of nucleoside analogues

The synthesis of macromolecular prodrugs of nucleoside analogues, the latter being the most common antiviral drugs, has been accomplished using both natural and synthetic polymers. As with most drugs, the main rationale behind these undertakings was to improve the pharmacokinetic properties of the therapeutic agent. Control of all the aspects of pharmacokinetics has been attempted, being absorption, distribution, metabolism, and excretion. Nucleoside analogues provide only scant opportunities for conjugation, usually through a 5′-hydroxyl group. Therefore the various examples of conjugation of these drugs to the carrier polymers were in fact variations on similar reaction schemes. Before the advent of controlled radical polymerization techniques, conjugation of antiviral drugs to naturally occurring bio-macromolecules was the dominant strategy, with the bio-macromolecule being either a protein or a polysaccharide. The benefits of using endogenous proteins as vehicles for drug delivery are a lower risk of immune response and the biodegradable nature of the carrier. When using natural polysaccharides, the issue of batch-to-batch variation of the polysaccharide of choice is prominent, as is the risk of immunogenic reactions.

(Pseudo)natural polymer carriers

Examples of conjugation of nucleoside analogues to polysaccharides consist mostly of AZT prodrugs, typically towards improving the pharmacokinetic profile of orally administered AZT. The linker most typically employed has been that of succinic ester formed through carbodiimide (*e.g. N*,*N*′-dicyclohexylcarbodiimide, EDC) mediated coupling reactions.[61,62] In the example shown in Scheme 3A, chitosan was functionalized with AZT succinic ester.[62] This macromolecular prodrug exhibited a controlled release of AZT in mouse plasma, with 40% released after 6 h, thus extending the blood residence time of the drug. An analogous conjugation approach with dextrin[61] significantly extended the half-life of AZT following intravenous (i.v.) administration in rats from 1.3 h to 23.6 h. Lamivudine has been conjugated to dextran in a similar approach.[63] The latter prodrug showed accumulation in the liver and kidney tissue of rats after i.v. administration without any targeting moieties. Accelerated release of lamivudine was observed in the presence of rat liver lysosomes compared to the corresponding buffer.

A study that stands out from the other efforts in the synthesis of macromolecular prodrugs of AZT is that by Vlieghe *et al.*, in which the aim was to have the polymer carrier work in concert with the drug.[64] κ-Carrageenan was chosen as the carrier so that this negatively charged polymer inhibits viral entry through a mechanism discussed in the previous section of this review. Carrageenan was chosen over heparin due to its low anti-coagulating effects. In cell culture with T cells,



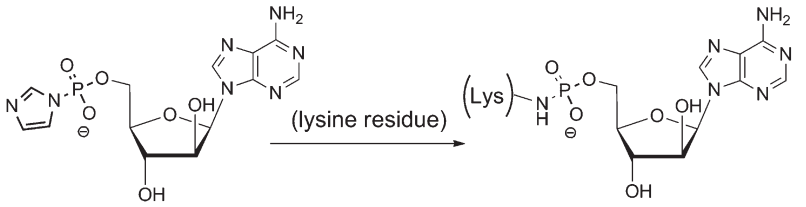
Scheme 4 Schematic illustration of PHEA conjugation with AZT succinate (A) and isothiocyanate derivative of mannose (B). In the latter case, mannose functionalization was performed with a view to the hepatic targeting of antiviral therapeutics. Adapted from ref. 65 (AZT conjugation) and 66 (mannose functionalization).

a concerted effect was observed for the conjugates with a high loading of AZT (for synthesis, see Scheme 3B), where the concentration required to inhibit the cytopathicity of HIV by 50% in T cells was lowered from 25 nM to 6.8 nM (expressed *via* the concentration of AZT) when bound to carrageenan. However, this effect was observed only when the cells were pre-treated with the polymer, *i.e.* in a setting addressing prevention of viral infectivity. The polymer prodrug was found to be less effective than free AZT if the cells were infected before the treatment. In an *in vitro* release assay, human serum was found to release AZT the fastest compared to buffered solutions at various pH values (in serum, 39% release of the drug over 48 h) suggesting an enzymatic cleavage of the succinic linker.

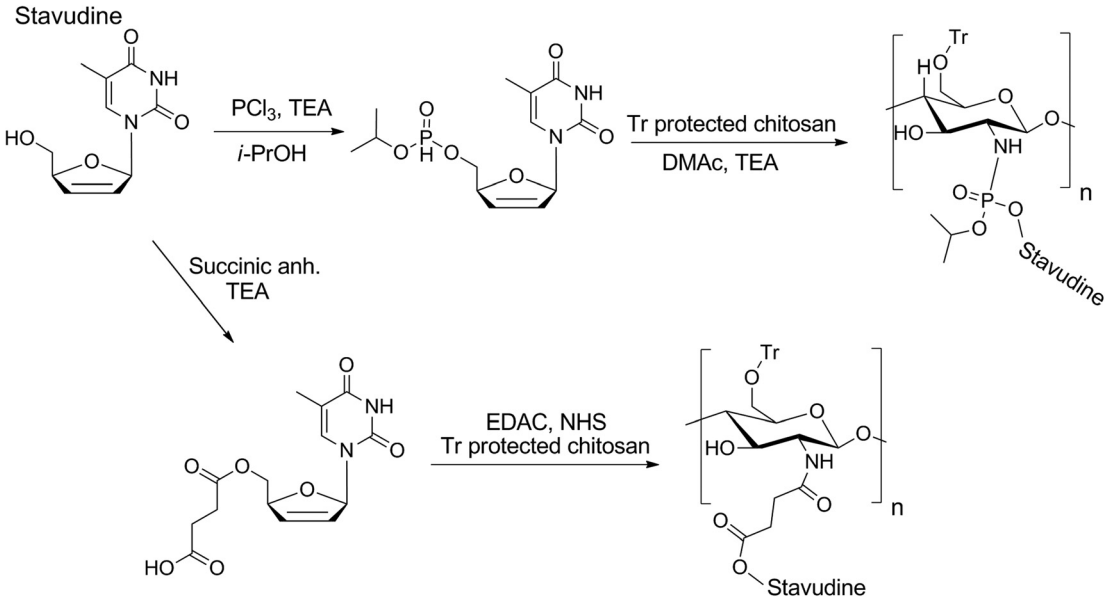
AZT-succinate has also been conjugated to poly(2-hydroxyethyl aspartamide) PHEA through a carbonyl diimidazole coupling, Scheme 4A.[65] The resulting polymer maintained good solubility in water and afforded a slow release of AZT, with 20% of the drug being released within 24 hours. Drug release was enhanced to 45% in the presence of chymotrypsin. This synthetic polypeptide was also used as a carrier for the hepatic delivery of acyclovir and ganciclovir.[66] Hepatic targeting was engineered through conjugation of the polymer to mannose and galactose using an isothiocyanate linker, Scheme 4B. Liver targeting was indeed observed in rats for the mannose functionalized polymer, *i.e.* exploiting the affinity of mannose for the Kupffer cells. In contrast, galactose functionalization afforded no increase in the liver uptake of the prodrug, despite the well documented affinity of galactose for the asialoglycoprotein receptor (AGPR) of hepatocytes. Unfortunately, none of these polymers were tested for antiviral activity.

The examples presented above made use of the biodegradable ester linkage to achieve release of the nucleoside analogue from its conjugate to a nominated polymer carrier. However, nucleoside analogues themselves are therapeutically inactive, and as such are prodrugs to their phosphorylated derivatives, the latter being the molecules exerting therapeutic benefit. With this knowledge, highly successful prodrugs of nucleoside analogues were designed to include a nature-inspired phosphoamide linkage.[67] The latter is biodegradable and, in contrast to the ester based prodrugs, its scission liberates a phosphorylated nucleoside analogue, *i.e.* a therapeutically active molecule. In the context of macromolecular prodrugs, this strategy has been accomplished to synthesize phosphoester-containing prodrugs of vidarabine (active against HSV, varicella zoster viruses, and HBV) based on poly-lysine[68] and lactosaminated serum albumin.[69,70] The reaction scheme includes activation of the phosphoester of vidarabine with imidazole and subsequent reaction with a lysine (Scheme 5). A formulation based on lactosaminated serum albumin afforded a marked decrease in HBV DNA *in vivo* and also aided in alleviating the side effects of vidarabine, such as neuro-muscular pain syndrome.[71] Using the same linker, Molema *et al.* made human serum albumin prodrugs of AZT.[72]

Among the studies of macromolecular prodrugs of nucleoside analogues, one report stands out in that the linkers between the drug and the polymer are compared side-by-side towards the optimization of drug release.[73] In this study, stavudine was conjugated to chitosan *via* two alternative strategies, namely a DCC-mediated coupling using a succinate diester spacer or using the phosphoramide linker (Scheme 6). The conjugates were compared to the parent drug with regard to their toxicity and antiviral activity. This experiment revealed that the phosphoamide-linked prodrug was superior to stavudine with regard to the therapeutic window. Specifically, the phosphoamide-based prodrug exhibited a greater potency and a concurrent reduction in cytotoxicity. In contrast, the succinyl ester prodrug was less active than stavudine, plausibly due to the incomplete drug release achieved over the course of the



Scheme 5 Synthetic pathway to macromolecular prodrugs of nucleoside analogues through the reaction of imidazole-activated phosphoesters of the drugs and lysine residues on proteins or polypeptides, resulting in bioconjugates in which antiviral agents are linked to the carrier macromolecule through a phosphoamide bond.



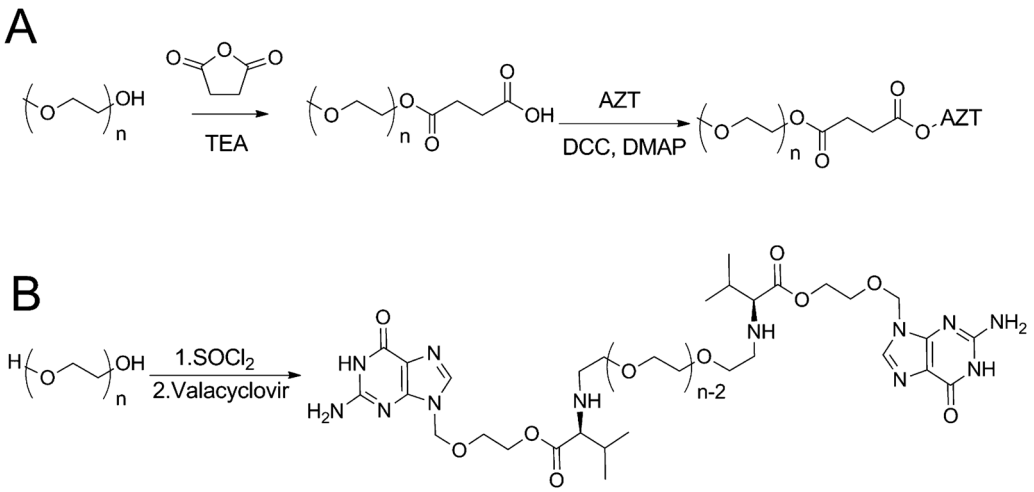
Scheme 6 Illustration of synthetic schemes employed to obtain stavudine conjugates with chitosan through either phosphoramide or succinic ester-amide linkages performed for a comparative investigation of these macromolecular prodrugs with regard to toxicity and antiviral activity. Adapted from ref. 73.

experiment. The superior activity of the phosphoamide-based polymer conjugates over the succinate counterpart is readily explained since the former releases an active form of the therapeutic, whereas the latter releases a nucleoside prodrug which needs to undergo intracellular phosphorylation. We strongly believe that this study is one-of-a-kind and demonstrates the power of synthetic chemistry to optimize the properties of macromolecular prodrugs using the existing knowledge of molecular and cell biology.

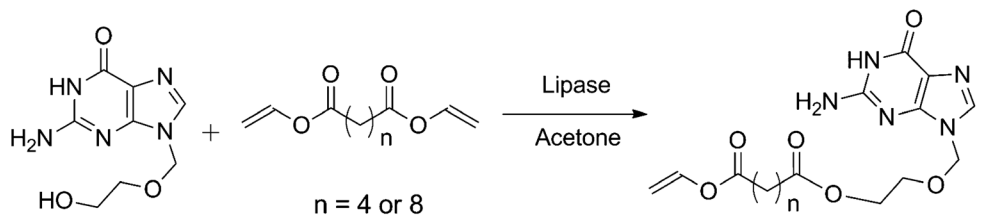
Fully synthetic macromolecular prodrugs of nucleoside analogues

Among the fully synthetic polymers, poly(ethylene glycol) stands out as a "golden standard" candidate carrier for drugs and – most successfully – proteins. For the latter, there are 9 FDA approved formulations on the market[5] with many more being tested at various stages of (pre)clinical trials. While the inert structure of this polymer is beneficial for its "stealth" properties and affords a lack of recognition by the human immune and reticulo-endothelial system, it also poses significant limitations with regards to chemical modification and bioconjugation strategies. Specifically, for linear PEG, synthetic opportunities are limited to the terminal groups of the polymer. De Clercq *et al.* used the terminal hydroxyl groups of PEG and the carbodiimide-mediated succinate ester coupling strategy to obtain PEG–AZT conjugates as shown in Scheme 7A.[74] This prodrug showed a lower activity compared to free AZT *in vitro*. This observation is rather typical for macromolecular prodrugs and reflects a delayed and plausibly incomplete release of the drug from its conjugate. The prodrug showed a faster release of the drug in gastric fluid compared to intestinal fluid. When administered orally in mice, the PEG–AZT prodrug showed a slower absorption and also a longer elimination half-life.[74] A similar conjugation approach was applied to obtain macromolecular prodrugs based on PEG and acyclovir and also valacyclovir, in the latter case activating the polymer terminal hydroxyl functionality using thionyl chloride (Scheme 7B).[75]

In a more ambitious study, AZT was conjugated to a peptide-agonist of CXCR4, a chemokine co-receptor used by



Scheme 7 Conjugation of AZT to PEG was accomplished using hydroxyl functionalities at the polymer terminus *via* a successive succinic anhydride activation and carbodiimide coupling strategy (top) as well as a thionyl chloride activation route (bottom). Adapted from ref. 74 and 75.



Scheme 8 Chemi-enzymatic synthesis of the vinyl esters of acyclovir was accomplished using a solid support immobilized lipase.

HIV to enter CD4+ T cells. This was performed in an attempt to achieve a synergistic effect between the two antiviral agents.[76] Conjugation of AZT was accomplished in the solid phase following the synthesis of the peptides. When tested in T cells, CXCR4 antagonist and AZT administered together indeed exhibited a synergy in a cytopathic assay. Regretfully, conjugation of the two conferred no added benefits. The authors speculated that this approach might be better *in vivo*, as the peptide would also confer targeting. While this study is not explicitly a polymer prodrug in the same sense as the other AZT conjugates, we believe that these results may provide inspiration for the development of macromolecular prodrugs for targeted delivery of the payload to the T-cells.

An alternative approach to the synthesis of macromolecular prodrugs consists of copolymerizing monomers, at least one of which has functionality as an antiviral agent, yet examples of this methodology put to practice are surprisingly few. Vinyl esters of acyclovir were obtained through a chemi-enzymatic functionalization of the 5′ hydroxyls of the nucleoside analogue, Scheme 8.[77] These were then copolymerized by free radical polymerization with their respective counterparts with a galactose functionality to facilitate association with the hepatic cells. The success of this was verified through visualization of the fluorescein-tagged polymer inside the cells by confocal microscopy.

In our recent work,[78] we synthesized a methacrylate derivative of AZT *via* a chemi-enzymatic approach (Fig. 4). In contrast to their vinyl ester counterparts, methacrylates have a wide range of co-monomers amenable to co-polymerization and the rational design of macromolecular prodrugs.[34,35] These include *N*-2-hydroxypropyl methacrylamide (HPMA), a monomer behind some of the most successful MPs in anticancer research.[2,79] It also includes methacrylic acid (MAA) which gives rise to PMAA – one of the very first polymers with inherent antiviral activity.[9,36] To obtain MPs, we employed a controlled radical polymerization technique, RAFT,[34,35] and obtained a range of polymers with independently varied average molar mass and drug loading. The obtained MPs were highly effective in preventing infectivity by the live HIV virus, as tested in both a model cell line (TZM-bl) and primary human CD4+ T cells. Using a model cell line, we showed that, following a single administration, MPs act for longer than AZT itself, the latter treatment becoming ineffective when the drug is removed from the cell culture medium. In contrast, internalized polymers provided a sustained anti-HIV effect, presumably through a slow release of the conjugated drug inside the cell over an extended period of time. For PMAA-based prodrugs, our data present a promising formulation for combination therapy in which the carrier prevents viral cell entry and the released nucleoside analogue fights replication of the

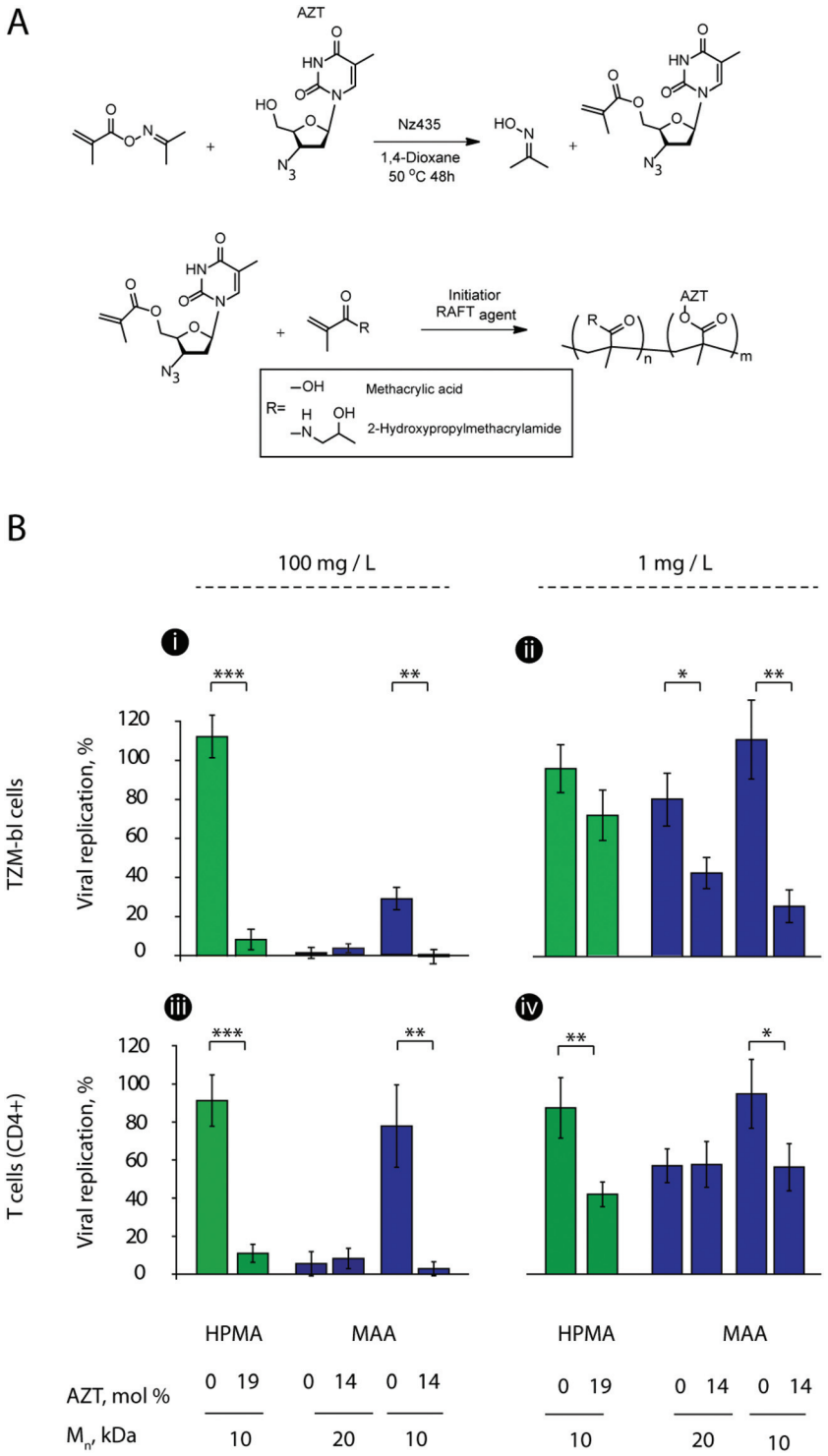
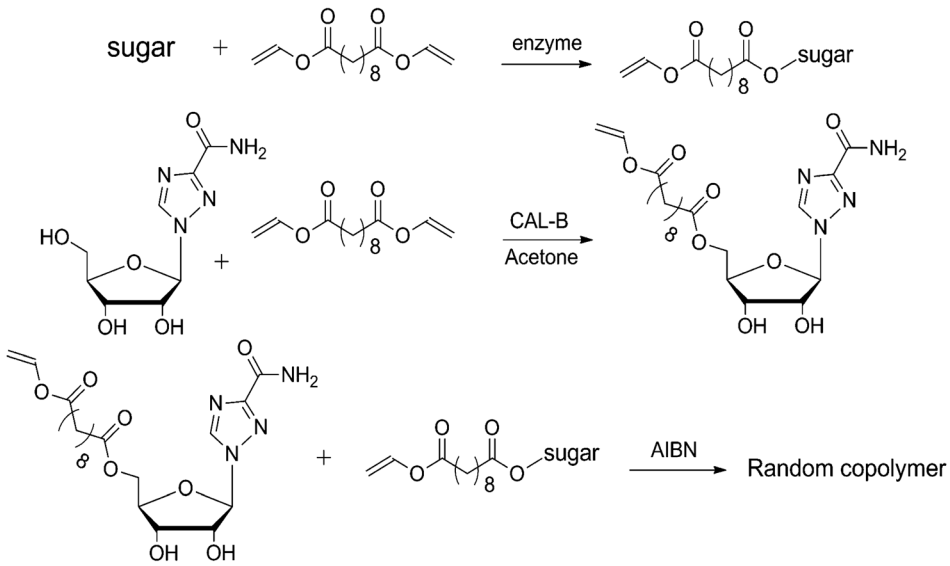


Fig. 4 (A) Schematic illustration of the synthesis of macromolecular prodrugs of AZT using a methacrylate derivative of the drug and the RAFT polymerization technique. (B) Prevention of viral infectivity of HIV using HPMA and MAA based MPs of AZT with different average molar mass and drug loading in model TZM-bl cells (i, ii) and primary human CD4+ T cells (iii, iv). Adapted with permission from ref. 78.

HIV intracellularly. Interestingly, in the T cells, MP showed no superior activity over the pristine PMAA and with matched molar mass, polymers with and without conjugated AZT revealed the same level of prevention of viral infectivity. In contrast, in TZM-bl cells, MPs were significantly more powerful in preventing viral infectivity than the pristine PMAA, and both the carrier and the drug were instrumental in fighting infectivity of the HIV virus. We are now further investigating the



Scheme 9 Enzymatic functionalization of RBV and appropriate carbohydrates (sugars) afforded monomers with functionality of the antiviral drug and of hepatic targeting. Copolymerization of these monomers *via* free radical polymerization produced polymers which self-assembled in aqueous solution into micellar aggregates as nanosized carriers of RBV. Adapted from ref. 87–89.

benefits of using a combination of polyanionic inhibitors of viral cell entry conjugated to antiretroviral drugs.

Macromolecular prodrugs of ribavirin

Ribavirin (RBV) is one of the few broad-spectrum antiviral agents. This drug is the first choice of treatment against HCV and is also prescribed as a part of the treatment of diverse viral diseases, including the treatment of infants.[80] While most antiviral agents are relatively safe and non-toxic, ribavirin has a well-documented dose-limiting side effect, namely its accumulation in red blood cells.[80,81] This phenomenon is reflected by an astounding value of the volume of distribution of RBV, 2000 L. A long term effect of this accumulation is hemolytic anemia. It has long been understood that the transport of RBV into red blood cells is assisted by specific nucleoside transporters, and thus can be suppressed if the drug is incorporated into a macromolecular prodrug. Indeed, there are several highly successful reports of having accomplished this, with favorable data from both *in vitro* and *in vivo* studies.[82,83]

Blood proteins, being water soluble and biodegradable macromolecules with a long circulation time, have been successfully used as scaffolds for macromolecular prodrugs of RBV. Conjugation was accomplished using imidazole phosphoamide to activate the nucleoside analogue followed by reaction with an amine, *e.g.* a lysine residue on hemoglobin (Hb), analogous to Scheme 5.[84] The functionalization of Hb with RBV and subsequent complexation of Hb with haptoglobin was performed to achieve targeted drug delivery to the liver Kupffer cells through the haptoglobin–hemoglobin degradation pathway.[84] *In vitro*, this targeting approach was tested in Chinese hamster ovary cells which express the appropriate receptor for the Hp–Hb complex. The functionalization of Hb with the imidazole activated RBV phosphate was gentle and effective, yielding a loading of 4–12 attached RBV phosphates per Hb tetramer.

A similar Hb–RBV prodrug was tested in mice infected with the murine hepatitis virus.[85] The macromolecular prodrug was shown to lower the viral titer and markedly increase the survival rate of the mice. Additionally, the prodrug-treated mice exhibited normal fur texture and behavior. Moreover, the prodrug was shown to produce a marked anti-inflammatory effect in macrophages, as evidenced by a reduction in secretion of pro-inflammatory cytokines, interferon-γ and tumor necrosis factor-α.[85] Further functionalization of the protein with lactose through reductive amination on lysine was performed to achieve hepatic targeting of the conjugate through the asialoglycoprotein receptor.[85] Similar design criteria were used by Di Stefano *et al.* who designed conjugates of RBV with lactosaminated poly-L-lysine.[86] When tested on mice with viral hepatitis, this prodrug exhibited a modest accumulation in liver tissue and afforded a decrease of the virus titer, while mostly avoiding association with RBCs.[86]

Towards the preparation of macromolecular prodrugs of RBV based on synthetic polymers, Li *et al.* performed a chemienzymatic synthesis of a vinyl-adipoyl ester of ribavirin.[87–89] This monomer was copolymerized by free radical polymerization with monomers bearing either a lactose or a galactose functionality, Scheme 9. In their aqueous solutions, the obtained copolymers self-assembled into micellar structures, and galactose-containing polymers caused aggregation of fluorescently labeled peanut agglutinin, as is expected for polymers with this functionality. The release of RBV *in vitro* was found to be accelerated at pH 7.4 compared to 1.2, with 50% of the drug released after approximately 150 h of incubation. Successful intracellular drug release was postulated based on

the observed cytotoxic effect of the polymers in hepatic cells, an effect which was reversed by the pre-treatment of cells with an excess of galactose, *i.e.* abolition of the receptor-mediated uptake. We note that, from the standpoint of polymer chemistry, this work leaves much room for improvement in that the polymer samples were found to have molar mass dispersities of >2.[87]

Controlled macromolecular prodrugs of RBV

Recently, we initiated a broad study to rationally design potent macromolecular prodrugs and achieve a safe delivery of RBV. We aimed to take full advantage of the tools for controlled radical polymerization, specifically the reversible addition–fragmentation chain transfer (RAFT) polymerization.[34,35] One of the main empowering attributes of RAFT is that a wide range of monomers are amenable for polymerization through this technique, including most of the monomers which are precursors to polymers with favourable characteristics in the context of polymer therapeutics. To accommodate the synthesis of macromolecular prodrugs of RBV through this route, we synthesized acrylate and methacrylate derivatives of RBV through a chemi-enzymatic synthesis.[90] In contrast to the previously described vinyl esters of RBV, (meth)acrylates have a wide range of potential co-monomers for the synthesis of MPs. Indeed, in our hands, RBV (meth)acrylate readily underwent copolymerization with monomers such as *N*-vinyl pyrrolidone,[91] acrylic and methacrylic acid,[92,93] and hydroxypropyl methacrylamide and afforded well-defined polymers with good control over the molar mass and drug content.

The rationale behind our efforts to formulate RBV into the form of macromolecular prodrugs was that, with appropriate choice of the structure, polymers can be designed to avoid association with red blood cells (RBC). We hypothesized that, when conjugated to these polymers, RBV will not be subject to internalization by RBC and thus will escape the origin of its main side effect. To test this, we used fluorescently labelled polymers and monitored their association with RBC using flow cytometry.[90,92] In accordance with the design, RBV-containing polymers revealed minor levels of association with RBC, and at the same concentration exhibited a pronounced degree of interaction with hepatocytes and macrophages (Fig. 5).

Having established the compatibility of RBV MPs with red blood cells, we next aimed to investigate if the polymer conjugates deliver their payload in a functional form to hepatic cells. However, a literature survey revealed that, to date, there is no readily available *in vitro* screen to ascertain the activity of the released RBV. Furthermore, the mechanism of action of RBV against HCV and the role of this drug in a broader context of fighting the virus and associated liver pathologies is still a subject of debate.[94] To establish a virus-free screening platform, we built on the hypothesis of the anti-inflammatory activity of RBV, specifically its capability to inhibit the synthesis of nitric oxide in macrophages.[95] The biochemical mechanism of this finds its origins in that phosphorylated RBV is an inhibitor of inosine-5′-monophosphate dehydrogenase (IMPDH). This activity results in a depletion of the intracellular pool of guanosine triphosphate followed by a decrease in the intracellular concentration of tetrahydrobiopterin. The latter is one of the cofactors which are required for the activity of inducible nitric oxide synthase, and an overall activity of RBV is therefore a suppression of the synthesis of nitric oxide (NO). Surprisingly, while the RBV–IMPDH connection has been well established and documented, the RBV–NO correlation has been experimentally verified in a single report and using an endothelial cell line,[96] *i.e.* with relevance to angiogenesis, and not using hepatic cells of relevance to hepatitis. In our work, we showed that indeed, RBV reveals a dose dependent inhibition of synthesis of NO by stimulated macrophages, with EC_{50} values (7 μM)[91] in close agreement with the concentration of this drug in the plasma of patients.[97] Our experiments also revealed that, with regard to this effect, RBV has a very narrow therapeutic window, with a toxicity-related IC_{50} of 19 μM (Fig. 6, top). Synthesized macromolecular prodrugs tremendously decreased toxicity of RBV and significantly broadened the therapeutic window of RBV (Fig. 6, bottom). Together with the favourable blood compatibility discussed above, these data strongly suggest that MPs represent a safer mode of delivery of this broad spectrum antiviral agent.

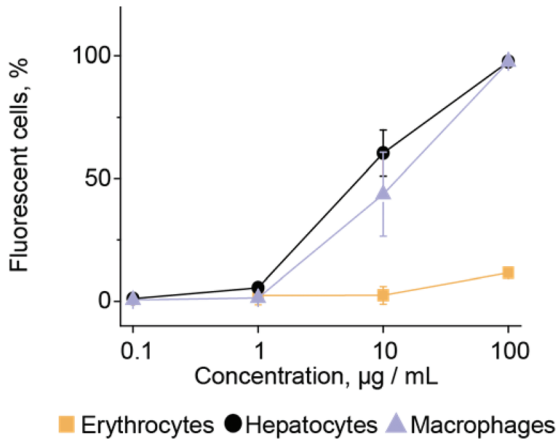


Fig. 5 Percentage of fluorescent cells of erythrocytes, hepatocytes and macrophages upon incubation with PAA-RBV for 24 h at polymer concentrations from 0.1 to 100 μg mL^{-1} quantified *via* flow cytometry. Results shown are the average of triplicate experiments, reported as mean ± SD (n = 3). Reprinted from ref. 90. Reproduced by permission of The Royal Society of Chemistry.

In a follow-up study, we aimed to probe the broad structure–function parameter space associated with macromolecular prodrugs and pinpoint the polymer composition (molar mass and drug content) constituting a potent carrier for the delivery of RBV. To accomplish the set goal, a robotic polymer synthesis platform was employed to accomplish a parallel synthesis of MPs based on poly(acrylic) and poly(methacrylic) acids.[93] A total of 48 polymers were synthesized and screened for their *in vitro* activities, and this study identified at least 10 formulations with a therapeutic response matching that of the free drug at no expense to cytotoxicity. The dataset further revealed a clear correlation between polymer molar mass and therapeutic activity, wherein polymer samples of lower

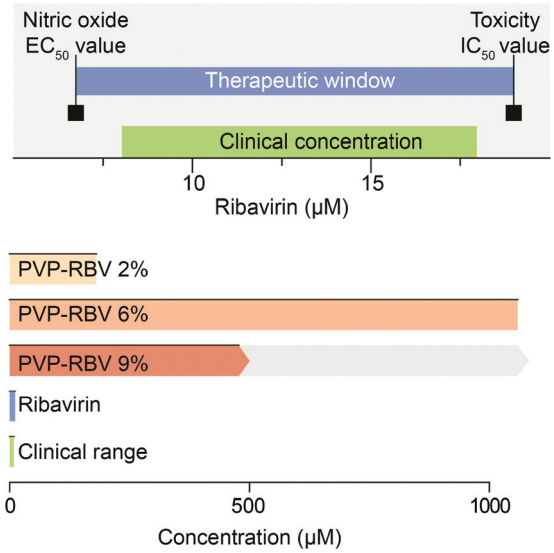


Fig. 6 The narrow therapeutic window of RBV with regards to inhibiting the production of nitric oxide in stimulated macrophages is effectively broadened with the use of macromolecular prodrugs of this therapeutic. Top: Graphic representation of the therapeutic window of RBV and clinically relevant concentrations of this drug. Bottom: Graphic representation of the therapeutic windows of macromolecular prodrugs of RBV and the free drug, as determined in ref. 91.

molecular weight elicited significantly stronger responses, plausibly due to an enhanced cellular uptake of chains with decreased molar mass. Interestingly, pristine PAA and PMAA also showed a statistically significant reduction in the production of NO. To the best of our knowledge, this constitutes the first report of polyanions acting as inhibitors of inducible nitric oxide synthase, and thus broadens their known therapeutic activity in the context of antiviral therapy beyond inhibition of viral cell entry.

Conclusions and perspectives

The presented overview of literature on the subject of antiviral polymer therapeutics makes it clear that these tools of biomedicine are highly promising in several aspects. It is well established that MPs allow for a slow sustained drug release due to a gradual degradation of the polymer–drug linkage, and markedly change the biodistribution of the drug.[61–63,65,66,75,77,84,98–101] The therapeutic efficacy of the drug *in vitro* is typically maintained upon conjugation to a polymer, although higher equivalents of the drug may be required to achieve a similar effect.[64,73,74,85,102] This shortcoming may be favourably compensated by an increased therapeutic index compared to the free drug, largely due to a decreased drug-associated toxicity.[90,91,99] Furthermore, *in vivo* studies suggest that a *lower* drug equivalent is able to produce an equipotent response, possibly due to an accumulation of the MP at the target tissue, an effect which also serves to decrease systemic side effects.[85,86]

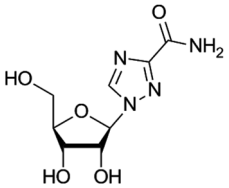
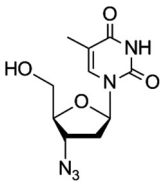
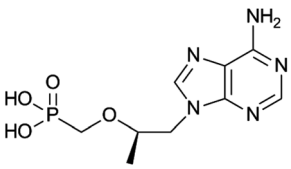
However, our survey also makes it evident that the development of antiviral MPs lags significantly behind their anticancer counterparts. This observation may come as a surprise given that the *in vivo* success of antiviral MPs was documented as early as the 1960s,[9,36] preceding the rise of activity in the development of anticancer MPs. The development of novel tools in chemistry such as self-immolative linkers,[3] drug targeting,[103] advent of controlled polymerization techniques, has failed to be as important for the accelerated optimization of antiviral MPs as it was and is for the anticancer MPs. What seems to make a large difference is that the majority of breakthroughs in the context of drug delivery originate in (bio-)chemical laboratories, and for anticancer research, this was made possible through routine validation of the therapeutic activity using a large number of commercially available viability assays. In contrast, chemical laboratories are not suited for antiviral research. Robust cell culture systems exist for a number of viruses, including HIV[64,73,74,102] yet it is the limited access to facilities to run these experiments which seems to keep the research activity in this field so low.

Perhaps, this should not be the case.

We believe that a plausible workflow in the development of antiviral MPs could include an activity screen preceding true antiviral characterization, and this screen can be conducted in a typical (bio)chemical laboratory. (Sub)genomic replicon systems[104,105] can be handled in routine cell culture facilities and lend themselves as a platform to verify the success of newly synthesized antiviral MPs. Taking a further step towards routine cell culture, characterization of MPs can be based on (sub)cellular effects elicited by the released antiviral drugs. A good case in point, for characterization of MPs for ribavirin, we developed a cell culture system structured around the intracellular activity of this drug as an inhibitor of inducible nitric oxide.[91] Being a convenient virus-free system, it also holds relevance to anti-HCV research. Finally, a literature survey reveals that a number of antiviral drugs (RBV, AZT, tenofovir, adefovir) possess immune-modulatory activity and regulate the production of various cytokines (*e.g.* IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, CCL3, CCL5).[85,106–118] Similarly to the involvement of NO in the pathogenicity of HCV, it has been proposed that the immune-modulatory activity of these nucleoside analogues constitutes part of their antiviral activity. More importantly, these cytokines can be readily quantified through established protocols (*e.g.* ELISA) and can serve as a quantification platform of drug activity. Table 4 summarizes the available literature reports on this subject and aims to facilitate research activities in the field through introducing these systems as reporter read-outs in the context of antiviral MPs.

The above discussion reveals that polymers are highly attractive as both antiviral drugs, where activity is inherited with the structure of the macromolecule, and as carriers for antiviral therapeutics. It appears fair to say that inhibition of the viral cell entry by polymers is rather well understood. Techniques in bioconjugation and polymer functionalization also allow the engineered delivery of antiviral drugs with optimized pharmacokinetics. We see that future developments in the field may relate to the identification of appropriate drug candidates and novel drug targets, and going well beyond the

Table 4 Immunomodulatory activity of antiviral nucleoside analogs. Modulatory activity of the drug is indicated by ↑ (upregulation) or ↓ (downregulation). Reported systems are amenable to direct quantification of intracellular drug activity through established biochemical assays in the absence of live viruses

Nucleoside analog	Cell line/type	Immunogenic stimulus	Observed effect	Ref.
Ribavirin (RBV) 	Human T cell	• Enterotoxin B • Phorbol ester and ionomycin	IL-2↑, IFN-γ↑, TNF-α↑ IL-4↓, IL-5↓, IL-10↓	106
	Murine macrophage cell line (RAW264.7)	• LPS	NO↓	91
	hPBMC	• PHA • recall antigen	IL-2↑ (only PHA) IFN-γ↓, TNF-α↓, IL-10↓	110
Azidothymidine (AZT) 	Human monocytic cell line (U937)	• TLR agonists (Pan3CSK4, LPS) • TNF-α	IL-8↑, CCL3↑	112
Tenofovir (TFV) 	U937	• TLR agonists (Pan3CSK4, LPS) • TNF-α	IL-8↓, CCL3↓	112
	hPBMC	• TLR agonists (Pan3CSK4, LPS, CL097) • TNF-α	IL-12↑ (only TLR) IL-10↓	112
	hPBMC	No stimulation	CCL3↑, CCL5↑	114
	Mouse peritoneal macrophage	No stimulation	IL-1↑, IL-10↑, TNF-α↑, CCL3↑, CCL5↑	116

hPBMC: humanPBMC: peripheral blood mononuclear cell, LPS: lipopolysaccharide, TLR: toll like receptor, PHA: phytohaemagglutinin, TNF: tumor necrosis factor, IL: interleukin, IFN: interferon, NO: nitric oxide, CCL: chemokine (C–C motif) ligand.

range of currently commercialized antivirals. After all, classic antiviral drugs are moderately toxic and typically have optimized bioavailability. Also, opportunities in combination therapy, whereby activity of the polymer and that of the drug are tuned to synergy, appear hardly explored but highly promising. Last but not least, the use of polymers as carriers in antiviral vaccine strategies has attracted little if any research attention, and may prove highly important in the overall quest to define safer and more efficacious treatments of viral diseases.

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