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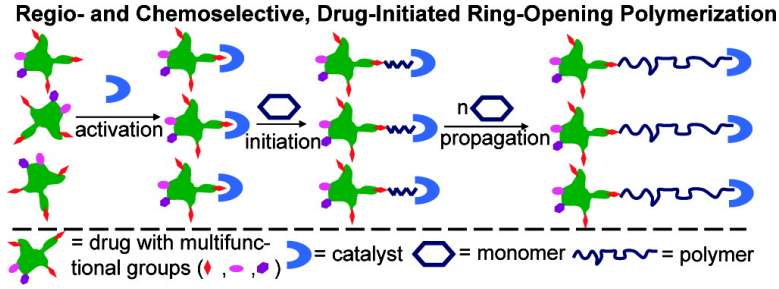

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Ring-Opening Polymerization-Mediated Controlled Formulation of Polylactide−Drug Nanoparticles

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Abstract: We report here a unique method for formulating doxorubicin−polylactide (Doxo-PLA) conjugate nanoparticles, known as nanoconjugates (NCs), through Doxo/(BDI)ZnN(TMS)$_2$-mediated [(BDI) = 2-((2,6-diisopropylphenyl)amido)-4-((2,6-diisopropylphenyl)-imino)-2-pentene], chemo- and regioselective polymerizations of lactide (LA) followed by nanoprecipitation. When Doxo/(BDI)ZnN(TMS)$_2$ was mixed with 1-pyrenemethanol (Pyr-OH) and 1-pyrenemethylamine (Pyr-NH$_2$) and the mixture was utilized for the polymerization of LA, remarkable chemoselectivity was observed. Pyr-OH was completely consumed and covalently linked to the terminus of the PLA, whereas the Pyr-NH$_2$ remained intact in the polymerization solution. When Doxo was used as the initiator to polymerize LA in the presence of (BDI)ZnN(TMS)$_2$, the polymerization was complete within hours, with nearly 100% Doxo-loading efficiency and 100% LA conversion. Doxo loading as high as 27% could be achieved at a LA/Doxo ratio of 10. Both the steric bulk of the chelating ligand and the metal catalyst had dramatic effects on the regioselectivity during the initiation step. When Doxo/(BDI)ZnN(TMS)$_2$ was mixed with succinic anhydride (SA) to mimic the initiation of Doxo/(BDI)ZnN(TMS)$_2$-mediated LA polymerization, Doxo-14-succinic ester (Doxo-SE) was the predominate product. When the steric bulk of BDI was reduced or when the BDI ligand was removed, significant amounts of Doxo-4′,14-bis-succinic ester (Doxo-2SE) and Doxo-4′,9,14-trisuccinic ester (Doxo-3SE) were formed. The use of (BDI)MgN(TMS)$_2$ in such a reaction also resulted in reduced regioselectivity and formation of both Doxo-SE and Doxo-2SE. Doxo/(BDI)ZnN(TMS)$_2$-mediated LA polymerizations yielded Doxo-PLA conjugates with well-controlled molecular weights and polydispersities (as low as 1.02). The nanoprecipitation of Doxo-PLA formed NCs less than 150 nm in size with narrow particle size distributions. The sustained release of Doxo from Doxo-PLA NCs was achieved without a burst release. This method may have widespread utility for controlled conjugation of hydroxyl-containing agents to polyesters and formation of corresponding nanoparticles.

1. Introduction

Polymeric nanomedicine, an emerging field that involves the use of drug-containing polymeric nanoparticles (NPs) for cancer treatment, is expected to alter the landscape of oncology.[1−5] Although the nomenclature of nanomedicine emerged only a few years ago,[6−11] the practice of applying nanotechnology to cancer drug delivery dates back to the 1970s.[8] Through numerous efforts, a handful of polymeric NPs have been developed and evaluated in various preclinical[4,11−27] or clinical

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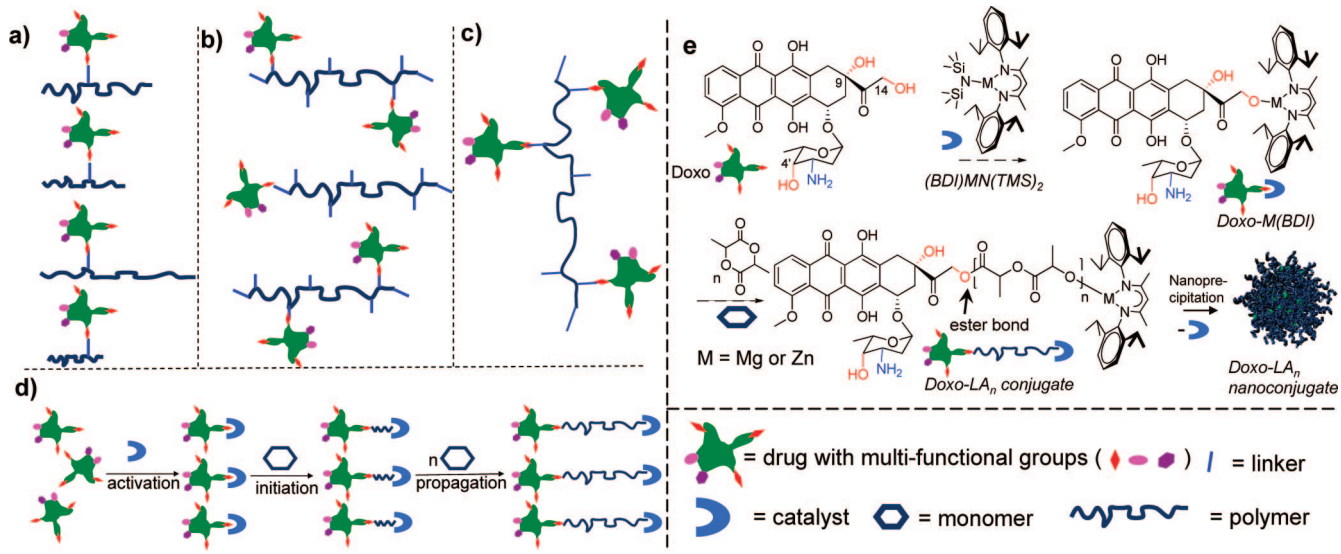
Scheme 1. (a) Polymer−Drug Conjugates with Variable Chain Lengths; (b) Polymer−Drug Conjugates with Uncontrolled Conjugation Site on Polymer Backbone; (c) Polymer−Drug Conjugates Lacking Regio- and Chemoselective Conjugation of Therapeutic Agents Containing Multiple Conjugation-Amenable Functional Groups; (d) Drug-Initiated, Controlled Polymerization for the Synthesis of Polymer−Drug Conjugates with Low Polydispersities, and with Regio- and Chemoselective Incorporation of Drug Molecules to the Termini of Polymers; (e) Doxo-Initiated LA Polymerization for Regio- and Chemoselective Doxo Conjugation to Form Doxo-PLA Conjugates Followed by Nanoprecipitation to Form Doxo-PLA Nanoconjugates



studies,[28−37] some of which have been approved for clinical cancer treatment.[38]

The application of nanotechnology to conventional chemotherapy has obvious benefits.[6] In general, the incorporation of chemotherapeutic agents in NP delivery vehicles has improved water solubility,[39,40] reduced clearance,[41] reduced drug resistance and enhanced therapeutic effectiveness.[7,42,43] However, the drawbacks of the incorporation of chemotherapeutic agents in delivery NPs are also obvious. Compared to conventional chemotherapy, where all therapeutic agents have identical structures and molecular weights, polymeric NPs are heterogeneous in their composition, structure and size.[44−47]

The heterogeneities of polymer−drug conjugates, a class of well-known polymeric NP delivery vehicles, may result from (1) the polydispersity of the polymer (Scheme 1a), (2) the lack of control over the site on the polymer backbone to which the drug is conjugated (Scheme 1b) and (3) the lack of control over the regio- and chemoselective conjugation of the therapeutic agents containing multiple conjugation-amenable functional groups (Scheme 1c). These heterogeneities are not easily addressable and may present bottlenecks to the clinical translation of polymeric NPs. Without eliminating the heterogeneities inherent in polymeric−drug conjugates, it will be difficult, if not entirely impossible, to achieve advanced drug delivery for targeted or even personalized cancer therapy.[8] In the past several decades, there have been numerous efforts to reduce these

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heterogeneities by developing polymers with low polydispersities,[48−52] conjugating therapeutic agents to the termini of polymers or to specific sites along polymer backbones[39,40,53,54] and blocking the undesirable conjugation of competing functional groups on therapeutic agents through the use of protection and deprotection chemistries.[55]

Ring-opening polymerization (ROP)[56−58] for the preparation of polyesters, such as polylactide (PLA), which is used in this study, has been investigated extensively.[59,60] This polymerization typically involves lactide (LA) ring-opening by a metal alkoxide (RO−M) to form a RO-terminated LA−metal alkoxide (ROOCCH(CH_3)O−M), followed by chain propagation to form RO-terminated PLA.[59−61] The RO is connected to the PLA terminus through an ester bond. This process is well understood and has been used extensively for the incorporation of hydroxyl-containing small molecules,[62] macromolecules[63] and NPs[64] to the termini of PLA. We hypothesized that the process could be utilized for the incorporation of hydroxyl-containing therapeutic agents and demonstrated the concept by using paclitaxel (Ptxl), a mitotic inhibitor that contains hydroxyl groups, as the initiator for the ROP of LA to allow efficient conjugation of Ptxl molecules to the termini of PLA.[65] This preliminary study set the foundation for further investigation of the incorporation of therapeutic agents with more complex structures.

In this article, we report the use of doxorubicin (Doxo), a therapeutic agent that contains multiple types of conjugation-amenable groups (three hydroxyl groups, one amine group and one ketone group), as an initiator for the ROP of LA. This approach allows for one-pot synthesis of Doxo-PLA conjugate and concurrently addresses all three of the heterogeneities observed in polymer−drug conjugates (Scheme 1d and 1e). This study demonstrated, for the first time, that Doxo can be incorporated into PLA in a regio- and chemoselective manner via a well-controlled polymerization process. Quantitative incorporation of Doxo and controlled polymerization (polydispersity (PDI) of Doxo-PLA as low as 1.02) was achieved simultaneously. Doxo was conjugated to the terminus of PLA through its 14-hydroxyl group specifically, without the need to protect its 3′-amine or 4′- and 9-hydroxyl groups (Scheme 1e). The nanoprecipitation of Doxo-PLA conjugates allowed for the formation of Doxo-PLA conjugate NPs, which are also called nanoconjugates (NCs) to differentiate them from NPs prepared by coprecipitating drugs and polymers, with narrow particle size distributions and controlled release kinetics. We demonstrated that this unique, polymerization-mediated drug conjugation method can be applied to the formulation of PLA NCs with a variety of hydroxyl-containing molecules.

2. Results and Discussion

2.1. Rationale behind the Use of Metal Catalysts for Chemoselective Ester Formation. The formation of amide bonds is favored in conventional carboxylate coupling reactions when both amine and hydroxyl groups are present. Therefore, the coupling of the terminal carboxylate of PLA with Doxo (Scheme 1e), a molecule bearing both amine and hydroxyl groups, predominately formed Doxo-PLA conjugates through the 3′-NH_2 of Doxo by creating an amide bond.[54,66] Such Doxo-PLA conjugates, however, cannot release Doxo in its original form by hydrolysis. Instead, Doxo-3′-lactamide, a prodrug of Doxo, is formed. In poly(ethylene glycol)-*b*-poly(aspartic acid) (PEG-*b*-PAsp) copolymer micelles in which Doxo was conjugated via its 3′-NH_2 to the pendant carboxylate groups of the PAsp block, Doxo may not be readily released from PEG-*b*-PAsp(Doxo) micelles to exhibit therapeutic effectiveness.[67]

In order for Doxo to be released in its original form, the hydroxyl groups of Doxo should be used for conjugation with the terminal carboxylate group of PLA to form an ester linker between Doxo and PLA. To facilitate such a reaction, protection and deprotection of the Doxo amine group before and after the conjugation, respectively, are thereby required. Although these protection and deprotection chemistries are possible, the manipulation of such chemistries on a multifunctional, unstable drug is difficult and may result in degradation of Doxo. Due to these challenges, efforts have been devoted mainly to the conjugation between the 13-ketone group of Doxo and hydrazine groups of polymeric carriers by forming an acid-labile hydrazone bond.[68−71] Some promising preclinical results of polymer-Doxo delivery vehicles with hydrazone linkers have been reported.[17,67] However, clinical studies of immunoconjugates with Doxo connected to monoclonal antibodies with hydrazone linkers gave unsatisfactory antitumor effects, which led to the termination of the clinical development of such immunoconjugates.[72]

Acylation of Doxo with its hydroxyl groups was achieved through a Subtilisin Carlsberg (a serine endopeptidase)-mediated reaction with vinyl butyrate.[73] Nevertheless, it is unlikely that this method can be used for the conjugation of Doxo to polymers. Diatos S. A. Laboratories recently reported the

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synthesis of Vectocell peptide-Doxo conjugates in which Doxo was linked to a peptide with an ester bond through its 14-OH group.[74] However, the synthesis was not achieved through the coupling of Doxo and the peptide. Multistep reactions starting from daunorubicin, rather than Doxo, were involved in the synthesis of the final product.[74] To our knowledge, there has been no report of effective acylation of Doxo via its hydroxyl groups for the formulation of polymer-Doxo conjugates.

In numerous previous studies involving the use of metal catalysts for LA polymerization, ring-openings of LA proceeded predominately by metal alkoxides (M-ORs) rather than by metal amides (M-NHRs).[60] Although there are only limited reports that describe the differences in the polymerization activities between these two classes of initiators, M-OR complexes typically have higher activities for LA ring-opening to form ester bonds than their amine analogues in a similar ring-opening reaction to form amide bonds. For instance, Coates and co-workers reported that (BDI)ZnOCH$(CH_3)_2$ [(BDI) = 2-((2,6-diisopropylphenyl)amido)-4-((2,6-diisopropylphenyl)-imino)-2-pentene] initiated and completed a LA polymerization within 20 min at a monomer/initiator (M/I) ratio of 200, while a similar polymerization mediated by (BDI)ZnN$(TMS)_2$ required 10 h to complete.[61] Based on this finding, we studied whether it was possible to use Zn catalysts for controlling Doxo conjugation to PLA preferentially with the hydroxyl groups of Doxo (Scheme 1e).

2.2. Metal Amide- and Metal Alkoxide-Initiated LA Polymerization. To evaluate metal amide- and metal alkoxide-initiated LA polymerization, we utilized 1-pyrenemethanol (Pyr-OH) and 1-pyrenemethylamine (Pyr-NH_2) as the corresponding model hydroxyl and amine initiators. Pyrene is UV-active and thus can be easily characterized by a UV detector equipped with a HPLC. (BDI)ZnN$(TMS)_2$, the Coates catalyst mentioned above, was chosen for our initial study.[61] Instead of preparing and isolating (BDI)ZnOPyr and (BDI)ZnNHPyr, we tested whether these complexes could be formed *in situ* and subsequently initiate LA polymerization. In the LA polymerization initiated by an equal molar mixture of Pyr-OH and (BDI)ZnN$(TMS)_2$, 100% consumption of LA was observed. Pyr-OH was not detectable in the polymerization solution after the reaction was complete. In contrast, a similar LA polymerization initiated by an equal molar mixture of Pyr-NH_2 and (BDI)ZnN$(TMS)_2$ produced no reaction; Pyr-NH_2 remained intact in the reaction solution (ii, Figure 1). To further verify the observed chemoselectivity, we used an equal molar mixture of Pyr-NH_2 and Pyr-OH with 1 equiv (BDI)ZnN$(TMS)_2$ to initiate a LA polymerization. The polymerization was initiated exclusively by Pyr-OH, whereas Pyr-NH_2 remained intact in the polymerization solution (i, Figure 1). This study demonstrated that the (BDI)ZnN$(TMS)_2$ catalyst had remarkable chemoselectivity and could specifically coordinate with hydroxyl groups to initiate LA polymerization, regardless the presence or absence of amine groups (Figure 1). It also should be noted that Pyr-NH_3^+, the protonated form of Pyr-NH_2, behaved similarly to Pyr-NH_2 in this polymerization (data not shown). Pyr-OH can be quantitatively recovered from the Pyr-PLA conjugate, the product of the Pyr-OH/(BDI)ZnN$(TMS)_2$-mediated LA polymerization, by treating Pyr-PLA with NaOH (0.1−1 M) overnight (data not shown). This study suggests that Pyr-OH molecules were linked to PLA termini through ester linkers that were subject to base-induced rapid hydrolysis (Figure 1).

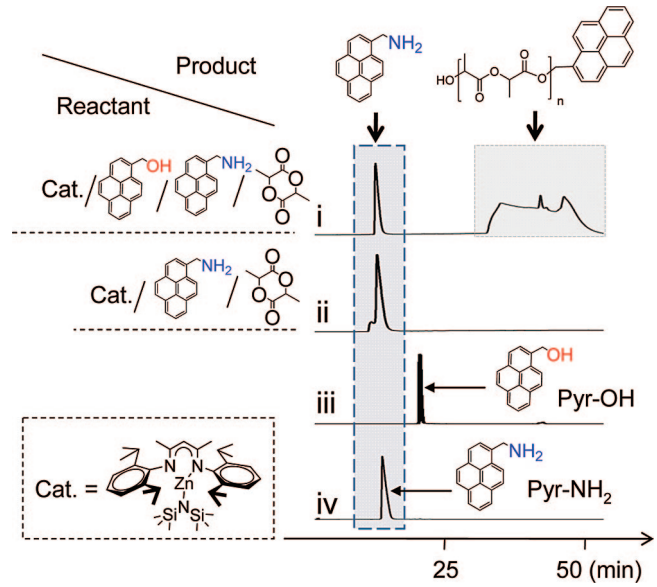


Figure 1. Reverse-phase (RP) HPLC analysis of (i) the mixture of Pyr-NH_2/Pyr-OH (1:1 molar ratio), (BDI)ZnN$(TMS)_2$ (1 equiv) and LA (25 equiv); (ii) the mixture of Pyr-NH_2, (BDI)ZnN$(TMS)_2$ (1 equiv) and LA (25 equiv); (iii) Pyr-OH; (iv) Pyr-NH_2. Note: The ill-defined peak of Pyr-PLA in (i) is largely due to the use of the reverse-phase HPLC column that is not suitable for the separation of Pyr-PLA. Substantially improved elution peaks were obtained when SEC columns were used for the analysis of Doxo-PLA conjugates (see Figure 5a).

2.3. Doxorubicin-Initiated LA Polymerization in the Presence of (BDI)MN$(TMS)_2$ (M = Zn, Mg).

2.3.1. Chemoselective Conjugation of Doxo to PLA. We first studied whether the metal catalyst would decompose Doxo during the initiation step and evaluated the coordination of (BDI)ZnN$(TMS)_2$ and Doxo without adding LA monomers. From the HPLC and MS analyses, it was evident that Doxo remained in its original form in the presence of the metal catalyst (Supporting Information, Figure S1). It was thus very unlikely that Doxo would be deleteriously affected during Doxo/(BDI)ZnN$(TMS)_2$-mediated LA polymerization.

We next used Doxo as the initiator in (BDI)ZnN$(TMS)_2$-mediated LA polymerization (Scheme 1e). When 100 equiv LA was added to a mixture of (BDI)ZnN$(TMS)_2$ and Doxo, the polymerization was complete within 12 h, with 100% Doxo incorporation efficiency (Figure 2a, ii) and 100% LA conversion. After Doxo-LA_{100}, the Doxo-PLA conjugate prepared with a LA/Doxo ratio of 100, was treated with 0.1 M NaOH, 88−92% of Doxo was recovered in its original form (Figure 2a, iii). Doxo is known to be unstable in NaOH;[73] therefore, it is not surprising that Doxo cannot be recovered quantitatively. This study suggests that Doxo molecules were likely linked to PLA through its hydroxyl group(s) by forming ester linker(s) with PLA and were subject to base-induced rapid hydrolysis. To demonstrate that Doxo was conjugated to PLA, we prepared Doxo-LA_{10} using Doxo/(BDI)ZnN$(TMS)_2$-mediated LA polymerization at a LA/Doxo ratio of 10 and analyzed its end group using MALDI-TOF MS. The MS analysis clearly showed that Doxo was covalently conjugated to PLA (Figure 3).

To evaluate whether Doxo can be released from Doxo-PLA in physiological conditions in its original form, we incubated Doxo-LA_{10} in PBS solution at 30 °C for 10 days and then analyzed the solution using HPLC. As shown in Figure 2b-ii, a

(74) Meyer-Losic, F.; Quinonero, J.; Dubois, V.; Alluis, B.; Dechambre, M.; Michel, M.; Cailler, F.; Fernandez, A. M.; Trouet, A.; Kearsey, J. *J. Med. Chem.* **2006**, *49*, 6908–6916.

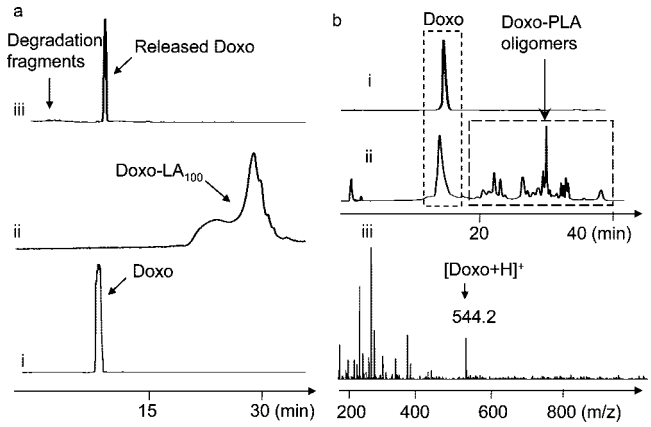
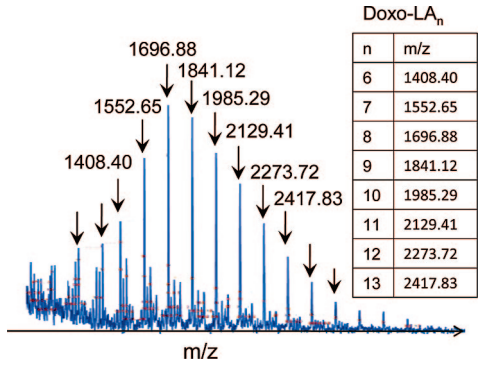


Figure 2. (a) RP-HPLC analysis of (i) Doxo, (ii) the polymerization solution of Doxo/(BDI)ZnN(TMS)$_2$ (1/3 molar ratio) mediated LA polymerization (LA/Doxo = 100), and (iii) Doxo-LA$_{100}$ treated with 1 N NaOH overnight followed by Doxo extraction with chloroform. HPLC analysis was mediated by an analytical pentafluorophenyl column (Curosil-PFP, 250 mm × 4.6 mm, 5 μ, Phenomenex, Torrance, CA) with acetonitrile/water (0.1% TFA) (50/50) at a flow rate of 1 mL/min. Note: the ill-defined peak of Doxo-LA$_{100}$ in ii is largely due to the use of the reverse-phase HPLC column that is not suitable for the separation of Doxo-LA$_{100}$. A substantially improved elution peak of Doxo-LA$_{100}$ was obtained when SEC columns were used for the analysis of this polymer−drug conjugate (see Figure 5a). (b) RP-HPLC analysis of (i) free Doxo incubated at 37 °C in 1 × PBS for 72 h and (ii) Doxo-LA$_{10}$ incubated in 1 × PBS at 37 °C for 10 days. (iii) High-resolution ESI (HR-ESI) MS analysis of the fraction in (Figure 2b-ii) that has the identical elution time as the authentic Doxo and was isolated using RP-HPLC. HPLC analysis and Doxo separation were mediated by an analytical RP-HPLC column (Luna C18, 250 mm × 4.6 mm, 5 μ, Phenomenex, Torrance, CA) with acetonitrile/water (0.1% TFA) (50/50) at a flow rate of 1 mL/min. MS (HR-ESI): calcd $C_{27}H_{30}NO_{11}$ $[M + H]^+$ m/z 544.1819, found m/z 544.1827.



Doxo-LA$_n$

n	m/z
6	1408.40
7	1552.65
8	1696.88
9	1841.12
10	1985.29
11	2129.41
12	2273.72
13	2417.83

Figure 3. MALDI-TOF MS analysis of Doxo-LA$_{10}$. The obtained m/z is identical to the calculated m/z of Doxo-LA$_n$ (543.52 + 144.13 × n). M_w = 2.0 × 10^3 g/mol, M_n = 1.7 × 10^3 g/mol, PDI = 1.17 (determined by MALDI-TOF MS).

peak with an elution time identical to that of the original Doxo was observed, along with other peaks that were presumed to represent the Doxo-PLA oligomers. The fragment with the same elution time as Doxo was isolated and confirmed to be Doxo by high resolution ESI-MS (Figure 2b-iii) and UV spectrometry (Supporting Information, Figure S2).

2.3.2. Effects of Metals and Ligands on Regioselective Conjugation of Doxo to PLA. Doxo has three hydroxyl groups at its C4′, C9 and C14 positions. Theoretically, LA polymerization can be initiated by any or all of these hydroxyl groups of Doxo. In the latter case, a lack of control over regioselective initiation will result in Doxo-PLA conjugates with heterogeneous structures. We next studied whether the initiation could be specifically controlled at one of the three hydroxyl groups of Doxo. Previous studies have indicated that the C14−OH of Doxo is most sterically accessible.[73] Due to the steric bulk of BDI, the catalyst likely forms an alkoxide complex with Doxo preferentially with its C14−OH, rather than with the more sterically hindered C4′− or the most sterically hindered C9−OH, to facilitate regioselective initiation and polymerization.

To evaluate the initiation regioselectivity, we mixed Doxo/(BDI)ZnN(TMS)$_2$ with succinic anhydride (SA) to mimic the initiation step of the Doxo/(BDI)ZnN(TMS)$_2$-mediated LA polymerization and characterized the reaction mixture by MS (Figure 4a) and NMR (Supporting Information, Figure S3). As expected, Doxo-14-succinic ester (Doxo-SE) was the predominate product (Figure 4a and Supporting Information, Figure S3). The chemical signal of the C14−H (peak g, Supporting Information, Figure S3b and S3c) was shifted downfield by 0.69 ppm (from 4.59 to 5.28 ppm), while the peaks corresponding to the C4′−H and C3′−H remained nearly unchanged (peaks i and e, respectively, Supporting Information, Figure S3b and S3c). It was thus evident that the SA ring was opened by the C14−OH of Doxo rather than by the C4′−OH or C3′−NH_2 of Doxo. C9−OH is the most sterically hindered and is thus unlikely to initiate polymerization. When (BDI)ZnN(TMS)$_2$ was replaced by (BDI′)ZnN(TMS)$_2$, a catalyst with a structure similar to that of (BDI)ZnN(TMS)$_2$ but having a less bulky diimine ligand (Figure 4b), the formation of Doxo-4′,14-bis-succinic ester (Doxo-2SE) was identified in conjunction with Doxo-SE (Figure 4b). When (BDI)ZnN(TMS)$_2$ was replaced by Zn(N(TMS)$_2$)$_2$, a Zn catalyst without ligands, the initiation regioselectivity completely disappeared. Doxo-4′,14-bis-succinic ester (Doxo-2SE) and Doxo-4′,9,4-trisuccinic ester (Doxo-3SE) were the predominant products (Figure 4c). Interestingly, the metal activity also had a profound effect on regioselectivity. When (BDI)MgN(TMS)$_2$ (which is more active than its Zn analogue) was used in a similar reaction, the formation of Doxo-2SE was also identified in conjunction with Doxo-SE (Figure 4d). Thus, by rationally designing ROP metal catalysts, Doxo-PLA conjugates with highly controlled regio- and chemoselectivity are achievable within one step without the need to protect the C3′−NH_2 and other competing hydroxyl groups of Doxo.

2.3.3. Metal and Ligand Effects on Doxo-PLA Molecular Weight. We next studied Doxo-initiated LA polymerization in the presence of these metal catalysts. Doxo/(BDI)ZnN(TMS)$_2$-mediated LA polymerization resulted in Doxo-PLA with low polydispersities (M_w/M_n less than 1.2) and the expected molecular weights (MWs) by adjusting the LA/Doxo feeding ratios (Table 1 and Figure 5a). For example, Doxo/(BDI)ZnN(TMS)$_2$-mediated LA polymerization at a M/I ratio of 200 resulted in Doxo-LA$_{200}$ with a polydispersity as low as 1.02 (Table 1). The obtained M_n of Doxo-LA$_{200}$ (3.38 × 10^4 g/mol) was close to the expected M_n (2.93 × 10^4 g/mol). A similar Doxo/(BDI)MgN(TMS)$_2$-mediated polymerization also produced Doxo-LA$_{200}$ with well-controlled MW (M_n = 3.12 × 10^4 g/mol). Nonetheless, a much higher polydispersity (M_w/M_n = 1.50) was observed, which was attributed presumably to the poorly controlled regioselectivity during initiation (Figure 4d), slow initiation relative to chain propagation and potential transesterification side reactions.[61,65] In general, Zn-alkoxides undergo slightly slower but better controlled polymerization reactions, as compared with their Mg analogues (Figure 5a,b).[61,75] (BDI′)ZnN(TMS)$_2$, the Zn catalyst with a less bulky

(75) Chisholm, M. H.; Gallucci, J.; Phomphrai, K. *Inorg. Chem.* **2002**, *41*, 2785–2794.

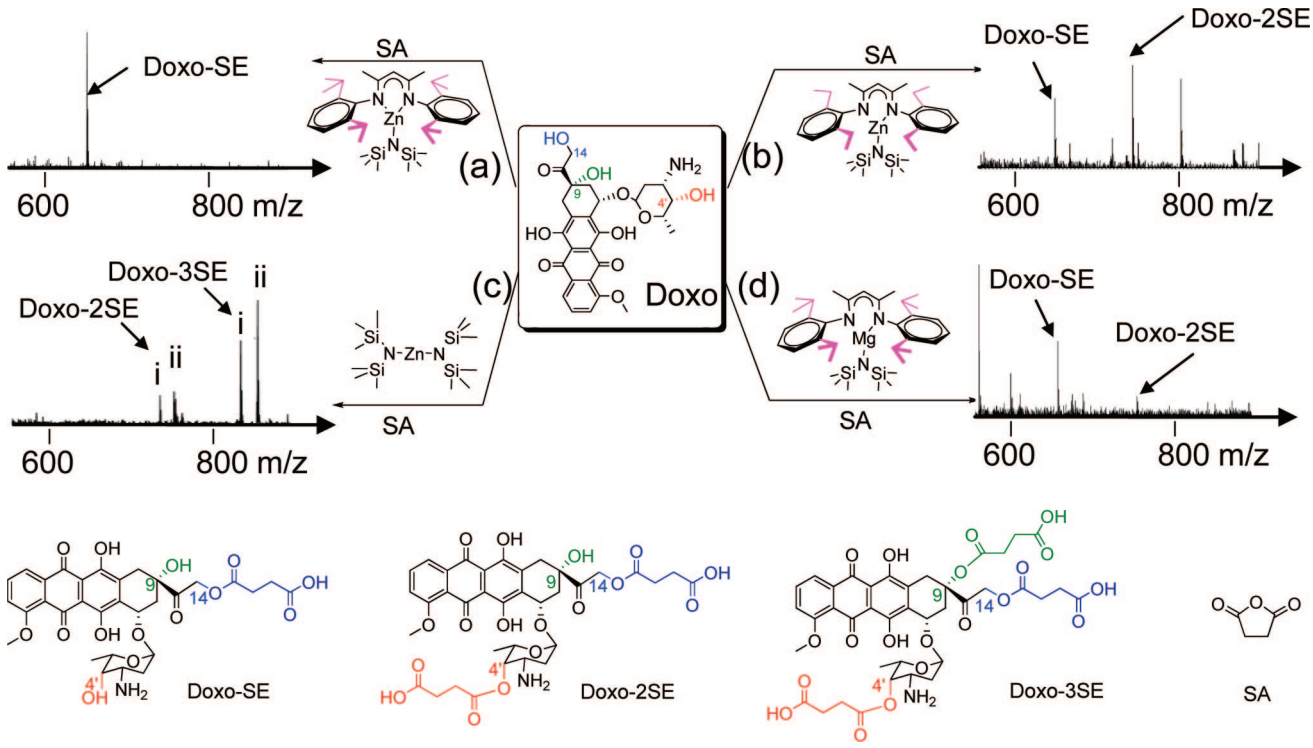


Figure 4. MS analysis of the mixture of (a) Doxo, 1 equiv (BDI)ZnN(TMS)$_2$ and 3 equiv SA, (b) Doxo, 1 equiv (BDI′)ZnN(TMS)$_2$ and 3 equiv SA, (c) Doxo, 1 equiv Zn(N(TMS)$_2$)$_2$ and 3 equiv SA, and (d) Doxo, 1 equiv (BDI)MgN(TMS)$_2$ and 3 equiv SA. Peak assignment: (a) Doxo-SE: ($M_{Doxo\text{-}SE}$ + H) 644.3; (b) Doxo-SE: ($M_{Doxo\text{-}SE}$ + H) 644.3; Doxo-2SE: ($M_{Doxo\text{-}2SE}$ + H) 744.2; (c) Doxo-2SE: (i = ($M_{Doxo\text{-}2SE}$ + H) 744.3, ii = ($M_{Doxo\text{-}2SE}$ + NH_4) 759.7; Doxo-3SE: (i = ($M_{Doxo\text{-}3SE}$ + H) 842.3, ii = ($M_{Doxo\text{-}3SE}$ + Na) 863.3); (d) Doxo-SE: ($M_{Doxo\text{-}SE}$ + H) 644.3; Doxo-2SE: ($M_{Doxo\text{-}2SE}$ + H) 744.3.

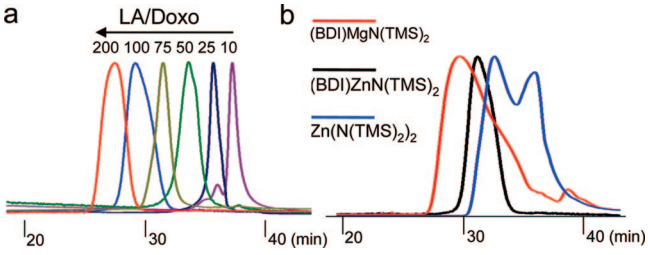


Figure 5. (a) SEC (equipped with a UV detector) analysis of Doxo-LA_n (n = LA/Doxo (M/I) prepared through Doxo/(BDI)ZnN(TMS)$_2$-mediated LA polymerizations at LA/Doxo ratios of 10, 25, 50, 75, 100 and 200, respectively; Note: Polymerizations at LA/Doxo ratio of 25, 50, 75, 100 and 200 resulted in corresponding Doxo-PLAs with monomodal SEC distribution patterns. Polymerization at a LA/Doxo ratio of 10, however, resulted in Doxo-LA_{10} with a multimodal SEC distribution pattern, which indicated that polymerization at such a low M/I ratio may not be as well-controlled as polymerization at higher M/I ratios. (b) SEC (equipped with a UV detector) analysis of the Doxo-LA_{100} prepared through Doxo/(BDI)ZnN(TMS)$_2$, Doxo/(BDI)MgN(TMS)$_2$, and Doxo/Zn(N(TMS)$_2$)$_2$ mediated LA polymerizations at a LA/Doxo ratio of 100.

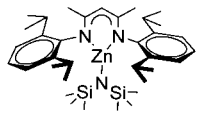
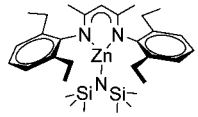
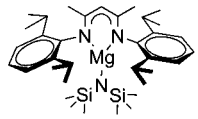
diimine ligand, also resulted in Doxo-LA_{200} with relatively high polydispersity (Table 1). These observations were in good agreement with the observed initiation regioselectivity of the corresponding Doxo/catalyst complexes for the ring-openings of SA (Figure 4).

2.4. Doxo-PLA Conjugated Nanoparticle (Nanoconjugate). Due to the excellent control over the Doxo-PLA structure and composition, NPs derived from these materials have well-controlled formulation parameters, which could potentially impact their *in vivo* performance and clinical translation. Doxo-PLA conjugated nanoparticles, termed nanoconjugates (NCs) in this paper to differentiate them from NPs prepared from the coprecipitation of drugs and polymers, were readily prepared through the nanoprecipitation of Doxo-PLA conjugates (Table 1e). NCs less than 150 nm in size with narrow, monomodal particle distributions were readily obtained (Table 2 and Figure 6), similar to the results that we reported previously for paclitaxel-PLA NCs.[65] The narrow size distributions of the NCs were in sharp contrast to the multimodal particle distributions frequently observed in conventional NPs prepared by coprecipitating polymers and drugs.[46,76] It is not clear why NCs derived from nanoprecipitation have such narrow size distributions. The multimodal particle distributions in conventional NPs have been attributed in part to the self-aggregation of non-encapsulated drugs,[46] and thus, the unimolecular structure of polymer−drug conjugates with reduced heterogeneities (low polymer polydispersities, the controlled site of conjugation on both polymer and Doxo and the absence of free Doxo) may contribute in part to the formation of NCs with low particle polydispersities (Table 2).

The surface compositions of Doxo-PLA NCs are unclear. Because the daunosamine sugar moiety of Doxo is hydrophilic, it is likely that Doxo molecules reside on or close to the surface of Doxo-PLA NCs and are subject to rapid release after the ester linkers between Doxo and PLA are hydrolyzed. The size of Doxo-PLA NCs can be fine-tuned within a range of 50−150 nm by adjusting the Doxo-PLA concentration in water-miscible organic solvents or by varying the types of water-miscible solvents present during nanoprecipitation. For instance, NCs prepared using DMF as solvent are typically 20−30 nm smaller than those prepared using THF or acetone as solvent, following the same trend that we observed previously in our study of docetaxel/poly(lactide-co-glycolide) nanoprecipitation.[46] We will

(76) Farokhzad, O. C.; Cheng, J.; Teply, B. A.; Sherifi, I.; Jon, S.; Kantoff, P. W.; Richie, J. P.; Langer, R. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 6315–6320.

Table 1. Polymerization of LA Initiated by the Complex of Doxo with $(BDI)ZnN(TMS)_2$, $(BDI')ZnN(TMS)_2$, or $(BDI)MgN(TMS)_2$

Catalyst	LA/Doxo (M/I)	*Expexted* M_n (x 10^3 g/mol)	*Obtained* M_n (x 10^3 g/mol)	*Obtained* M_w (x 10^3 g/mol)	M_w/M_n
 $(BDI)Zn(TMS)_2$	10[a]	2.0	1.7	2.0	1.17
	25	4.1	ND	ND	ND
	50[b]	7.7	6.3	7.4	1.18
	75[b]	11.3	12.7	13.6	1.07
	100[b]	14.9	18.3	19.8	1.08
	200[b]	29.3	33.8	34.5	1.02
 $(BDI')ZnN(TMS)_2$	200[b]	29.3	25.4	33.8	1.33
 $(BDI)MgN(TMS)_2$	200[b]	29.3	31.2	46.8	1.50

[a] MW determined by MALDI-TOF MS (see Figure 3). [b] MW determined by SEC (See Figure 5a). Note: The MW of Doxo-LA_{25} was too high to be analyzed by MALDI-TOF MS and too low to be determined by SEC equipped with a multiangle static light scattering detector. However, the SEC distribution pattern of Doxo-LA_{25} collected on a UV detector was monomodal and very similar as those of Doxo-LA_{50-200}. Therefore, it is very likely that the Doxo-LA_{25} also had the expected M_n and low polydispersity. ND = Not Determined.

Table 2. Doxo/$(BDI)ZnN(TMS)_2$-Initiated LA Polymerization followed by Nanoprecipitation to form NC[a]

M/I	LD (%)	CV (%)	IE (%)	NC[b]	NC size ± SD (nm)	PD ± SD
100	3.6	>99	>99	Doxo-LA_{100}	79.1 ± 0.7	0.08 ± 0.01
50	7.1	>99	>99	Doxo-LA_{50}	101.6 ± 0.7	0.07 ± 0.01
25	13.1	>99	98	Doxo-LA_{25}	90.8 ± 0.9	0.09 ± 0.01
10[c]	27.4	>99	94	Doxo-LA_{10}	125.2 ± 2.3	0.11 ± 0.01

[a] Abbreviations: M/I = LA/Doxo ratio; LD = Doxo loading in wt %; CV = conversion of LA; IE = incorporation efficiency of Doxo; SD = standard deviation; PD = polydispersity of NC. [b] NCs are denoted by Doxo-LAM/I ratio. [c] Prepared with (BDI)MgN(TMS)2.

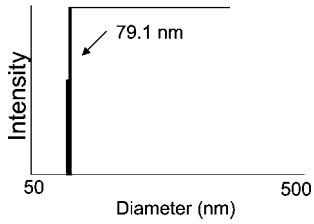


Figure 6. Analysis of Doxo-LA_{100} NC using dynamic light scattering. Doxo-LA_{100} was prepared using $(BDI)ZnN(TMS)_2$ by following the standard procedure as described in the Experimental Section.

report a comprehensive study of NC formulation, in particular for the control of NC sizes and formulation of NCs in solid form, in a separate paper.

Because both the monomer conversion and drug incorporation were nearly quantitative (Table 2), Doxo loadings in Doxo-PLA NCs could thus be predetermined by adjusting the LA/Doxo feeding ratios. At a low M/I ratio of 10, the drug loading was as high as 27.4% (Doxo-LA_{10}, Table 2). To our knowledge, this is by far the highest loading ever reported in Doxo-containing polymeric NPs. Even at this high drug loading, sustained release of Doxo from Doxo-LA_{10} NC was observed through the hydrolysis of the ester linker connecting the Doxo

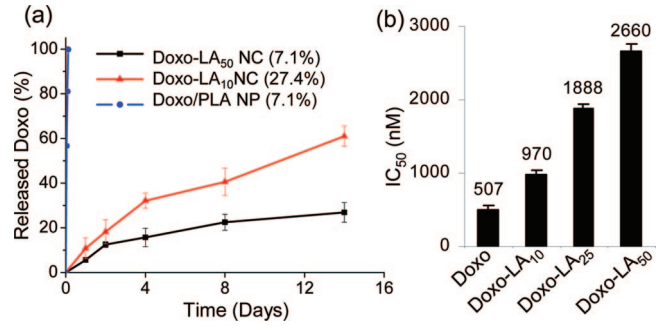


Figure 7. (a) Release of Doxo from Doxo-LA_{50} NC, Doxo-LA_{10} NC and Doxo/PLA NP ((Doxo/PLA (wt/wt = 1/13), ~7.1 wt % theoretical drug loading) prepared by coprecipitation of Doxo and PLA (MW = 15,000 g/mol) at 37 °C in 1 × PBS. Doxo-LA_{10} and Doxo-LA_{50} were prepared using $(BDI)ZnN(TMS)_2$ by following the standard procedure as described in the Experimental Section. (b) MTT assay for the analysis of cytotoxicity of Doxo-PLA NCs in PC-3 cells.

and the PLA (Figure 7a). No burst release of Doxo was observed in Doxo-LA_{10}. This observation was in sharp contrast to the burst release of PLA/Doxo NP prepared by coprecipitation (Figure 7a), in which Doxo release depended entirely on diffusion and over 90% was released within 3 h. The HPLC elution time and the MS spectrum of the Doxo released in PBS were identical to those of authentic Doxo, similar to the results we observed in the separate study mentioned above (Figure 2b). The MTT studies revealed that the toxicity of Doxo-PLA NC, which is directly related to the release kinetics of Doxo, could be tuned by adjusting the Doxo loading in the NCs (Figure 7b). In general, NCs with higher Doxo loadings released Doxo more rapidly (Figure 7a) and, therefore, showed higher toxicities (Figure 7b). This observation is presumably due to the fact that the NCs derived from the nanoprecipitation of higher-loading (lower MW) Doxo-PLA conjugates have more loosely packed structures, as compared to NCs derived from the lower-loading

(higher MW) Doxo-PLA conjugates. Therefore, the ester linkers between Doxo and PLA in NCs with higher drug loadings were more accessible to the aqueous phase and were subject to faster hydrolysis.

Conventional polymeric NPs prepared via coprecipitation of polymers and drugs have several formulation challenges that remain to be addressed.[77] NPs typically exhibit a "burst" drug release in aqueous solution; as much as 80−90% of the encapsulated drugs are rapidly released during the first few to tens of hours.[78] The rapid dose dumping may cause severe systemic toxicities.[32] In addition, drug loadings in conventional NPs can be very low, typically in a range of 1−5% in most NPs studied.[78−80] Drug loading of a delivery vehicle has been a critical measure of its utility in the clinic.[81,82] At lower drug loadings, a larger number of delivery vehicles are needed. Due to the limited body weight and blood volume of animals, the administered volumes are usually fixed. For instance, the volume of a solution intravenously administered to mice with 20- to 30-g body weights should be controlled to below 100 μL.[40] Intravenous administration of NPs with 1 wt % drug loading in a 100-μL solution at a dose of 50 mg/kg to a nude mouse with 20-g body weight requires the formulation of a concentrated, 1 g/mL NP solution, which is too viscous to formulate and inject intravenously. The third major challenge presented by NPs prepared via the encapsulation approach is the lack of a general strategy to achieve quantitative drug encapsulation. Depending on the amount of drug used, the hydrophobicity and hydrophilicity of the drug and the compatibility of the drug and polymer, the encapsulation efficiencies vary drastically over a range of 10−90%.[78,83] Unencapsulated drugs may self-aggregate[46] and can be very difficult to remove from the NPs. These formulation challenges significantly impact the processability and clinical translation of NP delivery vehicles prepared by drug−polymer coprecipitations.

Doxo has amine and multiple hydroxyl groups; it is therefore highly water soluble in its protonated form (10 mg/mL). Due to its high hydrophilicity, the encapsulation of Doxo in hydrophobic polymer can be very difficult. For instance, Yoo et al. reported a Doxo loading of 0.51% and a loading efficiency of 23% for micelles formed by coprecipitating Doxo and poly($_{DL}$-lactic-co-glycolic acid)-*b*-poly(ethylene glycol) (PLGA-PEG).[84] Doxo loadings of 0.6−8.7% and loading efficiencies of 11.4−43.6% were also reported by Hubbell and co-workers in their studies of encapsulating nonprotonated Doxo (which has increased hydrophobicity) into polymeric NPs using inverse emulsion polymerization.[83] The unencapsulated Doxo has to be removed from the NPs by column separation.[83] Burst releases of Doxo were reported in both systems.[83,84]

The Doxo-initiated ring-opening polymerization method that we have developed allows for the controlled incorporation of Doxo to PLA with drug loadings as high as 30% and up to 100% loading efficiency (Table 2). The Doxo-PLA NCs, derived from this Doxo-initiated ROP technique followed by nanoprecipitation, exhibit controlled release kinetics without any burst release effects and a precisely controlled Doxo-PLA structure, exactly as that depicted in Scheme 1d. This unique formulation technique allows for the formation of Doxo-containing PLA NCs with well-controlled formulation parameters.

2.5. Applicability of This Technique for the Conjugation of Other Hydroxyl-Containing Agents. To demonstrate that this technique can be broadly applied to the formation of NCs with agents that contain hydroxyl groups, we tested the capabilities of Cyanine 5 (Cy5), camptothecin (CPT), docetaxel (Dtxl) and cyclopamine (Cpa) to initiate LA polymerization in the presence of Zn or Mg catalysts. All of the therapeutic (Dtxl, CPT, Cpa) and dye (Cy5) molecules tested, when mixed with (BDI)ZnN(TMS)$_2$, initiated LA polymerization similar to that observed with Doxo, with nearly quantitative incorporation efficiencies and 100% LA conversions (Table 3). NCs with adjustable loadings (achieved by controlling the LA/drug (or dye) ratio) and low polydispersities were readily obtained (Table 3). Surprisingly, oligopeptides can even be used as initiators in Zn-catalyst-mediated LA polymerization. Goserelin (Gos), a luteinizing hormone-releasing hormone agonist,[85] initiated the successful ROP of LA in DMF. The resulting Gos-PLA conjugates and corresponding NCs could be prepared with extremely high drug loadings (≈50%) at low LA/Gos ratios and very low polydispersities (Table 3). Gos was recovered quantitatively through base treatment of Gos-PLA conjugates (Supporting Information, Figure S4). Comprehensive studies of the LA polymerization initiated by these agents and the resulting NCs (size, release kinetics, efficacy, etc.) are underway.

3. Conclusion

Preparations of Doxo-polymer conjugates with controlled loadings and release profiles have been previously reported using conventional coupling chemistry.[17,54,55,62,86] In this paper, we report a unique conjugation method accomplished via Doxo-initiated ROPs. This ROP-mediated Doxo conjugation method allows for facile regio- and chemoselective incorporation of Doxo into PLA and allows for the formation of PLA-Doxo conjugates with low polydispersity, predetermined drug loadings (up to ≈30%) and quantitative loading efficiencies. The BDI-metal chelating complexes do not have deleterious effects on Doxo and can be easily removed by solvent extraction. Because both Zn and Mg ions are biocompatible (as key elements in our dietary mineral supplements), there should not be significant safety concerns regarding the use of these two metal catalysts for the ROP and formulation of NCs for potential clinical applications. The multigram scale of PLA conjugates can be readily prepared within hours using this one-pot polymerization approach. Because drug molecules are covalently conjugated to PLA, the postreaction formulation process (precipitation, removal of catalysts, nanoprecipitation, sterilization, lyophilization, shipping and handling, etc.) can be much more readily handled with a minimum change of sample property, as compared to drug/polymer NPs prepared via encapsulation methods. This polymerization-mediated conjugation method may be utilized for the formulation of polymer−drug conjugates,

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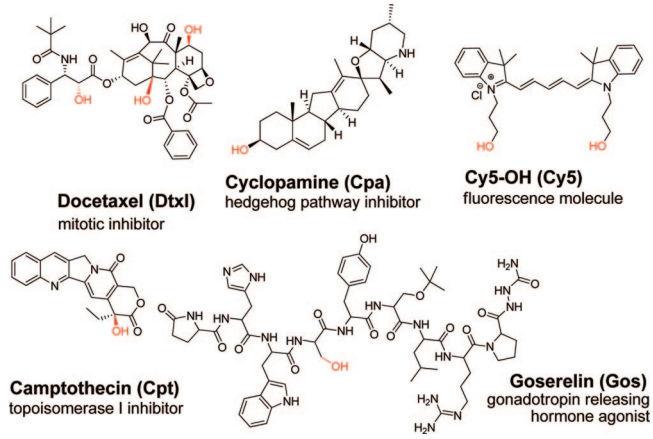
Table 3. Formulation of NCs with Hydroxyl-Containing Therapeutic and Dye Molecules



substrate	M/I	NCs[b]	loading g (%)	LA conv. (%)[c]	eff. (%)[d]	NC size ± SD (nm)[e]	PDI ± SD[e]
Dtxl	25	Dtxl-LA_{25}	18.3	>99	>99	64.5 ± 0.7	0.05 ± 0.02
Dtxl	10	Dtxl-LA_{10}	35.9	>99	97	77.9 ± 1.5	0.06 ± 0.02
Cpa	100	Cpa-LA_{100}	2.8	>99	>99	89.9 ± 1.8	0.04 ± 0.01
Cpa	50	Cpa-LA_{50}	5.4	>99	>99	78.0 ± 2.7	0.12 ± 0.01
Cy5	100	Cy5-LA_{100}	3.4	>99	98	95.1 ± 0.6	0.04 ± 0.02
Cy5	25	Cy5-LA_{25}	12.3	>99	95	76.3 ± 3.8	0.06 ± 0.01
CPT	100	CPT-LA_{100}	2.4	>99	>99	68.3 ± 0.8	0.08 ± 0.02
CPT	25	CPT-LA_{25}	8.8	>99	96	76.6 ± 0.9	0.09 ± 0.01
Gos	100	Gos-LA_{100}	8.1	>99	90	97.6 ± 2.3	0.05 ± 0.02
Gos	10	Gos-LA_{10}	46.8	>99	81	120.6 ± 2.7	0.01 ± 0.01

[a] Abbreviations: M/I = monomer/initiator ratio, NC = nanoconjugates, LA conv. = lactide conversion, incorp. eff. = incorporation efficiency, SD = standard deviation, PDI = polydispersity, Dtxl = docetaxel, Cpa = cyclopamine, Cy5 = cyanine 5, CPT = camptothecin, Gos = goserelin. [b] NCs are named as drug(or dye)-LA_n where n is the ratio of [LA] to [agent]. [c] Determined by analyzing unreacted lactide using FTIR (1772 cm^{-1}). [d] Based on RP-HPLC analysis of free molecules. We use incorporation efficiency instead of encapsulation efficiency because drug molecules are conjugated to, not encapsulated in, PLA. [e] Determined by dynamic light scattering.

not only for drug delivery but also for other controlled release applications (scaffolds, coatings of stents, etc.). Other cyclic esters (e.g., ε-caprolactone and β-valerolactone) are likely to replace LA and find use as monomers in such drug-initiated polymerizations.[87] The Doxo/(BDI)ZnN$(TMS)_2$-initiated polymerization of these cyclic esters at room temperature has recently been achieved in our laboratory, which will provide further tunability of the release profiles and other physicochemical properties. Given that the lack of a controlled formulation for nanoparticulate drug delivery vehicles is one of the bottlenecks to their clinical development, this unique, ROP-mediated conjugation methodology may contribute to the development of clinically applicable nanomedicines.

4. Experimental Section

4.1. General. $_{D,L}$-Lactide (LA) was purchased from TCI America (Portland, OR), recrystallized three times in toluene and stored at −30 °C in a glovebox prior to use. The BDI ligands and the corresponding metal catalysts ((BDI)MN$(TMS)_2$, M = Mg, Zn) were prepared by following the published procedures[61] and stored at −30 °C in a glovebox prior to use. All anhydrous solvents were purified by being passing through dry alumina columns and were kept anhydrous by using molecular sieves. Doxo·HCl was purchased from Bosche Scientific (New Brunswick, NJ) and used as received. Removal of HCl of Doxo·HCl was achieved by following the procedure reported in the literature.[73] Docetaxel (Dtxl) was purchased from LC Laboratories (Woburn, MA) and used as received. Cy5 was synthesized according to the published procedure.[88] All other chemicals were purchased from Sigma-Aldrich (St Louis, MO) and used as received, unless otherwise specified. The MWs of PLA and Doxo-PLA were determined by a size-exclusion chromatography instrument (SEC) equipped with an isocratic pump (model 1100, Agilent Technology, Santa Clara, CA), a DAWN HELEOS 18-angle laser light scattering detector (Wyatt Technology, Santa Barbara, CA) and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA). The wavelength of the HELEOS detector was set at 658 nm. The size exclusion columns used for the separation of PLA and Doxo-PLA conjugates were serially connected to a SEC (Phenogel columns 100 Å, 500 Å, 10^3 Å and 10^4 Å, 5 μm, 300 mm × 7.8 mm, Phenomenex, Torrance, CA) equipped with a 126P solvent module and a System Gold 128 UV detector (Beckman Coulter, Fullerton, CA). THF (HPLC grade) was used as the mobile phase of SEC with a 1-mL/min flow rate. The low-resolution electrospray ionization mass spectrometry (LR-ESI-MS) experiments were performed on a Waters Quattro II Mass Spectrometer. The high-resolution electrospray ionization mass spectrometry (HR-ESI MS) experiments were performed on a Micromass Q-TOF Ultima system. Matrix assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) spectra were collected on an Applied Biosystems Voyager-DE STR system. HPLC analyses were performed on a System Gold system (Beckman Coulter, Fullerton, CA) equipped with a 126P solvent module, a System Gold 128 UV detector and an analytical pentafluorophenyl column (Curosil-PFP, 250 mm × 4.6 mm, 5 μ, Phenomenex, Torrance, CA) or an analytical C18 column (Luna C18, 250 mm × 4.6 mm, 5 μ, Phenomenex, Torrance, CA). The UV wave-

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length for detecting Pyr-OH or Pyr-NH_2 was set at 300 nm. The UV wavelength for detecting Doxo was set at 450 or 500 nm, depending on the signal-to-noise ratio in corresponding studies. The NMR experiments were conducted on a Varian U500, a VXR500 or a UI500NB (500 MHz) NMR spectrometer. The sizes and particle polydispersities of the PLA-drug (dye) NCs were determined on a ZetaPlus Dynamic Light Scattering (DLS) detector (15-mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY). The PC-3 cells (ATCC, Manassas, VA) used in the MTT assay were cultured in Ham's F12K medium containing 10% fetal bovine serum, 1000 units/mL aqueous penicillin G and 100 μg/mL streptomycin.

4.2. 1-Pyrenemethylamine/(BDI)ZnN(TMS)$_2$ and 1-Pyrenemethylmethanol/(BDI)ZnN(TMS)$_2$-Mediated LA Polymerization. In a glovebox, 1-pyrenemethylamine (Pyr-NH_2) (2.3 mg, 10 μmol) was dissolved in chloroform (0.5 mL). The solution was mixed with a THF solution (0.5 mL) of (BDI)ZnN(TMS)$_2$ (6.3 mg, 0.01 mmol). A THF solution (400 μL) of LA (36 mg, 0.25 mmol) was added to a vigorously stirred mixture of Pyr-NH_2 and (BDI)ZnN(TMS)$_2$. The mixture was stirred for 3 h at room temperature. An aliquot of the reaction mixture (100 μL) was taken out of the glovebox for HPLC analysis (ii, Figure 1). Pyr-OH (2.1 mg, 0.009 mmol) in 300 μL of chloroform was added to the polymerization solution. The reaction solution was stirred for an additional 3 h and analyzed by HPLC (i, Figure 1).

4.3. Preparation of Doxo-LA$_{100}$ Conjugate. In a glovebox, Doxo (5.5 mg, 0.01 mmol) was dissolved in anhydrous THF (1 mL). (BDI)ZnN(TMS)$_2$ (18.3 mg, 0.03 mmol) was added to the Doxo solution. The mixture was stirred for 15−20 min at room temperature. LA (144 mg, 1.0 mmol) in DMF (1 mL) was added dropwise to a vigorously stirred mixture of Doxo and (BDI)ZnN(TMS)$_2$. The polymerization was monitored by following the lactone band at 1772 cm^{-1} using FTIR or by checking the methine (−C*H*−) peak of LA using ^{1}H NMR. After the polymerization was complete (usually within 12 h), an aliquot of the polymerization solution was injected to HPLC to quantify the unreacted Doxo, in order to determine the incorporation efficiency of Doxo to the Doxo-PLA conjugate. One drop of water was added to the polymerization solution to hydrolyze the Zn-Doxo alkoxide and thus terminate the polymerization. The resulting Doxo-LA$_{100}$ was precipitated with ethyl ether (10 mL), washed with ether and methanol/acetic acid (v/v = 100/1, 10 mL) to remove BDI ligand and metal catalyst and dried under vacuum. Complete removal of BDI was confirmed by NMR, HPLC and TLC. After the organic solvent was evaporated, the residue was dissolved in HPLC-grade THF (10 mg/mL) and analyzed by SEC to determine the MWs and polydispersities (Table 1, Figure 5b). Doxo-PLA conjugates prepared at various M/I ratios were also analyzed by a SEC equipped with a UV detector (Figure 5a).

Dtxl-PLA, CPT-PLA, Cpa-PLA, Cy5-PLA and Gos-PLCA conjugates (Table 3) were prepared by following the same procedure described above for Doxo-PLA, with the corresponding drug (or dye) as the initiator of the LA polymerization. Conversions of LA and drug-loading efficiencies in those polymerizations were also assessed in a manner similar to those for the polymerization of LA by Doxo/(BDI)ZnN(TMS)$_2$, as mentioned above. These conjugates were subsequently used for nanoprecipitation to prepare corresponding NCs without further characterization.

4.4. Doxo/(BDI)ZnN(TMS)$_2$-Mediated Ring-Opening of Succinic Anhydride. In a glovebox, Doxo·HCl (2.0 mg, 0.0035 mmol) was mixed with (BDI)ZnN(TMS)$_2$ (2.4 mg, 0.0042 mmol, 1.2 equiv) ((BDI′)ZnN(TMS)$_2$ 1.2 equiv, Zn(N(TMS)$_2$)$_2$, 1.2 equiv or (BDI)MgN(TMS)$_2$ 1.2 equiv) in THF (200 μL) for 30 min, followed by removal of the solvent under vacuum. Freshly crystallized succinic anhydride (SA) (0.41 mg, 0.0042 mmol, 1.2 equiv) in THF (42 μL) was added dropwise to the mixture of Doxo·HCl and (BDI)ZnN(TMS)$_2$. The reaction mixture was diluted with 200 μL of THF and stirred for an additional 60−90 min. The solvent was removed under vacuum. The residue was then dissolved using a mixture of acetic acid and methanol (v/v = 1/1, 300 μL). An aliquot of this solution was used for ESI-MS analysis.

4.5. Formation, Characterization and Evaluation of Doxo-LA$_{100}$ Nanoconjugate. A Doxo-LA$_{100}$ conjugate in DMF (100 μL, 10 mg/mL) was added dropwise to nanopure water (2 mL). The resulting Doxo-LA$_{100}$ NC was collected by ultrafiltration (15 min, 3000*g*, Ultracel membrane with 10,000 NMWL, Millipore, Billerica, MA) and was used for the characterization of particle size by DLS, drug loading by HPLC and release kinetics by HPLC. The NCs of Dtxl-PLA, CPA-PLA, CPT-PLA, Cy5-PLA and Gos-PLA were similarly formulated and characterized for their sizes and drug loadings.

4.6. Determination of Release Kinetics. The Doxo/PLA NPs were prepared through nanoprecipitation of PLA (1.5×10^4 g/mol) and Doxo by following the procedure reported in the literature.[46] Doxo-LA$_{10}$ and Doxo-LA$_{50}$ NCs were prepared by following the standard nanoprecipitation procedure described above using Doxo-LA$_{10}$ and Doxo-LA$_{50}$ conjugates, respectively. The NPs and NCs were collected and washed three times with water by ultrafiltration, using a 10,000 MWCO Amicon Ultra membrane (Ultracel YM-10, Millipore Inc., Billerica, MA), in order to completely remove DMF. The NCs (or NPs) collected from the ultrafiltration device were dispersed in 5 mL 1 × PBS solution; the PBS solution containing NCs (or NPs) was then divided into equal portions, added to five separate eppendorf tubes (1 mL per tube) and incubated at 37 °C. At selected time intervals, the corresponding eppendorf tubes were taken out of the incubator and centrifuged at 4000 rpm for 5 min. The supernatant (500 μL) was carefully transferred, using a micropipette, to a separate eppendorf tube without disturbing the precipitated NCs (or NPs). The supernatant was injected into HPLC to quantify the released Doxo from PLA/Doxo NP, Doxo-LA$_{10}$ NC and Doxo-LA$_{50}$ NC. An analytical RP-HPLC column (Luna C18, 250 mm × 4.6 mm, 5 μ, Phenomenex, Torrance, CA) was used for the quantification of the released Doxo. The area of the released Doxo peak was integrated and compared to a standard curve. To confirm that Doxo was released in its original form from Doxo-PLA NCs, we collected the fraction that had an elution time identical to that of the authentic Doxo on HPLC and analyzed it with high-resolution ESI-MS. The MS (HR-ESI) was calculated for $C_{27}H_{30}NO_{11}$ $[M + H]^+$ to be *m*/*z* 544.1819, and we found *m*/*z* 544.1827.

4.7. Determination of the Cytotoxicity of Doxo-PLA NCs. PC-3 cells were plated in a 96-well plate for 24 h (10,000 cells per well) before the addition of NCs. The cells were washed with prewarmed PBS. Freshly prepared Doxo-LA$_{10}$, Doxo-LA$_{25}$ and Doxo-LA$_{50}$ NCs (prepared in 1 × PBS, 100 μL) were added to the cells. The cells were incubated for 72 h in a 5% CO_2 incubator at 37 °C. Doxo was used as a positive control. Untreated cells were used as a negative control. After incubation for 72 h, the medium was removed from the cells. Standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay protocols were followed thereafter.[89]

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Supporting Information Available: Confirmation of the stability of Doxo in the presence of (BDI)ZnN(TMS)$_2$, confirmation of the release of Doxo from Doxo-PLA NC by UV spectroscopy, confirmation of Doxo-SE by NMR and MS, and confirmation of conjugation of Gos to PLA via Gos/(BDI)MgN(TMS)$_2$-mediated LA polymerization and confirmation of the release of Gos from Gos-PLA. This material is available free of charge via the Internet at http://pubs.acs.org.

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