Volume 50 | Number 93 | 4 December 2014 | Pages 14465–14684

ChemComm

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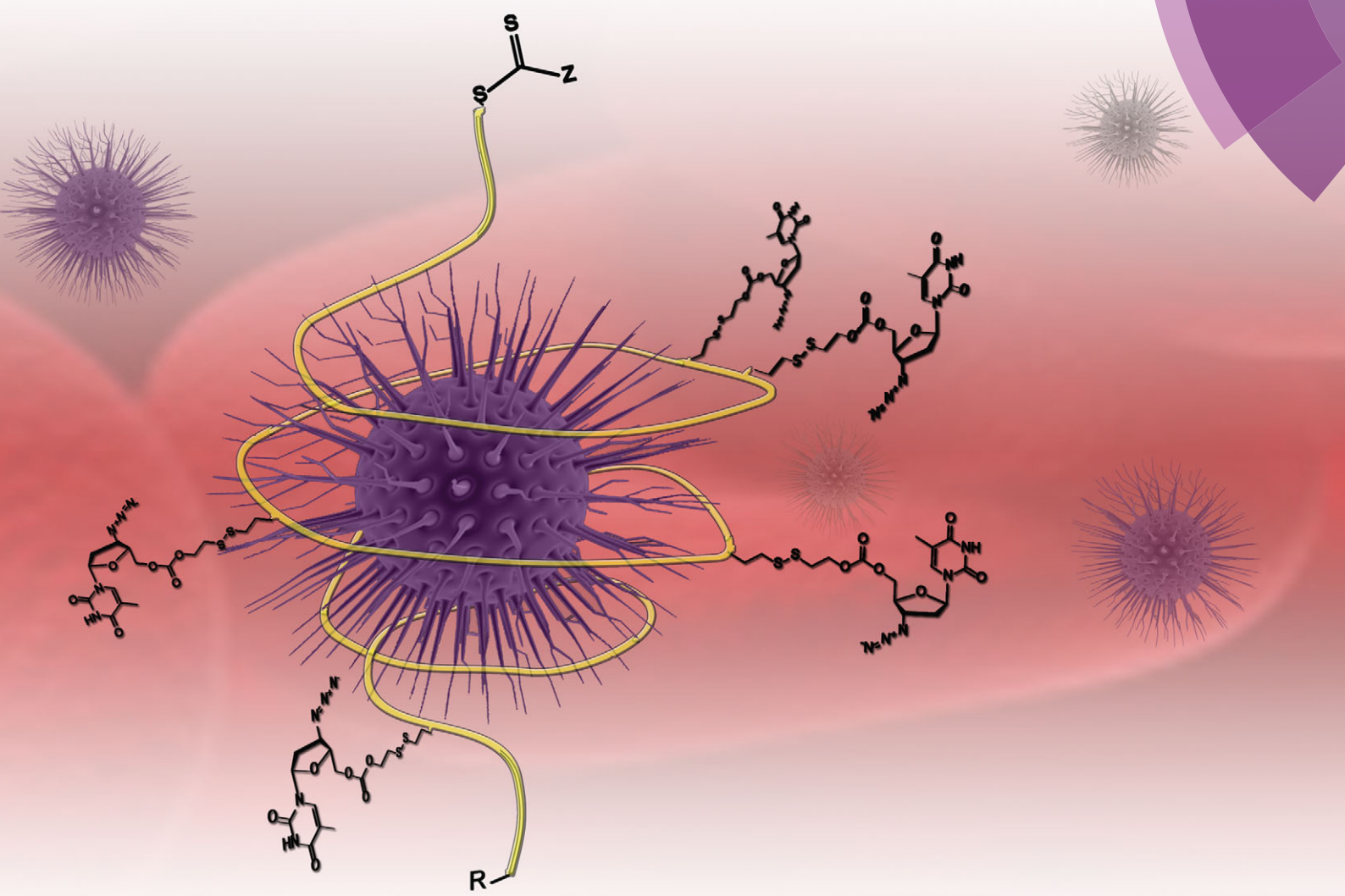

ISSN 1359-7345

ROYAL SOCIETY OF CHEMISTRY

COMMUNICATION

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ChemComm

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View Article Online
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Cite this: *Chem. Commun.*, 2014, 50, 14498

Received 4th June 2014,
Accepted 26th September 2014

DOI: 10.1039/c4cc04280h

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Disulfide reshuffling triggers the release of a thiol-free anti-HIV agent to make up fast-acting, potent macromolecular prodrugs†

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The release of azidothymidine from macromolecular prodrugs was designed to respond to the intracellular disulfide reshuffling. This drug has no thiol groups, and a response to this trigger was engineered using a self-immolative linker. The resulting formulations were fast-acting, efficacious, and highly potent with regards to suppressing the infectivity of the virus.

The successful design of macromolecular prodrugs (MP) relies on an intelligent choice of the polymer carrier, its average molar mass, and the linkage between the drug and the macromolecule.[1–4] For the former parameters, the preceding decades of development provide reliable guidance, and using the modern tools of polymer synthesis it is now possible to obtain MP with desired compositions for a vast range of therapeutic molecules. Regretfully, a similar flexibility cannot be stated with regard to binding of the drug to the carrier polymer. For each particular drug, ligation strategies are typically constrained by the limits of the "natural", inherent chemistry of the drug, *i.e.* availability of sites for conjugation.[5] The most warranted mechanisms of drug release are based on pH-responsive linkages, scissile peptide linkers, and thiol-disulfide chemistry,[4] yet these opportunities are available for a limited number of therapeutic entities. Notably, disulfide chemistry is highly specific to thiol containing molecules yet very few therapeutics contain sulfhydryl functionalities. In the past few years, a cunning tool of organic chemistry has entered the mainstream of prodrug design, namely "self immolative linkers" (SIL).[6,7] When placed between a "trigger" and a drug of interest, SIL spontaneously deconstructs upon cleavage of the trigger and releases the pristine drug. Specifically, it is possible to use thiol-containing SIL and use disulfide reshuffling to liberate thiol-free drugs and imaging reagents.[8] In very recent reports, this tool was applied to engineer drug release in response to the intracellular thiol–disulfide reshuffling for MP of anticancer agents such as camptothecin and SN-38.[9–12] In this work, we realize this advanced design of drug carriers outside the realm of anticancer research and develop MP of azidothymidine (AZT), an anti-HIV agent with limited opportunities for conjugation.

The design of MPs was based on the synthesis of appropriate monomers followed by their copolymerization through controlled radical polymerization technique, RAFT.[13,14] This approach is powerful and flexible at the same time and allows the synthesis of MPs with nominated chemistry and composition.[15,16] For the latter, average molar mass and drug content can be independently varied in broad respective range. A monomer equipped with an SIL and responsive to disulfide reshuffling trigger was synthesized starting with methacryloyl chloride, oxidized (dimeric) mercaptoethanol, and AZT, Fig. 1. The synthesis was accomplished in 3 steps and characterized with an overall yield (% of AZT) of 50%. The resulting monomer was used to obtain MPs based on *N*-(2-hydroxypropyl) methacrylamide, HPMA, the latter being used in the synthesis of MPs with advanced biomedical characterization and evaluation in clinical trials.[2,3] RAFT polymerization was used to obtain polymers with average molar masses of 10 and 20 kDa and AZT content of up to 20 mol%. The resulting polymers were characterized with dispersities at or below 1.2 revealing a uniform composition of MPs, a requisite for sound structure–function correlations.

To probe drug release in response to the nominated trigger, the synthesized MPs were exposed to 5 mM glutathione (GSH), conditions reminiscent of intracellular with regard to the content of this natural thiol-containing tripeptide.[17] In full agreement with the design, SIL equipped MPs released their payload in the presence of GSH, Fig. 2. Drug release was quantified using HPLC which revealed that drug release was fast, with $t_{1/2}$ of less than 30 minutes (ESI,† Fig. S3). In contrast, MPs synthesized based on AZT methacrylate[16] exhibited negligible drug release under these conditions. As a significantly more thorough test, the release of AZT from MP

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† Electronic supplementary information (ESI) available: Protocols for the syntheses, details of characterization (NMR, GPC), additional experimental results. See DOI: 10.1039/c4cc04280h

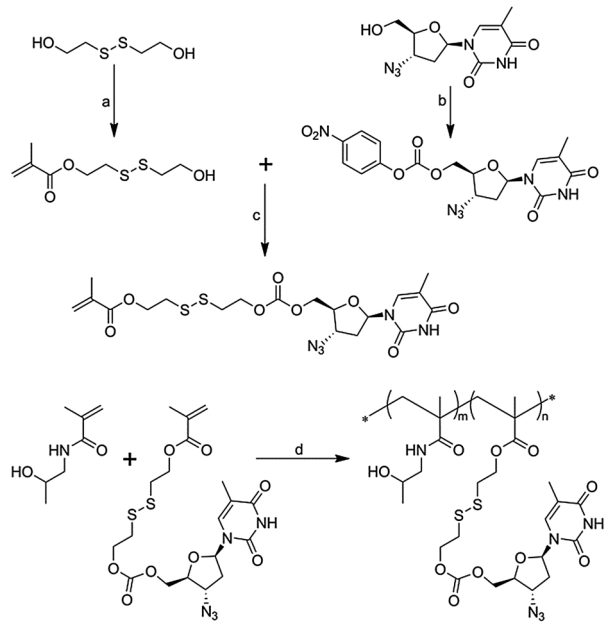

Fig. 1 Schematic illustration of the synthesis of MP of AZT with a thiol-triggered mechanism of drug release. (a) Methacryloyl chloride, TEA, DCM anh., 76%; (b) 4-nitrophenyl chloroformate, TEA, DCM, 82%; (c) DIEA, DMAP, DCM, 80%; (d) 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid, ACVA, DMSO.

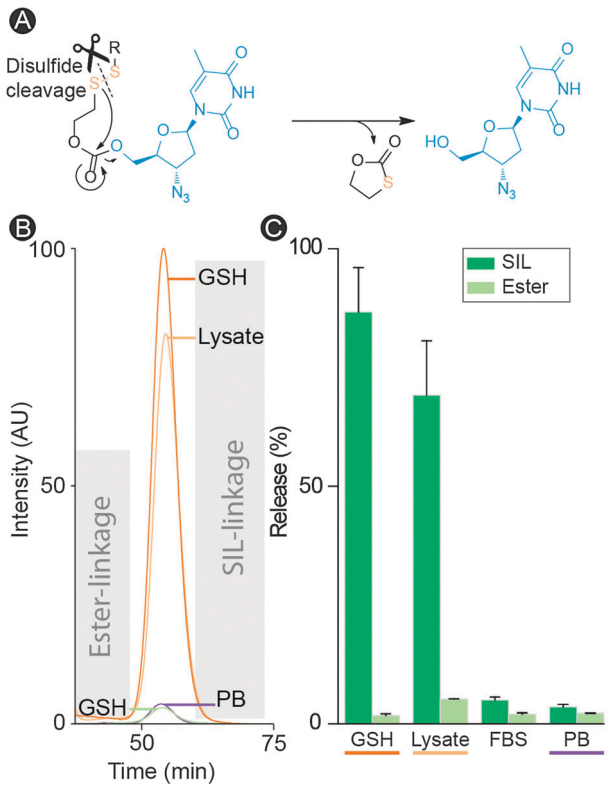

Fig. 2 (A) Schematic illustration of the mechanism of release of AZT from the macromolecular prodrugs as triggered by disulfide reshuffling. (B) HPLC elution profiles illustrating drug release from MP based on an SIL or an ester linkage between AZT and the polymer in phosphate buffer (PB), in PB in the presence of 5 mM GSH, and in the milieu consisting of lysate of mammalian cells. (C) Quantitative (HPLC) data on drug release from MP based on SIL or an ester linkage in specified solution conditions over 24 hours.

was quantified when triggered by the lysate of mammalian cells, TZM-bl, under which treatment the MPs released at least 70% of the conjugated AZT over 24 h of incubation. The spontaneous release of AZT in PBS or in the presence of serum during this time did not exceed 5%, Fig. 2C. These data reveal that the proposed design of AZT MPs affords a highly sensitive and fast release of the antiviral drug in conditions mimicking intracellular.

To investigate the therapeutic benefits afforded by the proposed conjugation strategy, TZM-bl cells were used to monitor infectivity of the HIV virus. These cells express CD4, CXCR4, and CCR5 (co)receptors and are appropriate hosts in developing anti-HIV agents.[18] Mammalian cells were incubated with the polymers at 1 mg L^{-1} concentration for 24 h prior to the viral challenge, and levels of HIV infectivity were quantified following further 48 h of incubation, Fig. 3A. Pristine polymers (PHPMA) revealed negligible antiviral activity. At the chosen low concentration of polymers, ester-based MPs afforded only a minor decrease of viral infectivity. In stark contrast, thiol-responsive MP decreased infectivity by as much as 80%. These data demonstrate superior activity of the MPs equipped with a sensitive mechanism of drug release in response to an intracellular trigger. Under these experimental conditions, activity related EC_{50} values of thiol-responsive MP were over 10-fold lower than the corresponding values for the ester-based counterparts with matched average molar mass and drug loading, revealing a significantly enhanced potency of the newly synthesized MPs (Table 1). Similar conclusions were made based on the experiments in which the polymers and the virus were

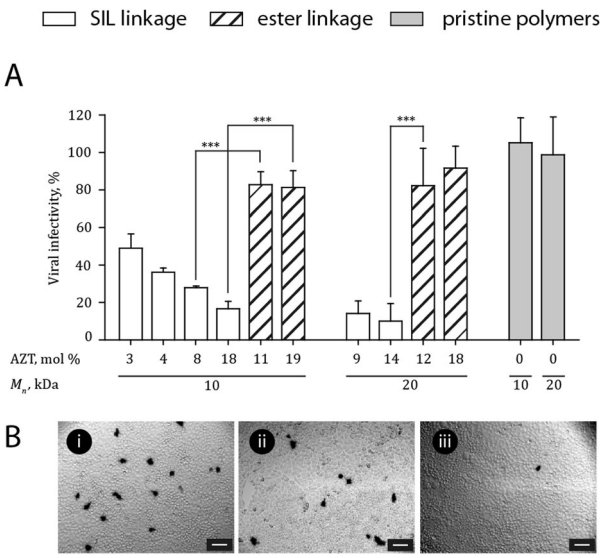

Fig. 3 (A) Viral infectivity inhibited by HPMA based macromolecular prodrugs of AZT administered at concentration 1 mg L^{-1}. Viral infectivity was quantified in TZM-bl cells with 24 h pre-incubation of the polymers with the cells and allowing further 48 h for viral infectivity. Results are average of three independent experiments and are presented as average ± standard deviation. (B) Staining of HIV-infected TZM-bl cells based on β-galactosidase assay. Cells were preincubated without the drug (i), with HPMA LMw 19% AZT 4 mg L^{-1} (ii), or SIL HPMA LMw 18% AZT 4 mg L^{-1} (iii). Infected cells are stained black. Scale bars: 100 μm.

Table 1 Pharmacodynamic properties of MPs of AZT based on ester and thiol-responsive, SIL containing linkages

Polymer (linkage, M_n, AZT content)	IC_{50} (mg L^{-1})	IC_{50} (AZT eq., nM)
Ester, 20 kDa, 12%	4.43	5000
SIL, 20 kDa, 14%	0.42	300
Ester, 10 kDa, 19%	3.07	3600
SIL, 10 kDa, 18%	0.27	230
AZT		85

administered onto the cells at the same time (see Fig. S4, ESI†). Under these conditions, SIL based polymers afforded at least 40% inhibition of viral infectivity thus demonstrating a fast-acting drug release. Potency data are supported by the images illustrating infectivity of HIV (Fig. 3B) in which case PHPMA and ester-based MP provide a low if any level of prevention of viral proliferation. At the same time, SIL-equipped MPs provide a near-complete suppression of HIV infectivity.

Our data present an approach to engineer intracellular drug release in response to a stimulus "un-natural" to the drug candidate. We present disulfide reshuffling as a trigger to release a thiol-free anti-HIV agent. MPs built around this technology are highly sensitive to the nominated trigger and as a result, are highly potent, specifically in the experiments addressing infectivity of the live immunodeficiency virus.

This work is supported by a Sapere Aude Starting Grant from the Danish Council for Independent Research, Technology and Production Sciences, Denmark.

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