Macromolecular Prodrugs of Ribavirin: Concerted Efforts of the Carrier and the Drug

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Ribavirin (RBV), a broad-spectrum antiviral agent, has limited efficacy of treatment and a narrow therapeutic window and is characterized by a dose-limiting side effect, namely accumulation in red blood cells with ensuing anemia.[1] Conjugation to polymers can help in delivery of RBV[2] and in particular, to escape the origin of this side effect.[3] However, the wide and complex structure–function parameter space for macromolecular prodrugs of RBV remains un-explored. To address this challenge, we developed the automated parallel synthesis of polymer–drug conjugates with varied molecular weights ($\overline{M}_n$) and drug content. Specific novelty of this work lies in that to the best of our knowledge, this study presents the first successful example of high-throughput synthesis (HTS) and screening of polymers applied for accelerated discovery of macromolecular prodrugs. Using this methodology, we identified several conjugates with efficacy of treatment matching that of the pristine drug and at the same time overcoming the main side effect of this drug.

The combinatorial approach has documented tremendous successes in identifying hit "answers" to diverse biomedical challenges. HTS and screening of materials has allowed identification of surfaces with enhanced suitability for stem cell culture,[4] optimized performance in preventing protein[5] or bacterial[6] adhesion, and assembly of anti-inflammatory polymer coatings.[7] For injectable formulations, HTS delivered rapid development of polymers and lipidoid compounds for gene delivery[8] and RNAi,[9] assembly of core–shell nanoparticles,[7] and design of synthetic enzyme-like catalysts.[10] Surprisingly, over the past 40 years of development, design of macromolecular prodrugs remained iterative[11] and this explains, in part, moderate overall success relative to invested efforts. Furthermore, development of polymer therapeutics largely considered anticancer treatment[12] and examples of macromolecular prodrugs of antiviral agents are solitary. We aimed to achieve accelerated selection of polymer $\overline{M}_n$ and drug loading to optimize in vitro delivery of one of the very few broad-spectrum antiviral agents, RBV.

For the synthesis of polymer libraries, we chose to explore copolymerization of RBV (meth)acrylate with the "base" monomer being either acrylic or methacrylic acid (**Figure 1**). Polymer analogous reactions[13] leave scant opportunities for facile, quantitative conjugation of nucleoside analogue drugs. In contrast, synthesis of (meth)acrylate derivatives of nucleosides is a well-established approach with excellent yields of the monomers achieved through chemi-enzymatic syntheses using lipases.[14] Further to this, in our previous report, we indicated that the content of RBV in the final polymer matched well the monomer feed ratio and this provided an excellent opportunity to control RBV loading onto the polymer.[2a] Finally, the main aspect that makes this approach feasible, is that with the advent of controlled radical polymerization techniques such as reversible addition–fragmentation chain transfer (RAFT), facile means to obtain polymers with well-defined, controlled $\overline{M}_n$ and narrow dispersity ($Đ$) have become available.

Polymerization reactions were conducted on an automated platform, which allows robotic sampling of liquids and solids, and parallel synthesis of polymers with rationally designed composition. For acrylic acid- and methacrylic acid-based polymers, we used cyanomethyl dodecyl trithiocarbonate and cyanomethyl dithiobenzoate as RAFT agents, respectively. All polymerizations were conducted using DMF as a solvent, as it accommodated the low solubility of the RBV monomers. Content of RBV in the polymer conjugates was systematically varied through a gradual increase of RBV (meth)acrylate monomer in the mixture with the carrier monomer, (meth)acrylic acid. Feed ratio of up to 20 mol% of RBV (meth)acrylate were used corresponding to ≈50% content of this monomer by weight.

An inherent feature of RAFT polymerization is the possibility to control the polymer $\overline{M}_n$ through monomer to RAFT agent ratio, [M]/[RAFT]. In this work, [M]/[RAFT] was varied from 20 to 350 to ensure a synthesis of polymers covering a broad range of $\overline{M}_n$, from ≈5 to over 30 kDa. With a change in [M]/[RAFT], kinetics of polymerization also exhibited a significant change, polymerization being significantly retarded at higher RAFT agent concentrations. For this reason, the entire array of polymer samples could not be synthesized within a single multi-vial polymerization experiment. Instead, polymers were obtained as series grouped by similar [M]/[RAFT] and using polymerization times appropriate for corresponding series. Purified polymer samples underwent a detailed characterization

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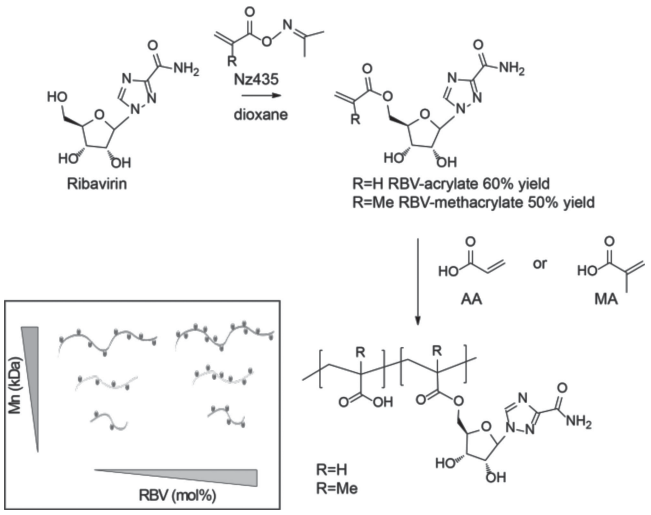

Figure 1. Schematic illustration of the synthesis of macromolecular prodrugs. Ribavirin (RBV) was concerted into its (meth)acrylate derivative via a chemi-enzymatic reaction using acetone oxime (meth)acrylate and a lipase enzyme. Synthesized monomers were then used in high throughout synthesis of polymers differing in their $\overline{M}_n$ and RBV content (schematically shown in the insert).

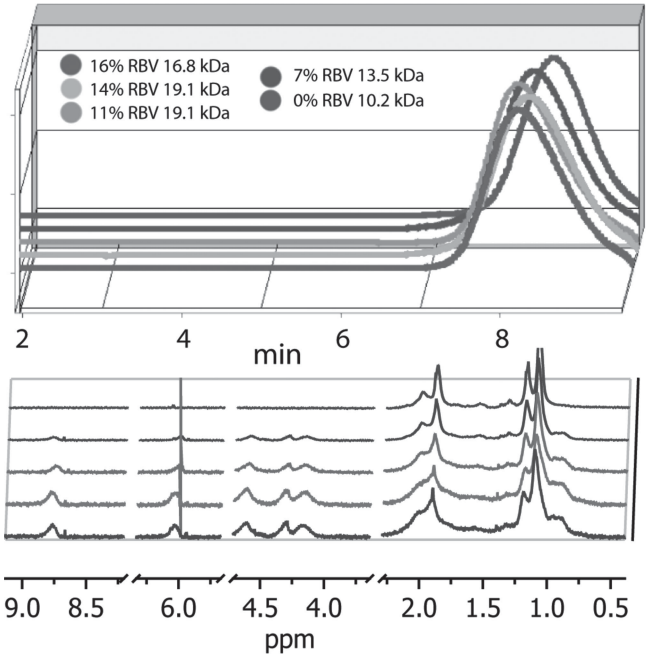

Figure 2. Gel permeation chromatography elution profiles (top) and ^{1}H NMR spectra (bottom) for a representative polymer series with similar $\overline{M}_n$ and increasing content of RBV on polymer chains.

using ^{1}H NMR and GPC to determine the content of RBV and values of absolute $\overline{M}_n$ and $Đ$.

One of the benefits of the automated polymer synthesis platform is that, at least within series, polymer samples are obtained using identical polymerization conditions, that is, the same polymerization times and temperature, as well as identical stock solutions of the monomers and RAFT agent. This minimizes sample-to-sample variability and ensures that macromolecular characteristics of the resulting polymers are closely matched—with the exception of variation in chosen parameters, in our case—drug loading. Indeed, GPC analyses of the polymer samples revealed that within the series, polymer samples had near-identical elution profiles indicative of closely matched $\overline{M}_n$ and $Đ$, **Figure 2**. We synthesized a total of 48 polymers, of which 23 were acrylate and 25 were methacrylate. Polymers were synthesized in five series using [M]/[RAFT] 350, 250, 50, 35, and 20, and RBV (meth)acrylate monomer feed up to 20 mol%. **Figure 3** graphically presents the resulting $\overline{M}_n$ and $Đ$ of the samples, along with the determined content of RBV, (for numerical values, see Supporting Information). With few exceptions, polymers were characterized with uniform and expected $\overline{M}_n$. For all of the polymer samples, $Đ$ was below 1.2 and for an overall majority of samples, below 1.1. This result is pivotal to draw reliable and meaningful structure–function correlations with regard to $\overline{M}_n$ and its impact on the therapeutic activity of the polymers.

To quantify the therapeutic effect of the synthesized macromolecular prodrugs, we took advantage of our recent finding on anti-inflammatory activity of RBV, specifically inhibition of production of nitric oxide in cultured macrophages.[15] Here, a mammalian macrophage cell line was cultured in the presence of 0.1 g L^{-1} polymers and subsequently stimulated with a potent pro-inflammatory agent, lipopolysaccharide (LPS). Following further 24 h in culture, supernatants were collected and used to quantify the levels of nitrite anion, a product of degradation of nitric oxide, using the colorimetric Griess assay.[16] Independently, metabolic activity of the cells was ascertained using a commercial viability kit. In these experiments, clinically relevant concentration of RBV, 10 μM RBV,[17] afforded a decrease in the levels of NO to ≈50%, accompanied by a ≈20% drop in the cell viability. 100 μM RBV afforded a pronounced decrease in the levels of NO to circa 25%, although this was attributed to cytotoxicity of the drug at this concentration.

Levels of nitric oxide measured for cells cultured in the presence of macromolecular prodrugs are graphically presented in Figure 3. The most important observation from these datasets is that macromolecular prodrugs elicit a pronounced therapeutic response and within the synthesized libraries, there existed at least 10 formulations with activity matching that of the pristine drug, with no expense to the cytotoxicity of the treatment. Furthermore, a striking observation is that reduction in the levels of nitric oxide was observed not only in the case of RBV containing polymers, but also when macrophages were cultured in the presence of pristine polymers, in particular—low $\overline{M}_n$ poly(acrylic acid), PAA. In separate experiments, we observed that this effect is also observed for the samples of PAA with higher $\overline{M}_n$ and is also pronounced for poly(methacrylic acid) (PMAA) when administered at higher concentrations (1 g L^{-1}) (see Figure SI1, Supporting Information). Negatively charged polymers are well-studied in the anti-HIV research as agents to prevent association of the virus with mammalian cells.[18] However, to the best of our knowledge, polyanions have not been previously shown to have an inhibitory effect on inducible nitric oxide synthase (iNOS) or to interfere with the synthesis of NO in other ways. Our data reveal that PAA and PMAA function

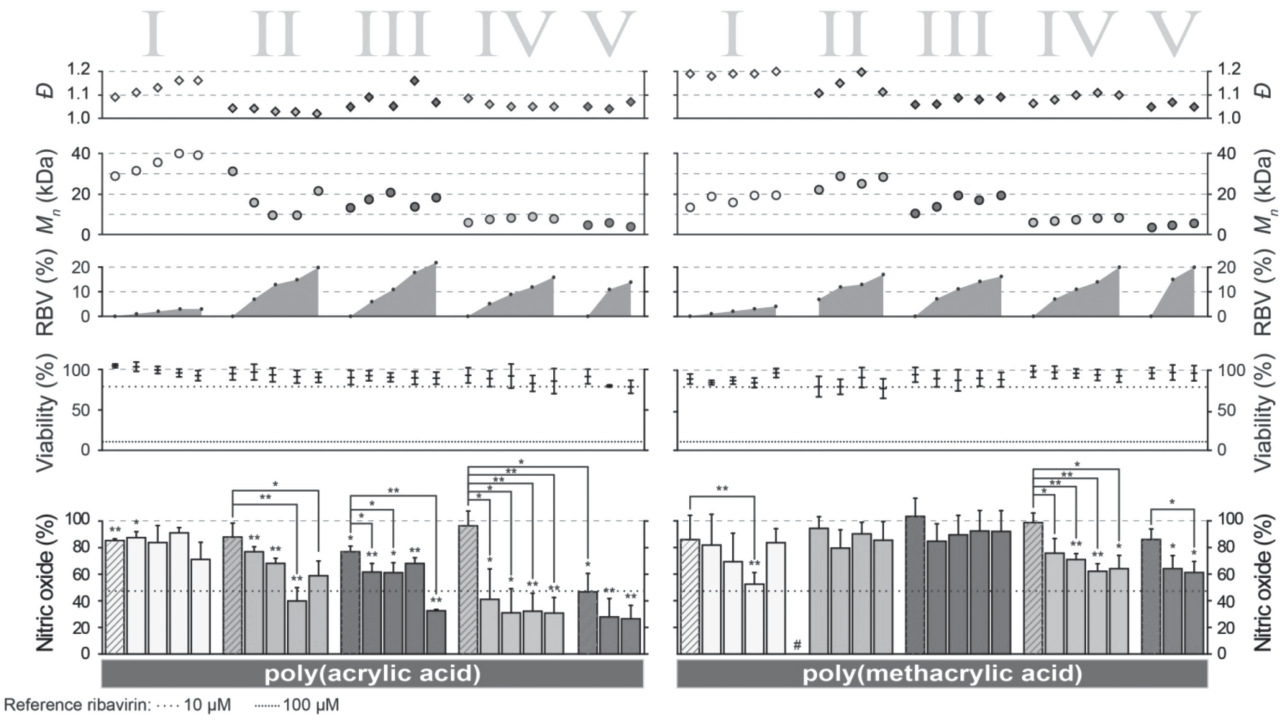

Figure 3. Graphic representation of the macromolecular characteristics of the synthesized macromolecular prodrugs (from top to bottom: dispersity *Đ*, number-average molecular weight $\overline{M}_n$, and drug loading (mol% of RBV (meth)acrylate)), as well as viability of cultured macrophages and levels of nitric oxide produced by these cells upon cell culture in the presence of 0.1 g L^{-1} of polymers and pro-inflammatory stimulation using LPS. For full experimental details, see Main text and Supporting Information. For cell culture experiments, results shown are the average of three independent experiments, reported as mean ± SD (n = 3). Statistical significance is given compared to the negative control, unless indicated otherwise. *p < 0.05, **p < 0.01.

as more than just inactive carrier polymers for delivery of RBV. The carrier and the drug appear to make concerted efforts and either constituent contributes to the overall therapeutic effect.

The first important observation with regard to structure-function analysis of the synthesized polymers is that, for both acrylates and methacrylates, it holds true that polymers with the lowest $\overline{M}_n$ had the most pronounced therapeutic effect (Spearman ρ = 0.58, ***). Within individual polymer series, increased content of RBV resulted in enhancement of the therapeutic activity. Acrylate polymers with $\overline{M}_n$ 5–10 kDa (series IV) proved to be the most effective carriers for RBV and with the drug content of 5 mol% these polymers were as effective in suppressing production of NO as the pristine drug. In separate experiments, we used fluorescently labeled polymer samples and observed that with similar RBV content (15 ± 2 mol%), polymer chains with the lowest $\overline{M}_n$ (≈5 kDa) afforded significantly higher mean fluorescence of the macrophages suggesting that these chains exhibit a more pronounced association with these cells compared to their higher $\overline{M}_n$ counterparts (Figure SI2, Supporting Information). Indeed, these samples were also characterized with the highest therapeutic effect (Figure 3). Similar analysis with regard to the RBV content revealed no such correlation (data not shown). Together, these data suggest that the efficiency of cell entry is the limiting step in the overall therapeutic effect of macromolecular prodrugs of RBV and indicates that polymer $\overline{M}_n$ is the primary means to control this process. We further observed that regardless of $\overline{M}_n$ and drug loading, polymers escape association with red blood cells thus effectively overcoming the origin of the main side effect of RBV, namely accumulation in erythrocytes (see Figure SI3, Supporting Information).

Altogether, this work presents the first example of using tools of HTS for accelerated synthesis, optimization, and structure-function analysis in the field of polymer therapeutics and specifically for the synthesis of antiviral macromolecular prodrugs. Analysis of therapeutic activity was accomplished using an assay built around the ability of RBV to inhibit the production of nitric oxide in stimulated macrophages. Unexpectedly, our results revealed that pristine PAA and PMAA in absence of the conjugated drug exhibit a pronounced anti-inflammatory activity. With conjugated RBV, therapeutic activity of the polymers significantly increased, thus revealing concerted activity of the carrier and the drug in the overall therapeutic effect. From the structure-function analysis of the polymers emerged an understanding of the criteria/factors relevant for a successful design of polymeric prodrugs of RBV. Most importantly, polymer conjugates with $\overline{M}_n$ exceeding 20 kDa proved to be largely ineffective in delivering RBV and exhibited minor activity in suppressing production of nitric oxide. In contrast, oligomeric samples with $\overline{M}_n$ ≈5 kDa were highly effective and exhibited inhibition of NO synthesis matching the effect achieved using the pristine drug. At the same time, conjugation to polymers afforded formulations of RBV that overcome association with red blood cells. We believe that this work

contributes significantly to the development of a safer delivery of this broad spectrum antiviral agent.

Supporting Information

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

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