Polymers Fight HIV: Potent (Pro)Drugs Identified Through Parallel Automated Synthesis

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Efficacious, non-toxic, potent macromolecular (pro)drugs (MPs) acting against the human immunodeficiency virus (HIV) were identified in this work using high-throughput experimentation techniques. We synthesized a new monomer with the functionality of a well-established anti-HIV drug, azidothymidine (AZT) and obtained polymer libraries with systematic variations in the structure of the polymer backbone, polymer molar mass, and content of AZT. Anti-HIV activity was screened and visualized in a model cell line and human primary $CD4^+$ T cells. MPs were superior to the parent drug in the longevity of antiviral effect following a single administered dose. MP comprised a unique combination therapy whereby the polymer and the conjugated drug act in concert and retain activity in the primary human T cells over at least 72 h.

Macromolecular (pro)drugs are a powerful tool of biomedicine with successful applications in extending circulation of protein therapeutics,[1] delivery of anti-cancer drugs,[2] anti-viral treatments,[3] etc. For anti-HIV treatment, polymers have been investigated as inhibitors of viral cell entry and negatively charged polymers hold particular promise.[4,5] Surprisingly, while conjugation of active therapeutics to polymers has entered the mainstream of anti-cancer research,[2] antiviral therapies based on polymeric prodrugs is a field that remains in its infancy.[6] A further surprise lies in that for anti-HIV therapies, macromolecular (pro)drugs are typically based on natural polymers[7] and to date, the highly powerful arsenal of tools of synthetic polymer chemistry, that brought several anti-cancer formulations to advanced clinical trials,[2] for anti-HIV therapies remains mostly untouched.

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The origin of polymers as antiviral agents goes back to the 1960s.[8,9] Developments in this field well preceded the advent of polymer therapeutics (that is, conjugation of drugs to polymers) and controlled radical polymerization techniques, specifically reversible addition–fragmentation chain transfer (RAFT) polymerization.[10,11] The latter offers several fundamental advantages over the use of free radical polymerization or natural polymers, specifically the possibility to obtain polymers with controlled molar mass and narrow molar mass dispersity,[10,11] which is pivotal to ensure reproducibility and predictability of results and is of paramount importance when advancing to clinical trials. Also highly important is the opportunity to synthesize MPs through copolymerization of monomers taken at a defined ratio.[12] In our recent work, we used this approach to obtain MPs of a broad-spectrum antiviral agent, ribavirin, with a systematic variation in the drug content, up to 50 wt%.[13] In this work, for the first time, we use this strategy to design the MP of AZT, an antiretroviral agent with an extensive history of use and which remains an integral part of the anti-HIV treatment in many areas of the Developing World. Towards this goal, we synthesized a methacrylate derivative of AZT using a chemi-enzymatic approach, **Figure 1**A. The resulting monomer was then used in copolymerization with two monomers, methacrylic acid (MAA) and 2-hydroxypropyl methacrylamide (HPMA). HPMA is the most well characterized and widely used monomer in the context of polymer therapeutics with MP derived thereof having reached phase III clinical trials.[2] In turn, poly(methacrylic acid) (PMAA) has been shown to inhibit viral cell entry and thus presents itself as a polymer carrier with an inherent antiviral activity[8] thus offering opportunities for combination therapy. To probe the structure–function parameter space associated with MP, we employed RAFT polymerization to prepare polymer libraries using an automated parallel synthesizer.[14] Automated, high-throughput techniques (HTT) have become highly successful in biomaterials science.[15] In contrast, to our knowledge, this approach has not been used for the synthesis, analysis, and optimization of polymer therapeutics (with exception to our recent report on the use of HTT for optimization of MP of ribavirin).[13]

In RAFT controlled polymerization, molar mass of the polymer chains is defined by the ratio of concentrations of the monomer and the RAFT agent, [M]/[RAFT].[11] For HPMA and MAA, a total of 20 polymers were synthesized using [M]/[RAFT] ratio of 250, 125, and 50, and AZT methacrylate varied from 0 up to 15 mol% (up to 40 wt% for PMAA, 34 wt% for HPMA). Within series based on the same monomer (HPMA or MAA), monomer mixtures were robotically prepared using the same stock solutions and polymerizations were carried out concurrently, at identical conditions (freeze–pump–thaw cycles,

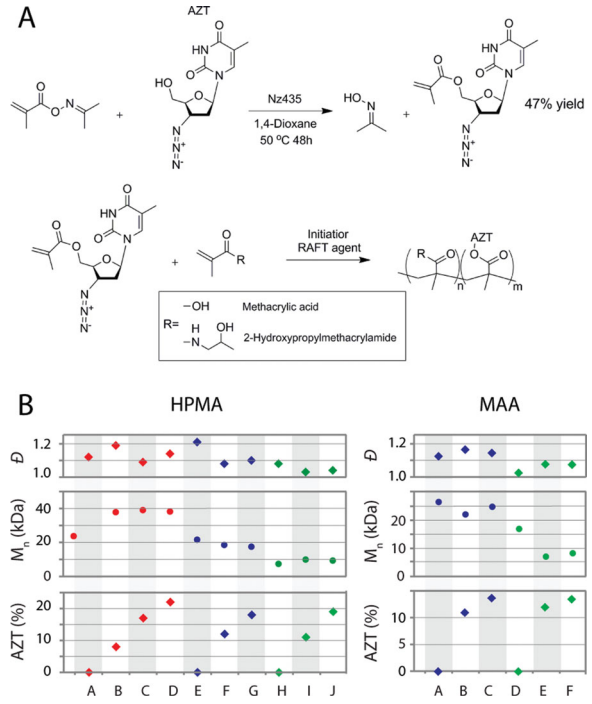

Figure 1. Schematic illustration of A) synthesis and B) macromolecular characteristics of the synthesized macromolecular prodrugs (MPs) of AZT. For experimental details, see Supporting information. $\bar{M}_n$—number average molar mass, *Đ*—dispersity, AZT (%) — mole % content of AZT methacrylate in the MPs.

temperature, and duration of polymerization, for details, see Supporting Information). These procedures are poised to minimize the batch-to-batch variation, ensure uniformity of polymers and adherence to the nominated design criteria (molar mass of the polymer and AZT content). Isolated polymers were then characterized using ^{1}H NMR to estimate the drug loading and using size-exclusion chromatography set-up with multi-angle light scattering detector for absolute molar mass characterization. Characteristics of the polymers are graphically presented in Figure 1B.

For HPMA-based polymers, all the synthesized polymers showed dispersity (*Đ*) at or below 1.2 demonstrating a good degree of control over the polymerization. Molar masses of the polymers were close to the designed values and clustered in series around number-average molar mass ($\bar{M}_n$) of 10, 20, and 40 kDa. Within series, AZT content on the polymer chains was gradually increasing from 0 for pristine polymer to 20 mol%. Drug content in the polymer was found to be very similar to the monomer feed ratio and this finding makes it easy to fine-tune the composition of the MP. For MAA-based polymer series, *Đ* values were also below 1.2 yet with regards to $\bar{M}_n$, synthesized series were relatively less controlled as compared to their HPMA counterparts. Lower [M]/[RAFT] series afforded polymers within the expected range of $\bar{M}_n$ yet a looser fit around the average $\bar{M}_n$ within this series. Highest [M]/[RAFT] series failed to produce polymer samples with anticipated $\bar{M}_n$ (not shown). Nevertheless, the data in Figure 1 illustrate the suitability of automated parallel synthesis of MP to obtain libraries of polymers with diverse, rationally programmed $\bar{M}_n$ and drug loading.

Initial screen of the synthesized series of HPMA- and MAA-based MP of AZT for their utility in delivery of antiretroviral drugs was conducted using a HeLa-derived cell line, TZM-bl. These cells stably express CD4 receptor and CCR5/CXCR4 co-receptors, which are indispensable for HIV to infect the cell. The TZM-bl cell line is also engineered with a luciferase/β-galactosidase reporter system under control of an HIV long terminal repeat promoter.[16] Hence, the reporter genes are only expressed upon viral infection. To ensure significant polymer internalization and allow polymers to release their payload inside the cells, TZM-bl cells were incubated with the polymers at concentration 0.1 g L^{-1} for 24 h, following which cells were infected with HIV-1 Bal strain. After 48 h, levels of viral infection were quantified via the luciferase reporter system (**Figure 2**).

Regardless of molar mass, pristine HPMA polymers showed no antiviral activity. In contrast, AZT-containing HPMA polymers at this concentration proved to be highly efficient in delivering their payload. Upon treatment with these polymers, viral replication did not exceed 10% illustrating that these MP are potent inhibitors of HIV infection ($p < 0.0001$, Figure 2A-i). Lowering the concentration of the copolymers by 100-fold to 1 mg L^{-1} resulted in a significant decrease in the antiviral activity of the polymers but aided in revealing the structure–function correlation and polymers with increased AZT content exhibited a higher degree of inhibition of viral infection (Figure 2A-ii). Interestingly, no correlation was observed with regard to the polymer molar mass and antiviral activity. In the range 10–40 kDa, decrease in the polymer chain length afforded a minor if any change in the effectiveness of the HPMA-based prodrugs to deliver their payload to the TZM-bl cells.

For MAA-based polymers, structure–function correlation differed significantly from the above-discussed HPMA-based counterparts. Pristine polymers bearing no nucleoside analogue antiviral drug were highly efficient in preventing viral infection. Indeed, at 0.1 g L^{-1}, 20 kDa PMAA homopolymer afforded an inhibition of the viral replication to a non-detectable level and 10 kDa polymer decreased viral replication by ca. 70%. These results are not unexpected. Negatively charged polymers bind the positively charged amino acids clustered in the V3 loop of the gp120 and in doing so prevent interaction of the virus with the host cell, and this behavior has previously been observed for a range of polyanions, both synthetic and natural.[4,5,17,18] Decreasing the polymer concentration 100-fold lowered the inherent antiviral effects of PMAA alone and revealed a pronounced structure–function correlation with AZT-containing polymers being significantly more effective in inhibiting HIV replication than the pristine polymer samples (Figure 2A-iv). Increasing the drug content on the polymer chain was followed by a pronounced increase in the antiviral activity of the polymers. At this low concentration (1 mg L^{-1}), PMAA sample with molar mass 10 kDa and drug content of 14 mol% afforded an 80% inhibition of viral replication. These data reveal that PMAA–AZT conjugates comprise a combination anti-retroviral therapy in which the carrier polymer and the conjugated drug counter HIV infectivity through distinctly different mechanisms of action, prevention of viral interaction with the host cell (PMAA), and blocking of the viral reverse transcriptase (AZT). Importance of this lies in that multiple polyanions has

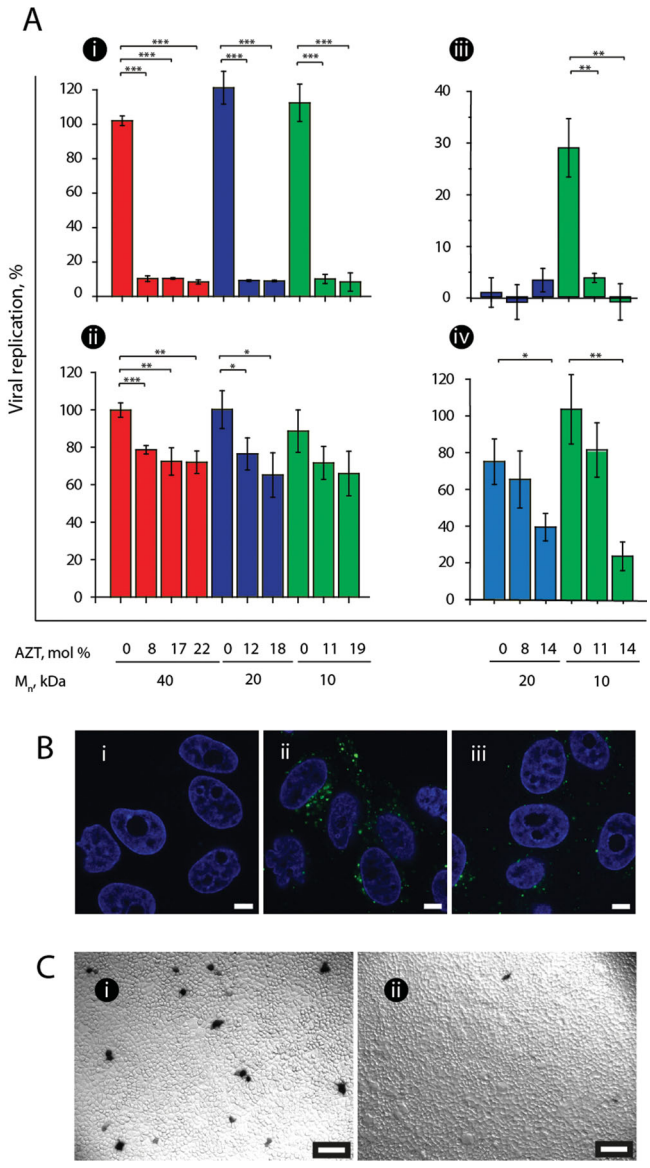

Figure 2. A) Viral replication inhibited by i,ii) PHPMA and iii,iv) PMAA-based macromolecular prodrugs of AZT administered at concentration i,iii) 0.1 g L^{-1} or ii,iv) 0.001 g L^{-1}. Viral replication was quantified in TZM-bl cells with 24 h preincubation of the polymers with the cells and allowing further 48 h for viral proliferation. Results are average of three independent experiments and are presented as average ± standard deviation. B) Confocal laser scanning microscopy images of TZM-bl cells preincubated with FITC-conjugated polymers: i) control, ii) 10 kDa PHPMA with 19 mol% AZT, iii) 10 kDa PHPMA without AZT. Scale bars B i-iii): 5 μm. C) β-Gal based staining of HIV infected TZM-bl cells (i) and the cells which were incubated with PMAA-based prodrug (ii, 20 kDa, 14% AZT) Infected cells are stained black or dark grey. Scale bars C i,ii): 100 μm.

been tested in clinical trials as topical microbicides to prevent infectivity of HIV, and despite the overwhelming success in vitro, all clinical trials to date have failed.[5,17] In contrast, topical formulations containing anti-retroviral drugs (e.g., tefonovir) have shown initial success in the clinical trials.[17] Drug-equipped polyanions may therefore be highly advantaged over their parent, drug-free macromolecules and build on both the success of polyanions and that of the drug depots to comprise a more effective topical microbicide formulation.

To provide visual support for the above data, uptake of fluorescently labeled polymers in TZM-bl cells was analyzed using confocal laser scanning microscopy. For PMAA, our attempts to visualize internalization of MPs were met with failure, possibly due to the quenching of fluorescein fluorescence by the adjacent acidic groups of the polymer (data not shown). HPMA-based samples with and without conjugated AZT revealed a pronounced level of polymer internalization, as evidenced by a pronounced fluorescein signal (Figure 2B). Control samples revealed no signal in this channel, indicating that the registered fluorescence is indeed due to the internalized polymer.

To visualize antiviral activity of the polymers, we made use of the β-galactosidase (β-Gal) component of the TZM-bl reporter system. Upon viral infection, the cells start to express this enzyme, and its localization is conveniently identified using a substrate X-gal. In presence of β-Gal, X-Gal is converted to insoluble, dark blue product. In the absence of MP, administration of the virus causes a pronounced protein expression, as is evidenced by multiple plaques within each field of view, indicating viral infection, Figure 2C-i. In contrast, viral challenge of the cells which were preincubated with the PMAA-based MP (20 kDa, 14% AZT) results in few if any plaques indicating minor levels of viral infection (Figure 2C-ii). Together, images in Figure 2 support the above data on well-pronounced interaction of the polymers with TZM-bl cells and activity of the polymers in preventing viral infectivity.

To put the macromolecular (pro)drugs to a more thorough test, the next set of experiments was performed using HIV Bal strain and TZM-bl cells preincubated with MP but removing the polymers from the incubation media prior to administering the virus. In these experiments, degradation and elimination of the MP from the cells prior to administration of the virus would render the treatment ineffective. This was indeed the case for the pristine drug, AZT: removal of the polymerase inhibitor from the incubation media prior to administering the virus abolished effectiveness of the drug and viral infectivity was not statistically different from that in the absence of the drug (**Figure 3**). The virus was administered immediately following the removal of the drug or 48 h after removing the drug, and in both cases, the level of viral infection was ascertained 48 h after the viral challenge. Lack of therapeutic activity of the drug indicates that over this time, internalized AZT is removed from the cell, thus depleting its intracellular concentration to a level below therapeutic. In contrast, preincubation with MP afforded a statistically significant decrease in the viral infectivity by as much as ≈50% (Figure 3, for brevity, only samples with statistically significant effect and respective control samples are shown). For the HPMA-based MP, this effect was minor and only significant for the AZT-containing polymer sample with an average molar mass of 10 kDa. For PMAA-based samples, unexpectedly, sustained antiviral activity was equally preserved for the drug-containing polymers as well as the parent, pristine PMAA. For 10 kDa PMAA with and without the conjugated drug, inhibition of HIV infectivity was statistically significant even when the virus was administered following an additional 48 h incubation of the cells in the absence of polymers.

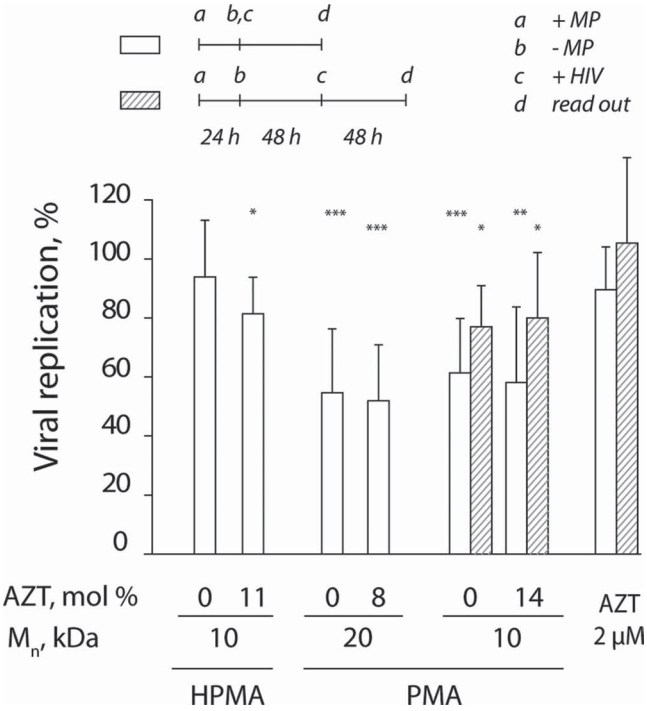

Figure 3. Viral replication inhibited by PHPMA and PMA-based macromolecular prodrugs of AZT administered at concentration 0.1 g L^{-1} and incubated with cells for 24 h. After 24 h, polymers were washed away and cells were infected with HIV-1 Bal strain at time 0 and 48 h after polymers were washed away. Results are average of at least four independent experiments and are presented as average ± standard deviation.

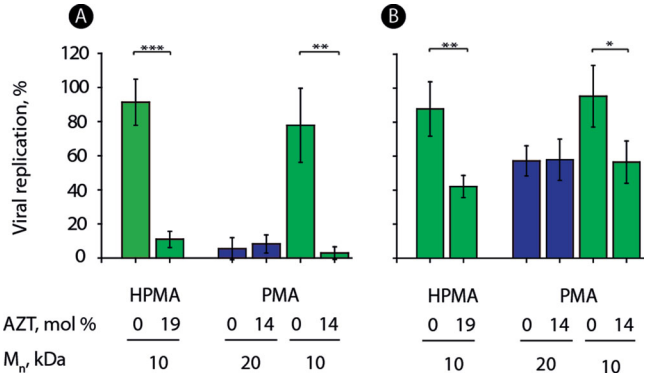

Figure 4. Viral replication in HIV-infected human T cells preincubated with AZT polymers taken at concentration A) 100 mg L^{-1} and B) 1 mg L^{-1}. Each bar represents average of level of infection in three different donors related to control sample with virus only.

Taken together, Figure 3 demonstrates a significantly extended duration of activity of the MP as compared to AZT. We note that removal of the polymers before administration of the virus is expected to limit the mode of activity for the polymers to intracellular effects. "Time of addition" protocol as employed herein is poised to identify the target for the therapeutic intervention, i.e., interference with the viral proliferation.[19] Results in Figures 2 and 3 reveal that PMAA has a dual activity, inhibition of viral adsorption and an intracellular effect, plausibly acting on the viral transcriptase and/or integrase.[20]

Encouraged by the data in a model cell line, macromolecular (pro)drugs were further tested as inhibitors of viral replication in human primary CD4+ T cells, which are the main reservoir for HIV infection in humans. Primary T cells were isolated from blood of three healthy donors, preincubated with polymers for 24 h, and infected with HIV-Bal. Following viral challenge, polymers and the virus were removed and inhibition of viral proliferation after this time point was therefore due to intracellular effects exerted by the polymers. Supernatants from the infected cells were harvested on day 3 post-infection, and the level of viral replication was quantified by measuring level of viral p24 protein with ELISA. Pristine HPMA polymer and 10 kDa sample of PMAA exhibited a minor if any effect on the viral proliferation regardless of polymer concentration (**Figure 4**). In contrast, 20 kDa PMAA and each of the three AZT containing polymers taken at 0.1 g L^{-1} concentration were highly effective in blocking viral proliferation with levels of p24 not exceeding 10% of untreated control. Furthermore, even at a 100-fold lower concentration, the polymers exhibited at least 40%–60% inhibition of viral proliferation. With regard to structure–function correlation, data in Figure 4 demonstrate that in primary cells and at a low concentration of the polymers, HPMA-based prodrugs are the most effective. With 20 kDa PMAA, the presence of AZT afforded no additional activity as compared to the pristine, drug-free polymer. Unexpectedly, 10 kDa PMAA polymer revealed minor if any inhibition of the HIV proliferation in T cells. At the same time, its drug-containing counterpart was highly effective in suppressing proliferation of HIV. Most importantly, results in Figure 4 demonstrate the ability of MP to inhibit HIV replication in human primary T cells over at least 72 h. We are currently optimizing MP with regard to kinetics of intracellular drug release towards making "fast acting" and/or "long-lasting" formulations.

Together the data presented herein demonstrate a set of MP, which fight HIV with efficacy matching that of AZT, longevity of treatment following a single administered dose (as identified in a model cell line) exceeding that of the pristine drug, and activity in primary human T cells over at least 72 h. Extended duration of treatment revealed no toxic effects of the polymers (see Supporting Information) making the synthesized MP both potent and safe. Designed combination therapy composed of PMAA and conjugated AZT is unique in that it interferes with the viral life cycle in two distinctly different stages, extracellular (adsorption) and intracellular (reverse transcription). Furthermore, our data point towards a novel, previously unknown mode of intracellular antiviral activity of the pristine PMAA. We strongly believe that presented opportunities in the design of MPs are unique and will aid in establishing novel combination treatments with enhanced potency and duration of treatment.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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