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Conjugation Chemistry through Acetals toward a Dextran-Based Delivery System for Controlled Release of siRNA

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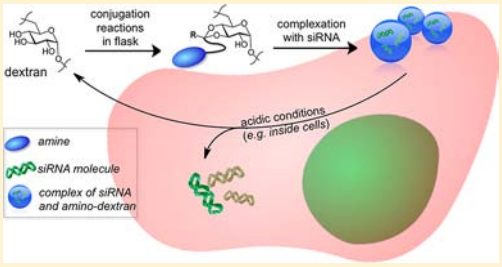
Supporting Information

ABSTRACT: New conjugation chemistry for polysaccharides, exemplified by dextran, was developed to enable the attachment of therapeutic or other functional moieties to the polysaccharide through cleavable acetal linkages. The acid-lability of the acetal groups allows the release of therapeutics under acidic conditions, such as that of the endocytic compartments of cells, regenerating the original free polysaccharide in the end. The physical and chemical behavior of these acetal groups can be adjusted by modifying their stereoelectronic and steric properties, thereby providing materials with tunable degradation and release rates. We have applied this conjugation chemistry in the development of water-soluble siRNA carriers, namely acetal-linked amino-dextrans, with various amine structures attached through either slow- or fast-degrading acetal linker. The carriers with the best combination of amine moieties and structural composition of acetals showed high *in vitro* transfection efficiency and low cytotoxicity in the delivery of siRNA.



INTRODUCTION

Although theoretically able to knock down all diseased genes, RNAi therapy has limited application in clinical use.[1] One of the major barriers is the lack of suitable systemic carriers that can facilitate the effective delivery of RNAi inducing molecules, such as plasmid DNA and small interfering RNA (siRNA).[2] The development of ideal systemic delivery systems for RNAi must address many criteria, including the biocompatibility of the carrier, sufficient protection of the genetic cargo as it travels to its target site, minimization of opsonization by reticuloendothelial system (RES), ability to distinguish diseased and healthy tissue, suitable carrier size to avoid rapid kidney filtration, efficiency in cellular uptake, and release of the negatively charged genetic materials from the carrier.

A large number of siRNA carriers have been explored, including viral vectors, cationic lipids, and polymers.[3] Viral vectors have generally been quite effective in transfection, but safety concerns have limited their clinical use due to possible mutagenicity, oncogenesis, and the potential for unwanted immune responses.[4] Therefore, alternative efforts have been directed toward the use of cationic lipids and polymers, which can trap genetic materials via electrostatic interactions.[5] Natural polymers, such as polysaccharides, are potentially attractive as delivery vehicles due to their biodegradability, low toxicity, and low immunogenicity.[6] One prominent example is chitosan, which has been used successfully to deliver DNA and has demonstrated sufficient transfection and low toxicity.[7] However the effectiveness of chitosan in the delivery of siRNA proved to be limited with transfection efficiency significantly lower than those of polyethylenimine (PEI) or lipofectamine.[8] Chitosan modified through the introduction of secondary and tertiary amines showed enhanced transfection efficiency, but the lack of degradability of the amide-modified material could prove to be a liability.[9] Other polysaccharides, such as dextran, starch, cellulose, pullulan, alginate, and hyaluronan, have been explored as drug conjugates for systemic and oral administration to increase plasma half-life and tumor accumulation. However, the conjugation methods used to date have mainly involved ester, amide, carbonate, and carbamate linkages with frequently slow release of therapeutics *in vivo*.[10]

Acetals are commonly used as temporary protecting groups for alcohols in organic synthesis.[11] The acid-lability of acetals has made them attractive for the release of therapeutic cargoes under acidic conditions, which exist at various diseased sites such as tumor and inflammation, as well as inside endocytic compartments.[12] We have previously reported an acetalated dextran (Ac-DEX) material, which was able to encapsulate therapeutics via physical entrapment during fabrication of nanoparticles and release the cargo once internalized by cells.[13] Such a system has been proven to be efficient in successful delivery of protein antigens, vaccine adjuvants, and genetic materials.

Here we describe a facile conjugation method for the functionalization of polysaccharides via acetals (Figure 1). Functional handles can be introduced to either ketones or aldehydes, which are then activated as dimethyl acetals for reaction with the hydroxyl groups of polysaccharides.

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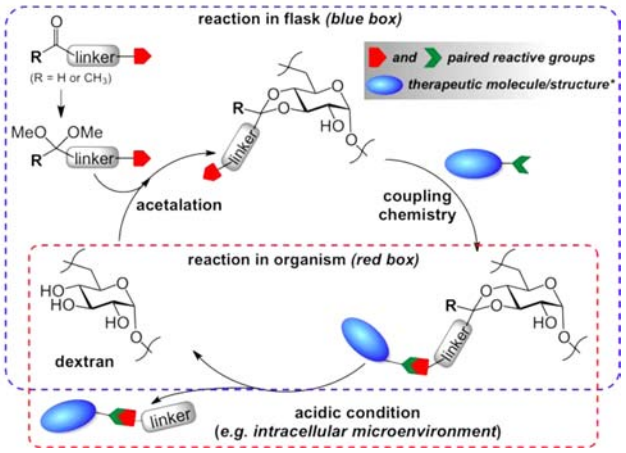

Figure 1. General scheme for the conjugation chemistry through acetals. Polysaccharide can be acetalated with a functional linker, which can be conjugated to therapeutic molecules or functional structures through appropriate reactions. Once endocytosed by cells, the acetal linker can be cleaved under acidic conditions to release the therapeutic cargo, while regenerating the free polysaccharide. *Note: the blue oval represents amine structure, which can complex with genetic material (the complexation step is not shown in the figure).

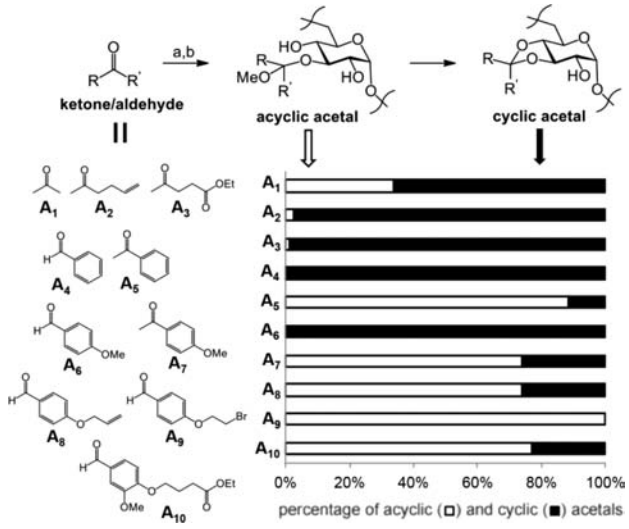

Figure 2. Formation of acetals on dextran leading to varying ratios of cyclic to acyclic acetals, depending on the linker molecule ($\mathbf{A_1}$-$\mathbf{A_{10}}$). Reaction conditions: a. Anhydrous methanol, $CH(OMe)_3$, *p*-TSA, MS4 Å, reflux, 1 h; b. Anhydrous DMSO, dextran, *p*-TSA, MS5 Å, 50–80 °C, 2 h to overnight. Note: the attachment of a cyclic acetal group to a glucose moiety may be at each two adjacent hydroxyl groups (only a 3,4-*O*-cyclic acetal is shown in the scheme), and an acyclic acetal may be tethered to any hydroxyl group (only a 3-*O*-acetal is shown). Dimethyl acetal derivatives of $\mathbf{A_1}$-$\mathbf{A_{10}}$ are referred to as $\mathbf{A_1}'$-$\mathbf{A_{10}}'$.

Therapeutic molecule or functional structure bearing a properly matched reactive group can be introduced to the polysaccharide and then released under acidic conditions, regenerating the original natural polysaccharide in the end. In our study, we used dextran, an $\alpha(1,6)$-linked polymer of glucose, which is approved by the FDA as a plasma expander and shows an excellent biodistribution profile when administered systemically in a tumor model.[14] We applied our conjugation strategy in the development of siRNA delivery vehicles with controlled release rates and investigated its utility for gene knockdown studies in live cells.

RESULTS AND DISCUSSION

Chemical Synthesis and Degradation Studies. In organic synthesis, acetals are common protecting groups for adjacent hydroxyl groups, such as those in 1,2- (vicinal) or 1,3-diols. Modern carbohydrate synthesis has taken a great advantage of acetal protecting group strategies to form temporary blockages of specific diols to achieve selective modification of other hydroxyls. Usually acetalation of alcohols is accomplished by treatment with an aldehyde or ketone under slightly acidic conditions in anhydrous solvents. To develop a general method for polysaccharide modification via acetals, we started with commercially available aldehydes or ketones. However we found treatment of dextran with aldehyde or ketone under similar conditions could only form negligible amounts of acetals with nearly all free dextran recovered.

We then turned our attention to more reactive derivatives of aldehydes or ketones. Dimethyl acetals can be easily installed to aldehydes or ketones and are proven to undergo trans-acetalation with alcohols. The initial attempts involved the use of a couple of commercially available dimethyl acetalated aldehydes and ketones, 2,2-dimethoxypropane ($\mathbf{A_1}'$), and benzaldehyde dimethyl acetal ($\mathbf{A_{10}}'$) (Figure 2). Indeed, under anhydrous conditions, the transacetalation reaction could lead up to 0.8 acetal per glucose unit on dextran, suggesting the practicability of using acetalation as conjugation method. We then investigated the generalization of dimethyl acetal formation on various aldehydes and ketones ($\mathbf{A_1}$-$\mathbf{A_{10}}$), some of which contained functional handles that could be used for further modification (Figure 2). All tested aldehydes or ketones could form dimethyl acetals under standard conditions in high isolated yields (usually around 90%).

Due to their acid-lability and reasonable purity (usually over 80%), these dimethyl acetals, containing small amounts of aldehyde or ketone precursors, were not further purified and were used directly in reactions with dextran. The trans-acetalation of dextran with these dimethyl acetals ($\mathbf{A_1}'$-$\mathbf{A_{10}}'$) proved facile leading to the introduction of 0.4 to 0.8 acetals per glucose repeat unit on dextran (Figure 2 and SI). The use of ketone or aldehyde molecules containing other reactive groups such as esters, alkenes, or bromides provides access to the tethering of a variety of functional moieties onto dextran.

The acetalation of dextran generally proceeds with the rapid formation of acyclic acetal on one hydroxyl group; subsequent reaction with a vicinal hydroxyl then leads to a cyclic acetal (Figure 2). Interestingly, ketone derivatives (both aliphatic and aromatic) gave mostly cyclic acetals, while aldehyde derivatives predominantly generated acyclic acetals. The amount of acyclic and cyclic acetals were determined by ^{1}H NMR (SI). As previously observed,[13a] acyclic acetals have a shorter half-life in water (pH 5.0 or pH 7.4) than their cyclic analogs. Therefore, the release rate of the cargo can be fine-tuned by selecting ketones or aldehydes linkers with different peripheral groups to adjust the ratio of acyclic to cyclic acetals.

In gene delivery, release of the nucleic acid from its carrier is key to successful transfection. Most of the existing polymer-based cationic gene delivery systems are said to achieve endosomal escape via the "proton sponge effect". However how the release of nucleic acid from the carrier affects the transfection outcome is still the object of debate. The chemistry described herein is able to provide useful structure property

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relationship data as carriers differing only in amine structures or degradation rates can be compared.

In our study, we selected materials **1** and **2** (Figure 3). The two linkers ($\mathbf{A_3}$ and $\mathbf{A_{10}}$) both have an ester group that allows

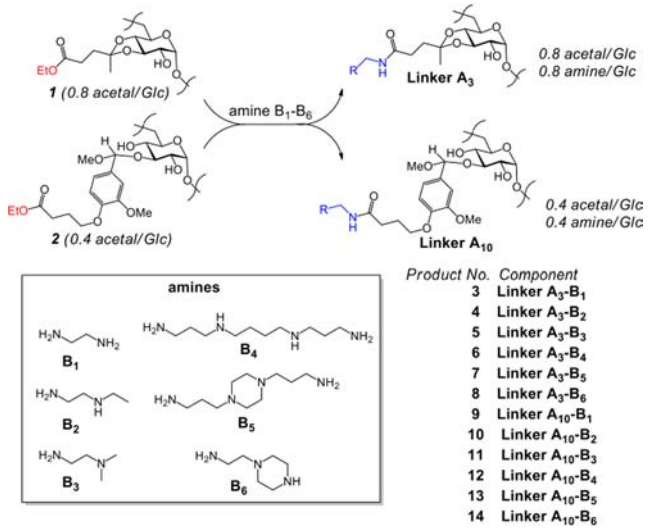

Figure 3. Synthesis of gene delivery carriers bearing linkers with tunable degradation rates (linker $\mathbf{A_3}$ and linker $\mathbf{A_{10}}$) and various amine functionalities ($\mathbf{B_1}$ to $\mathbf{B_6}$).

further one-step reaction with amines. The two linkers have different acyclic and cyclic acetal composition, which showed drastically different degradation rates. The levulinate cyclic acetal (linker $\mathbf{A_3}$) required days to degrade at both pH 5.0 and pH 7.4 at 37 °C (SI), while the half-life of the vanillin acyclic acetal (linker $\mathbf{A_{10}}$) was only 2 h at pH 5.0 vs 120 h at pH 7.4 (SI). These two features allow the generation of two series of acetal-linked amino-dextrans with primary, secondary, and tertiary amine functionalities and different degradability profile.

Amine modification of compounds **1** and **2** is straightforward with simple workup steps, which allows large scale and library synthesis of amine-modified materials. Replacement of ethyl ester by an amino group occurs upon heating, and the product can be isolated simply by precipitation in ethyl acetate. The starting materials **1** and **2** were water-insoluble, while the amine-modified products were water-soluble, indicating the replacement of ester groups by amino groups. Interestingly, the replacement of ethyl ester groups was only observed for primary amines, on the other hand no secondary or tertiary amines reacted with ethyl esters on dextran. This was confirmed by parallel reactions of compound **1** or **2** with an amine containing only secondary or only tertiary amine. Only starting material was obtained over the same time span as the other reactions (observed by ^{1}H NMR). Because multiple amino groups were present in each amine structure, cross-linking of the dextran materials was a concern. However, we observed little or no cross-linked material by ^{1}H NMR and size exclusion chromatography (SI). Importantly, the amount of acetals, both cyclic and acyclic, on dextran did not change obviously after amidation, suggesting relative stability of acetals under these reaction conditions.

Degradation studies of all materials were performed in pH-adjusted deuterated buffer (pH 5.0 and pH 7.4) using ^{1}H NMR (Figure 4). The progress of degradation was monitored by comparing the ratio of integration of signature functional groups in ^{1}H NMR (exemplified in Figure 5). For compounds **3** to **8**, there was no obvious degradation (no resonances from the small molecule) at both pH 5.0 and pH 7.4 (Figure 4, Figure 5a, and SI). In contrast, compounds **9** to **14** degraded and released the small molecule aldehydes at pH 5.0 starting from 30 min, and the quantity of this aldehyde increased remarkably as the incubation time proceeded (Figure 4, Figure 5b, and SI). These compounds reached nearly complete degradation after 48 h of study. However, at pH 7.4, compounds **9** to **14** did not show appreciable degradation

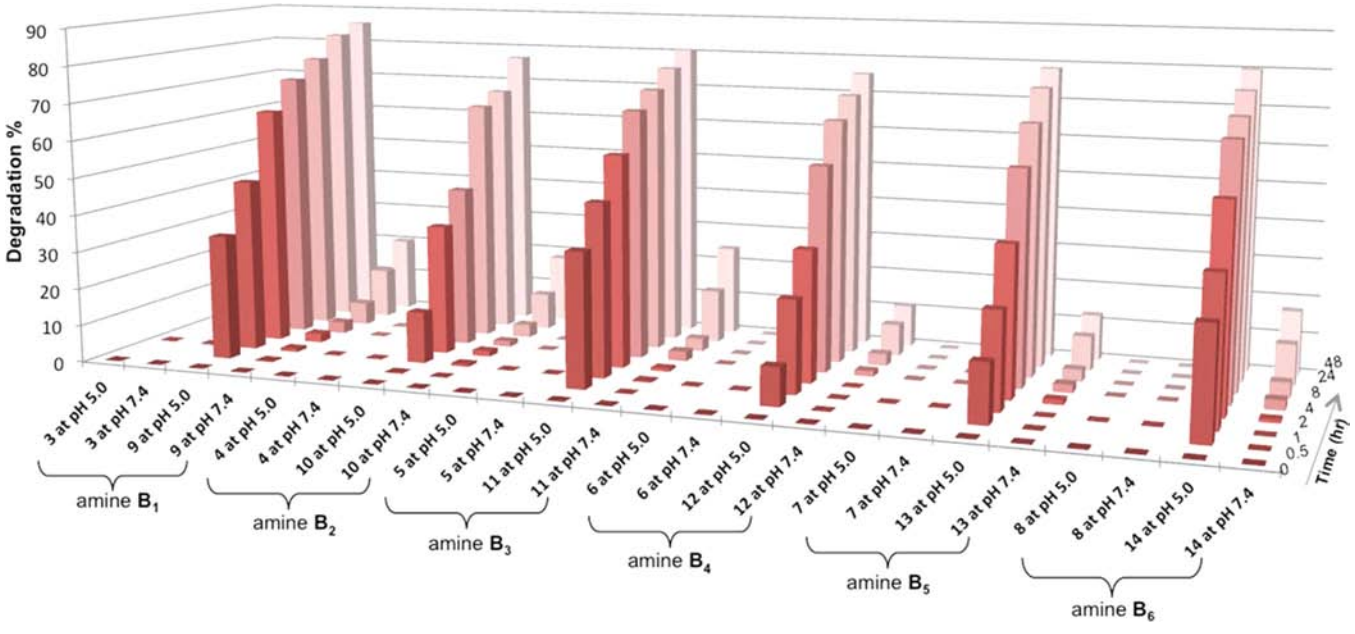

Figure 4. Degradation study of slow- (linker $\mathbf{A_3}$) and fast- (linker $\mathbf{A_{10}}$) degrading amine-dextrans **3** to **14** at 37 °C at pH 5.0 and pH 7.4 over a course of 48 h. Degree of degradation was calculated using integration of signature peaks in ^{1}H NMR and plotted against incubation time.

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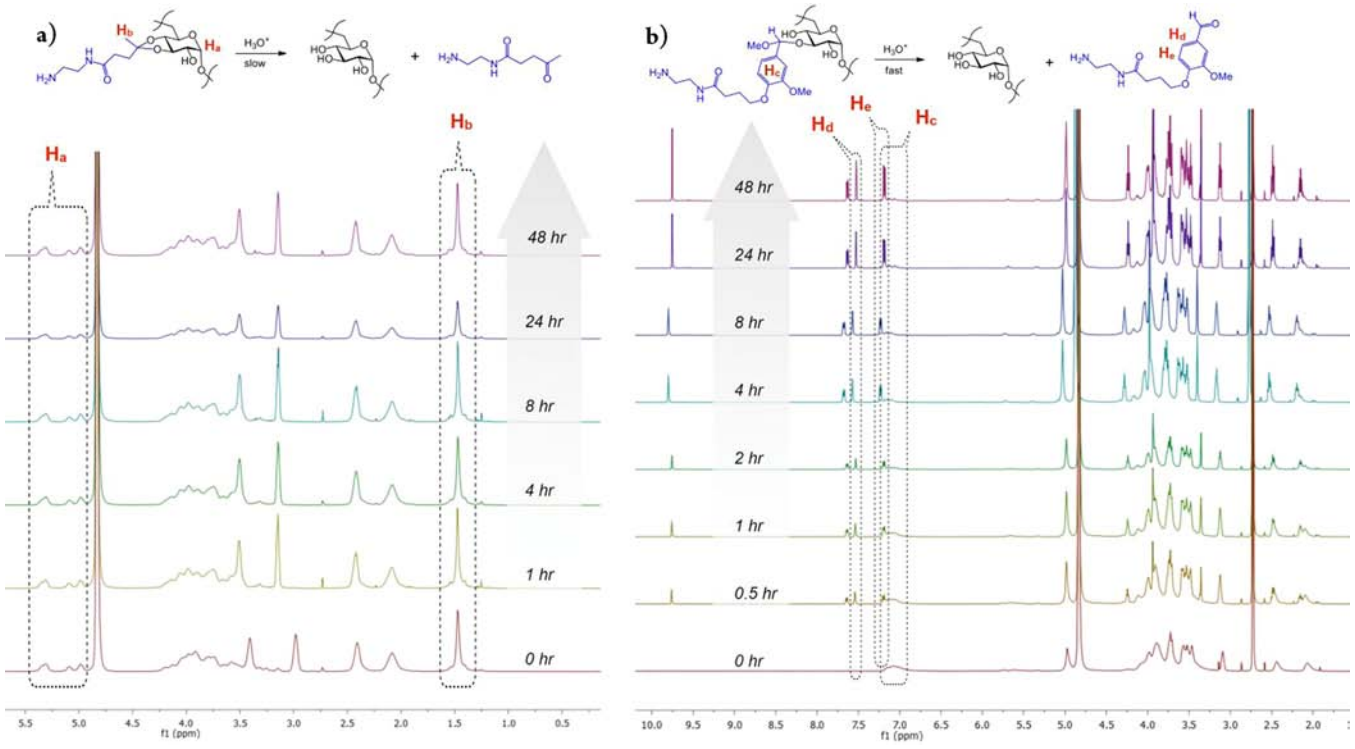

Figure 5. Representative arrays of ^{1}H NMR spectra obtained during degradation study of amino-dextran **3** (a) and **9** (b). Time labels indicate incubation time of each compound in deuterated pH 5.0 buffer at 37 °C. For compounds **3** to **8**, the degradation rates were calculated by comparing the ratio of the integrations of H_a (anomeric proton of glucose residue on dextran) and H_b (methyl group of the acetal). If the compound degrades completely, H_b will disappear. For compounds **9** to **14**, all aromatic protons on the polymer (H_c) appear as a broad peak around 7.1 ppm. When the material degrades, small molecules appear to give sharp resonance peaks. The degradation rate of compound **9** was calculated using the ratio of integration of H_c and H_d.

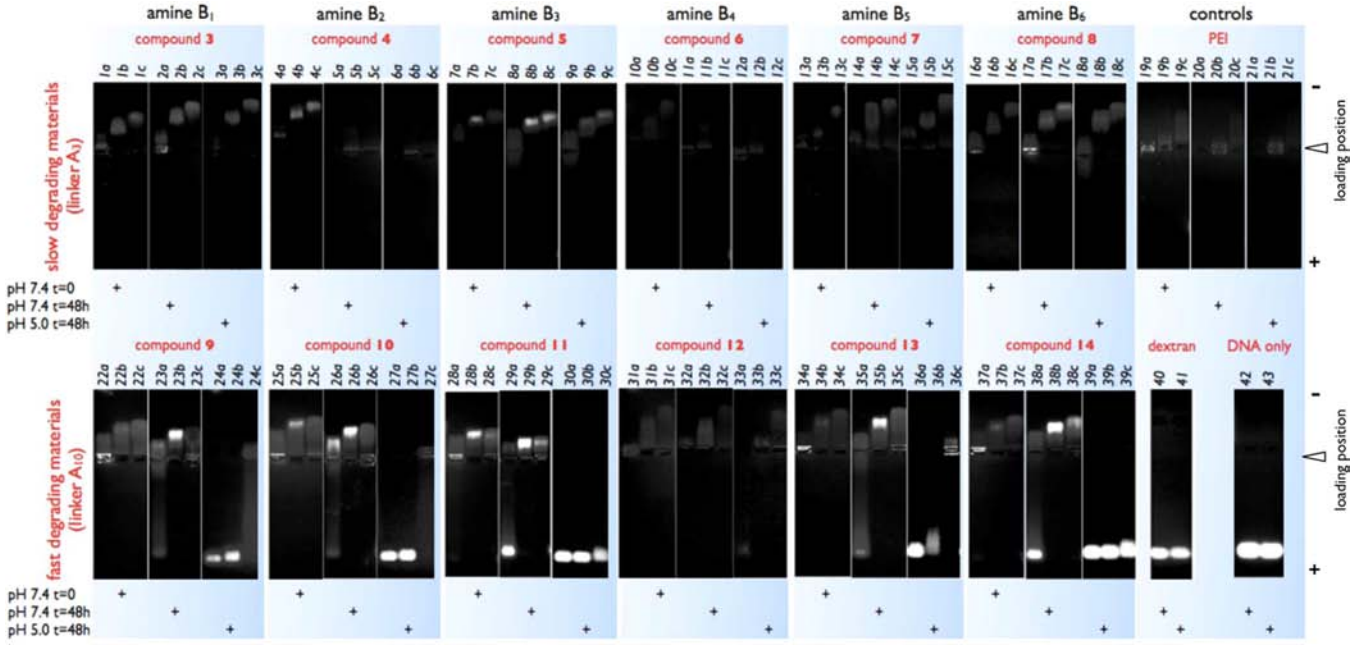

Figure 6. Gel electrophoresis to study the complexation of genetic material and amino-dextrans. Top row: slow-degrading materials (compound **3** to **8**); bottom row: fast-degrading materials (compound **9** to **14**). Compounds with the same amine structure are placed in the same blue column for easy comparison. Direction of the electrical field is labeled by negative (-) and positive (+) signs. Loading position for the complexes is indicated by an open arrow on the right. Lanes 1a, 2a, 3a, etc. are loaded with complexes of N/P = 1; Lanes 1b, 2b, 3b, etc. are loaded with complexes of N/P = 10; Lanes 1c, 2c, 3c, etc. are loaded with complexes of N/P = 100. Incubation condition of the complexes (pH 7.4 t = 0; pH 7.4 t = 48 h; pH 5.0 t = 48 h) is indicated below each panel or lane by "+". Each sample contains 5 μg firefly luciferase dsDNA. Gel was visualized by fluorescence of ethidium bromide under UV excitation (254 nm).

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until 4 h, and there was only a very minor amount of degraded products after 48 h.

As presented in Figure 4, compounds **3** to **8** and compounds **9** to **14** showed similar degradation profile as their precursor compounds **1** and **2** respectively, suggesting the degradation was caused dominantly by the linker used ($\mathbf{A}_3$ and $\mathbf{A}_{10}$).

Complexation of Nucleic Acid to Amino-Dextrans and Its Release at Different pH's. Successful gene delivery requires efficient binding of the cargo to the carrier during transportation and the capability to release the cargo at the optimal time. Therefore, we examined the complexation between genetic materials and our various amino-dextrans (**3** to **14**) using gel electrophoresis. We used a short firefly luciferase dsDNA (21-bp) as model material in this study. We selected hyperbranched PEI as a positive control, as it has been used as a gene delivery carrier and is known to form tight polyplexes with siRNA. All amino-dextrans (**3** to **14**) formed firm complexes with dsDNA at nitrogen/phosphate (N/P)[15] ratios of 1, 10, and 100 when compared to PEI-DNA complexes (Figure 6, pH 7.4 at time 0). Weaker fluorescence of ethidium bromide from all complexes was observed than that of free DNA. Since the same amount of dsDNA was applied in all loaded samples, this phenomenon suggests some extent of exclusion of ethidium bromide from the DNA due to the binding between DNA and positively charged materials. Complexes formed by compound **6** (at N/P = 1, 10, and 100) showed very faint fluorescence compared to others, suggesting possible tighter binding between the DNA molecule and compound **6** than other materials. This phenomenon was further confirmed by an ethidium bromide exclusion assay, and indeed compound **6** showed stronger binding strength to DNA than compound **7** and PEI (SI).

After 48 h incubation at 37 °C at pH 7.4 (Figure 6), all slow-degrading materials stayed as tight complexes at all N/P ratios, and all of the fast-degrading amino-dextrans, except for compound **12** started to release small amounts of DNA at N/P = 1. This is in alignment with the degradation profile of these fast-degrading materials. Interestingly, compound **12** shares the same amine structure, spermine, as compound **6**, providing more evidence of stronger binding between DNA and spermine-based amino-dextrans than other amino-dextrans. After 48 h of incubation at 37 °C in pH 5.0 buffer (Figure 6), all complexes of slow-degrading materials stayed intact at all N/P ratios. On the other hand, all fast-degrading materials, except for compound **12**, reached complete release of DNA at N/P = 1 and 10 and partial to complete release of DNA at N/P = 100. Compound **12** only started partial release of DNA at N/P = 1 and 10 and stayed nearly unaffected at N/P = 100.

As discussed previously, monitoring degradation by NMR showed that all amino-dextrans with the same acetal linker degraded at similar rates (Figure 4). Therefore, in the case of the fast-degrading amino-dextrans, the difference in DNA release was probably caused by the different binding affinities of various amino-dextrans to DNA. Although not a direct measurement, the DNA release studies taken together with the findings of the amino-dextran degradation study suggests that the release rate of DNA is inversely proportional to the binding energy of the complex. Among all materials, spermine-based amino-dextrans **6** and **12** are the tightest binder for DNA, as suggested by the DNA release study and ethidium bromide exclusion assay (SI).

Delivery of siRNA Using Amino-Dextrans. The structure and pK_a of amines on a polymeric gene delivery carrier can significantly affect complexation and transfection.[16] We examined the effects of amine structures on the transfection efficiency of our amino-dextrans by comparing compounds **3** to **8** *in vitro* using a luciferase-expressing HeLa cell line (HeLa-*luc*). In these experiments, antifirefly luciferase siRNA was complexed with each polymer at various N/P ratios and incubated with HeLa-*luc* cells for 48 h (Figure 7). Compound

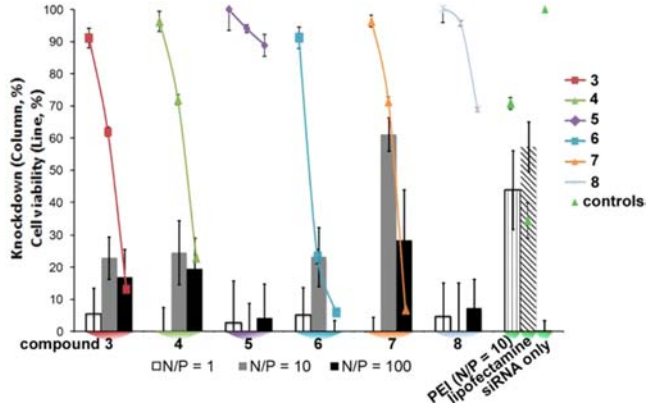

Figure 7. *In vitro* delivery of siRNA by amino-dextrans **3**–**8** bearing different types of amines. HeLa-*luc* cells were treated with polymers complexed with 0.5 μg siRNA/well. Complexes were prepared at various N/P ratios. Results were compared to untreated cells and the percentage knockdown of luciferase expression was calculated. The final percentage knockdown values in the graph were obtained after subtracting the knockdown result from cells treated with scrambled siRNA control. Transfection results (columns) are presented with concurrently performed cell viability assay (symbols and lines).

7, a bis(amino-propyl)-piperazine modified dextran, showed a gene knockdown efficiency over 60%. This is better than that of PEI, which showed a similar toxicity profile. While comparing to lipofectamine, compound **7** exhibited similar transfection efficiency and lower toxicity to cells. Compounds **3**, **4**, and **6** showed some gene knockdown (around 20%) at N/P = 10; however, the materials became toxic at N/P = 100. Compound **5** and **8** only achieved minimal gene knockdown at all N/P ratios tested.

Since all these materials have the same polymer backbone, linker, and degree of modification, the only difference is the pendant amine structure. Compounds **3**, **4**, and **5**, with primary, secondary, and tertiary amines respectively, formed complexes with DNA similarly (Figure 6) however, after 48 h incubation at both pH 7.4 and pH 5.0, more DNA was released from compound **11** (a fast-degrading analog of **5**) than from **9** or **10** (a fast-degrading analog of **3** and **4** respectively), suggesting a slightly weaker binding strength of tertiary amine-based material. Therefore, the low transfection of **5** may be caused, at least partially, by combined effects of relatively loose packing of siRNA as well as difference in "proton sponge" strength that may exist. The low transfection efficiency of compound **6** may be caused by failure to release the siRNA sufficiently due to its tight binding with genetic materials as observed in the complexation study. Compound **8**, bearing only secondary and tertiary amines, gave minimal gene knockdown, and this might suggest an important role of primary amine in siRNA delivery.

To investigate the effects of degradation rate of the material on siRNA delivery, we compared the transfection efficiency of slow-degrading amino-dextrans (**3** to **8**) to their fast-degrading

analogs (**9** to **14**). Although the amine structure was retained, the fast-degrading compound **13** was less efficient in delivering siRNA than its slow-degrading analog **7** (Figure 8). On the other hand, fast-degrading compound **12** led to more efficient transfection and less toxicity than its slow-degrading analog **6**. None of the remaining fast-degrading amino-dextrans showed improved overall transfection efficiency than their slow-degrading analogs. Indeed, degradation rate of the carrier has a significant impact on gene delivery. As discussed above, spermine-based compound **6** and **12** are tighter binders of DNA than other amino-dextrans. Therefore when spermine was linked to dextran through slow-degrading linker $\mathbf{A_3}$, the transfection efficiency was low due to inefficient release of siRNA; while spermine was conjugated through fast-degrading linker $\mathbf{A_{10}}$, transfection was greatly improved. As a comparison, other weaker binding amino-dextrans were unable to achieve comparable or higher transfection efficiency when a fast-degrading linker was used, presumably due to earlier release of siRNA than desired during transportation.

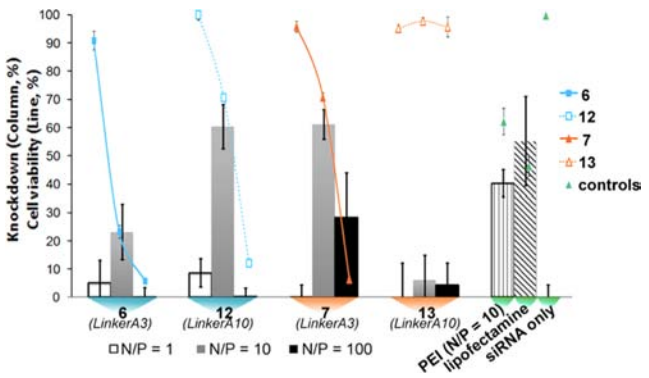

Figure 8. *In vitro* delivery of siRNA by amino-dextrans of different degradation rates (compound **6** vs **12** and compound **7** vs **13**). Transfection results (columns) are presented with concurrently performed cell viability assay (symbols and lines).

Hence, together with amine structure on the carrier, degradation rate of the carrier also affects transfection efficiency. Faster degradation rate of siRNA delivery carrier does not necessarily lead to higher transfection efficiency, rather a balance of association (binding) and dissociation (release) between the carrier and siRNA is crucial. When the binding affinity between the carrier and siRNA is low, a fast degradation rate of the carrier can abolish their association/dissociation balance, thereby reducing the transfection efficiency, as seen in the case of compounds **7** and **13**. On the other hand, tighter binding materials (such as compound **6**) are harder to dissociate from siRNA; therefore, introduction of a fast-degrading linker (e.g., compound **12**) facilitates the release of siRNA while maintaining sufficient binding, thereby achieving high transfection efficiency. Besides these two pairs of examples, other amino-dextrans, although not very efficient in transfection, may be possible carriers for siRNA when linkers of proper degradation rates are selected to maintain the association and dissociation balance.

CONCLUSION

In summary, we have developed new conjugation chemistry for the attachment of functional structures or therapeutics to polysaccharides, exemplified by dextran, through the formation of acetals on its hydroxyl groups. The properties of the acetal groups can be tuned by changing their stereoelectronic effects, therefore providing materials with tunable degradation rates that allow the cargo to be released at a variety of rates. Our synthetic approach allows for the modular exploration of various acetals, functional handles, and amine structures to find optimal materials for the delivery of siRNA. Amino-dextrans **7** (slow-degrading) and **12** (fast-degrading), which possessed different amine structures, demonstrated comparable or higher transfection efficiency and less cytotoxicity than the known positive controls PEI and lipofectamine. Clearly, successful delivery of siRNA can be achieved by careful selection of amine structures and degradation profile of the carriers to maintain the balance between binding and dissociation of the carrier and siRNA. Moreover, the modular conjugation chemistry via acetals offers new opportunities for polysaccharide-based materials in the delivery of therapeutics.

EXPERIMENTAL SECTION

Chemical Synthesis. *Synthesis of Ethyl 4-(4-Formyl-3-methoxy)-phenyl Butyrate (A_{10}).* Vanillin (20.0 g, 0.131 mol), ethyl 4-bromobutyrate (22.5 mL, 0.157 mol), and potassium carbonate (36.3 g, 0.263 mol) were heated at 50 °C in dry DMF (200 mL) for 1 h. (Sodium hydride was not suitable for the reaction due to the formation of byproduct ethyl acrylate.) Potassium carbonate was then filtered, and the DMF solution was extracted with ethyl acetate (1 L) and water (1 L) twice. The ethyl acetate layer was collected, dried over anhydrous magnesium sulfate, and removed by rotary evaporation. The residue was dried under high vacuum to obtain the pure vanillin-scented product as white powder (32.7 g, 93%). IR (KBr, cm^{-1}): 3008, 2976, 2869, 2350, 1766, 1710, 1620, 1565, 1530, 1493, 1433, 1366, 1301, 1222. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.84 (s, 1H, HC═O), 7.49−7.34 (m, 2H, Ar), 6.98 (d, 1H, J = 8.1 Hz, Ar), 4.34−3.99 (m, 4H, OCH_2, $COOCH_2$), 3.92 (s, 3H, OCH_3), 2.54 (t, 2H, J = 7.2 Hz, CH_2C═O), 2.20 (dddd, 2H, J = 6.8, 6.8, 6.8, 6.8 Hz, $CH_2CH_2CH_2$), 1.25 (t, 3H, J = 7.1 Hz, CH_2CH_3). ^{13}C NMR (126 MHz, $CDCl_3$): δ 191.10, 173.14, 154.02, 150.06, 130.29, 126.95, 111.73, 109.44, 68.07, 60.70, 56.17, 30.72, 24.42, 14.39. ESI HRMS calcd for $C_{14}H_{18}O_5Li$ (M + Li): 273.1309, found: 273.1310.

The General Procedure for the Synthesis of Dimethyl Acetal or Ketal (Figure 2) Is As Follows. Aldehyde or ketone (1 to 100 mmol) and trimethyl orthoformate (2.0 equiv) were refluxed in anhydrous methanol (2−200 mL, freshly distilled) in the presence of activated molecular sieves (4 Å, 0.2−20.0 g) and *p*-toluenesulfonic acid monohydrate (2−200 mg) for 1 h, then heat was removed, and the reaction was quenched by addition of triethylamine. Molecular sieves were filtered off, and solvents were removed by rotary evaporation. The residue was redissolved in dichloromethane (10 to 200 mL) and was washed with sodium bicarbonate solution (pH 8.0, 10−200 mL). The dichloromethane layer was isolated and dried over anhydrous sodium sulfate powder, the solvent was evaporated, and the residue was dried under vacuum. The product was not further purified and was used directly in the next step syntheses.

Ethyl Levulinate Dimethyl Ketal (A_3'). Obtained as colorless oil (88% in yield, containing 86% dimethyl ketal and 14% ketone, calculated by NMR integration). IR (cm^{-1}): 2987, 2945, 2909, 2831, 1739, 1448, 1380, 1294, 1174, 1128, 1106, 1046, 859. ^{1}H NMR (400 MHz, CD_2Cl_2): δ 4.09 (q, 2H, J = 7.1 Hz, CH_2CH_3), 3.14 (s, 6H, OCH_3), 2.30 (t, 2H, J = 7.8 Hz, CH_2C═O), 1.90 (t, 2H, J = 8.3 Hz, CH_2CH_2C═O), 1.23 (t, 3H, J = 7.8 Hz, CH_2CH_3,), 1.21 (s, 3H, CH_3C). ^{13}C NMR (126 MHz, CD_3CN): δ 174.14, 101.83, 61.11, 48.44, 32.37, 30.25, 21.21, 14.61. ESI HRMS calcd for $C_9H_{18}O_4Li$ (M + Li): 197.1360, found: 197.1360.

Ethyl 4-(4-Formyl-3-methoxy)phenyl Butyrate Dimethyl Acetal (A_{10}'). Obtained as colorless oil (95% in yield, containing 99% dimethyl acetal and 1% aldehyde, calculated by NMR integration). IR (cm^{-1}): 3077, 2941, 2831, 2756, 1737, 1685, 1595, 1511, 1467, 1418, 1375, 1348, 1268, 1137, 1102, 864, 809. ^{1}H NMR (400 MHz, CD_3CN): δ 6.99 (d, 1H, J = 1.5 Hz, Ar), 6.95−6.92 (m, 2H, Ar), 5.29

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(s, 1H, $CH(OCH_3)_2$), 4.11 (q, 2H, J = 7.1 Hz, $COOCH_2$), 4.03 (t, 2H, J = 6.3 Hz, OCH_2), 3.82 (s, 3H, $ArOCH_3$), 3.30 (s, 6H, $CH(OCH_3)_2$), 2.48 (t, 2H, J = 7.3 Hz, $CH_2C{=}O$), 2.05 (dddd, 2H, J = 7.2, 7.2, 6.4, 6.4 Hz, $CH_2CH_2CH_2$), 1.23 (t, 3H, J = 7.1 Hz, CH_2CH_3). ^{13}C NMR (151 MHz, CD_2Cl_2): δ 173.73, 150.22, 149.24, 132.21, 119.90, 113.49, 111.09, 103.99, 68.72, 61.07, 56.61, 53.35, 31.49, 25.51, 14.79. ESI HRMS calcd for $C_{16}H_{24}O_6Li$ (M + Li): 319.1727, found: 319.1730.

The General Procedure for Modification of Dextran by Dimethyl Acetals or Ketals (Figure 2). Dextran of desired molecular weight (100 mg) was dissolved in anhydrous DMSO (1 mL), followed by addition of dimethyl acetal or ketal (2 eq. per hydroxyl group), molecular sieves (5 Å, 100 mg), and *p*-toluenesulfonic acid monohydrate (5 mol % per hydroxyl group). The reaction mixture was heated for 2 h to overnight at 50 or 80 °C, and the reaction was quenched by addition of triethylamine. Molecular sieves were then filtered, and the DMSO solution was dripped into a mixture of isopropyl alcohol or isopropyl alcohol and hexanes (1:1) to obtain white precipitate. The white product was collected by centrifugation, purified by redissolving in DMSO and precipitating in isopropyl alcohol or isopropyl alcohol and hexanes (1:1), and dried under vacuum to obtain a water-insoluble white powder.

Compound 1. To a solution of dextran (MW = 35000−45000 g/mol, 500 mg, 3.09 mmol glucose residue) in anhydrous DMSO (5 mL) were added ethyl levulinate dimethyl ketal $\mathbf{A_3}'$ (3.55 g, 18.5 mmol), molecular sieves (5 Å, 500 mg), and *p*-toluenesulfonic acid monohydrate (8.8 mg). The reaction mixture was heated for 2 h at 50 °C. Following the general procedure during workup produced compound **1** as a white powder (802 mg, 0.8 ester per glucose residue as calculated by its ^1H NMR, and confirmed by the ^1H NMR of its derivatives compound **3** to **8**, yield 99%). Due to the rather complex and uninterpretable ^1H NMR spectrum in d_6-DMSO (a good solvent for **1**), the ^1H NMR of compound **1** was obtained in acidic D_2O (pH 2.0), where the attached ketals were cleaved to give water-soluble free dextran and ethyl levulinate. ^1H NMR (500 MHz, D_2O−DCl-$(CD_3)_2SO$) δ 5.22−4.76 (m, 1H, glucose-H_1), 3.98 (q, 0.8 × 2H, J = 7.2 Hz, $COOCH_2$), 3.90−3.21 (m, 5H, glucose-H_{2-6}), 2.73 (t, 0.8 × 2H, J = 6.3 Hz, CH_2COO), 2.42 (t, 0.8 × 2H, J = 6.3 Hz, CH_3COCH_2), 2.06 (s, 0.8 × 3H, CH_3COCH_2), 1.05 (t, 0.8 × 3H, J = 7.2 Hz, CH_2CH_3). As a reference, the ^1H NMR resonances of a less densely functionalized (0.5 ketal per glucose residue) water-soluble analogue **1′** is provided as follows: ^1H NMR (500 MHz, D_2O) δ 5.43−4.91 (m, 1H, glucose-H_1), 4.29−4.11 (m, 0.5 × 2H, $COOCH_2$), 4.17−3.25 (m, 6H, glucose-H_{2-6}), 2.56 (m, 0.5 × 2H, CH_2COO), 2.21 (m, 0.5 × 2H, ketal-CH_2), 1.68−1.36 (m, 0.5 × 3H, ketal-CH_3), 1.37−1.22 (m, 0.5 × 3H, $COOCH_2CH_3$) (also see S-1).

Compound 2. To a solution of dextran (MW = 35000−45000 g/mol, 2.0 g, 12.3 mmol glucose residue) in anhydrous DMSO (20 mL) were added ethyl 4-(4-formyl-3-methoxy)-phenyl butyrate dimethyl acetal $\mathbf{A_{10}}'$ (23.1 g, 74.1 mmol), molecular sieves (5 Å, 2.0 g), and *p*-toluenesulfonic acid monohydrate (35.2 mg). The reaction mixture was heated overnight at 80 °C. Following the general procedure during workup produced compound **2** as a white powder (3.35 mg, 0.4 ester per glucose residue, yield 99%). Due to the same reason as that described for compound **1**, the ^1H NMR of compound **2** was also obtained in acidic D_2O, and the formation of acyclic ketal was confirmed by the generation of methanol in acidic solution: ^1H NMR (500 MHz, D_2O−DCl-$(CD_3)_2SO$) δ 9.77 (s, 0.4 × 1H), 7.65 (dd, 0.4 × 1H, J = 8.3, 1.7 Hz, Ar), 7.51 (d, 0.4 × 1H, J = 1.7 Hz, Ar), 7.22 (d, 0.4 × 1H, J = 8.4 Hz, Ar), 5.34−4.90 (m, 1H, glucose-H_1), 4.24 (t, 0.4 × 2H, J = 6.1 Hz, $ArOCH_2$), 4.12 (q, 0.4 × 2H, J = 7.1 Hz, $COOCH_2$), 4.04−3.39 (m, 6H, glucose-H_{2-6}), 3.33 (s, 0.4 × 3H, CH_3OH), 2.55 (dd, 0.4 × 2H, J = 9.8, 4.2 Hz, $CH_2C{=}O$), 2.15 (p, 0.4 × 2H, J = 6.5 Hz, $CH_2CH_2CH_2$), 1.21 (t, 0.4 × 3H, J = 7.2 Hz, CH_2CH_3).

The General Procedure for Introduction of Amines to Dextran (Figure 2). Compound **1** or **2** was dissolved in anhydrous DMSO, followed by addition of various amines (20−40 eq. amino group per ester group). The reaction mixture was then warmed at 50 °C for two weeks for completion (monitored by ^1H NMR, until the disappearance of the resonance of ethyl ester group (**S-3**)). The reaction was worked up by carefully dripping the DMSO solution into ethyl acetate to have the product precipitated. The suspension was centrifuged for 10 min at 10000 × g to obtain the white precipitate, which was purified by three times reprecipitation in ethyl acetate. After removal of the residual solvents under high vacuum, the amino-modified dextran was dissolved in dd-H_2O (pH 8.0) and lyophilized to get a white foamy product (Table S-2). Amine modification of dextran was quantified by ^1H NMR.

Compound 3. Reaction of compound **1** (50 mg, 0.150 mmol ester) and amine $\mathbf{B_1}$ (201 μL, 3.0 mmol) in anhydrous DMSO (0.5 mL) gave compound **3** as a white powder (45 mg, 87% yield, 0.8 amine $\mathbf{B_1}$ per glucose unit). ^1H NMR (500 MHz, D_2O) δ 5.48−4.94 (m, 1H, glucose-H_1), 4.34−3.11 (m, 6H, glucose-H_{2-6}), 3.42 (br. app. s, 0.8 × 2H, $CH_2NHC{=}O$), 2.85 (br. app. s, 0.8 × 2H, CH_2NH_2), 2.42 (br. app. s, 0.8 × 2H, $CH_2C{=}O$), 2.11 (br. app. s, 0.8 × 2H, ketal-CH_2), 1.70−1.30 (m, 0.8 × 3H, ketal-CH_3).

Compound 4. Reaction of compound **1** (50 mg, 0.150 mmol ester) and amine $\mathbf{B_2}$ (632 μL, 6.0 mmol) in anhydrous DMSO (0.5 mL) gave compound **4** as a white powder (42 mg, 75% yield, 0.8 amine $\mathbf{B_2}$ per glucose unit). ^1H NMR (500 MHz, D_2O) δ 5.44−4.92 (m, 1H, glucose-H_1), 4.31−3.12 (m, 6H, glucose-H_{2-6}), 3.43 (br. app. s, 0.8 × 2H, $CH_2NHC{=}O$), 2.87 (br. app. s, 0.8 × 2H, CH_2NH), 2.87 (br. app. s, 0.8 × 2H, CH_2NH), 2.39 (br. app. s, 0.8 × 2H, $CH_2C{=}O$), 2.08 (br. app. s, 0.8 × 2H, ketal-CH_2), 1.63−1.35 (m, 0.8 × 3H, ketal-CH_3), 1.10 (br. app. s, 0.8 × 2H, $NHCH_2CH_3$).

Compound 5. Reaction of compound **1** (50 mg, 0.150 mmol ester) and amine $\mathbf{B_3}$ (656 μL, 6.0 mmol) in anhydrous DMSO (0.5 mL) gave compound **5** as a white powder (40 mg, 72% yield, 0.8 amine $\mathbf{B_3}$ per glucose unit). ^1H NMR (500 MHz, D_2O) δ 5.46−4.92(m, 1H, glucose-H_1), 4.30−3.16 (m, 6H, glucose-H_{2-6}), 3.46 (br. app. s, 0.8 × 2H, $CH_2NHC{=}O$), 2.89 (br. app. s, 0.8 × 2H, CH_2N), 2.57 (br. app. s, 0.8 × 6H, $(CH_3)_2N$), 2.40 (br. app. s, 0.8 × 2H, $CH_2C{=}O$), 2.07 (br. app. s, 0.8 × 2H, ketal-CH_2), 1.70−1.32 (m, 0.8 × 3H, ketal-CH_3).

Compound 6. Reaction of compound **1** (50 mg, 0.150 mmol ester) and amine $\mathbf{B_4}$ (647 μL, 3.0 mmol) in anhydrous DMSO (0.5 mL) gave compound **6** as a white powder (65 mg, 89% yield, 0.8 amine $\mathbf{B_4}$ per glucose unit). ^1H NMR (500 MHz, D_2O) δ 5.44−4.92 (m, 1H, glucose-H_1), 4.32−3.03 (m, 6H, glucose-H_{2-6}), 3.25 (br. app. s, 0.8 × 2H, $CH_2NHC{=}O$), 3.04−2.65 (m, 0.8 × 10H, CH_2NH, CH_2NH_2), 2.37 (br. app. s, 0.8 × 2H, $CH_2C{=}O$), 2.08 (br. app. s, 0.8 × 2H, ketal-CH_2), 1.88 (br. app. s, 0.8 × 2H, $NHCH_2CH_2CH_2NHC{=}O$), 1.80 (br. app. s, 0.8 × 2H, $NHCH_2CH_2CH_2NH_2$), 1.65 (br. app. s, 0.8 × 4H, $NHCH_2CH_2CH_2CH_2NH_2$), 1.65−1.30 (m, 0.8 × 3H, ketal-CH_3).

Compound 7. Reaction of compound **1** (50 mg, 0.150 mmol ester) and amine $\mathbf{B_5}$ (618 μL, 3.0 mmol) in anhydrous DMSO (0.5 mL) gave compound **7** as a white powder (63 mg, 87% yield, 0.8 amine $\mathbf{B_5}$ per glucose unit). ^1H NMR (500 MHz, D_2O) δ 5.42−4.89 (m, 1H, glucose-H_1), 4.31−3.24 (m, 6H, glucose-H_{2-6}), 3.20 (app. br. s, 0.8 × 2H, $CH_2NHC{=}O$), 3.11−2.14 (m, 0.8 × 16H, CH_2N, CH_2NH_2, $CH_2C{=}O$), 2.07 (br. app. s, 0.8 × 2H, ketal-CH_2), 1.86−1.75 (m, 0.8 × 2H, $NCH_2CH_2CH_2NHC{=}O$), 1.68 (br. app. s, 0.8 × 2H, $NCH_2CH_2CH_2NH_2$), 1.57−1.27 (m, 0.8 × 3H, ketal-CH_3).

Compound 8. Reaction of compound **1** (50 mg, 0.150 mmol ester) and amine $\mathbf{B_6}$ (786 μL, 6.0 mmol) in anhydrous DMSO (0.5 mL) gave compound **8** as a white powder (60 mg, 97% yield, 0.8 amine $\mathbf{B_6}$ per glucose unit). ^1H NMR (500 MHz, D_2O) δ 5.40−4.92 (m, 1H, glucose-H_1), 4.29−3.18 (m, 6H, glucose-H_{2-6}), 3.37 (m, 0.8 × 2H, $CH_2NHC{=}O$), 3.09 (br. app. s, 0.8 × 4H, $NHCH_2CH_2N$), 2.70 (br. app. s, 0.8 × 4H, $NHCH_2CH_2N$), 2.60 (br. app. s, 0.8 × 2H, $NCH_2CH_2NHC{=}O$), 2.38 (br. app. s, 0.8 × 2H, $CH_2C{=}O$), 2.08 (br. app. s, 0.8 × 2H, ketal-CH_2), 1.65−1.30 (m, 0.8 × 3H, ketal-CH_3).

Compound 9. Reaction of compound **2** (200 mg, 0.292 mmol ester) and amine $\mathbf{B_1}$ (195 μL, 2.92 mmol) in anhydrous DMSO (2 mL) gave compound **9** as a white powder (180 mg, 88% yield, 0.4 amine $\mathbf{B_1}$ per glucose unit). ^1H NMR (500 MHz, D_2O) δ 7.28−6.69 (m, 0.4 × 4H, Ar), 6.17−5.41 (m, 0.4 × 1H, acetal-H), 5.40−4.90 (m, 1H, glucose-H_1), 4.36−3.18 (m, 6H, glucose-H_{2-6}; 0.4 × 2H, $ArOCH_2$; 0.4 × 3H, $ArOCH_3$; 0.4 × 2H, $CH_2NHC{=}O$; 0.4 × 3H,

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acetal-OCH_3), 3.09 (br. app. s, 0.4 × 2H, CH_2NH_2), 2.35 (br. app. s, 0.4 × 2H, $CH_2C{=}O$), 2.07 (br. app. s, 0.4 × 2H, $ArOCH_2CH_2$).

Compound **10**. Reaction of compound **2** (200 mg, 0.292 mmol ester) and amine $\mathbf{B_2}$ (614 μL, 5.84 mmol) in anhydrous DMSO (2 mL) gave compound **10** as a white powder (185 mg, 87% yield, 0.4 amine $\mathbf{B_2}$ per glucose unit). ^{1}H NMR (500 MHz, D_2O) δ 7.24−6.75 (m, 0.4 × 4H, Ar), 6.12−5.43 (m, 0.4 × 1H, acetal-H), 5.38−4.92 (m, 1H, glucose-H_1), 4.37−3.32 (m, 6H, glucose-H_{2-6}; 0.4 × 2H, $ArOCH_2$; 0.4 × 3H, $ArOCH_3$; 0.4 × 2H, $CH_2NHC{=}O$; 0.4 × 3H, acetal-OCH_3), 3.17−2.99 (m, 0.4 × 4H, CH_2NH), 2.48 (br. app. s, 0.4 × 2H, $CH_2C{=}O$), 2.08 (br. app. s, 0.4 × 2H, $ArOCH_2CH_2$), 1.37−1.11 (br. app. s, 0.8 × 2H, $NHCH_2CH_3$).

Compound **11**. Reaction of compound **2** (200 mg, 0.292 mmol ester) and amine $\mathbf{B_3}$ (637 μL, 5.84 mmol) in anhydrous DMSO (2 mL) gave compound **11** as a white powder (176 mg, 83% yield, 0.4 amine $\mathbf{B_3}$ per glucose unit). ^{1}H NMR (500 MHz, D_2O) δ 7.28−6.65 (m, 0.4 × 4H, Ar), 6.22−5.42 (m, 0.4 × 1H, acetal-H), 5.39−4.90 (m, 1H, glucose-H_1), 4.38−3.20 (m, 6H, glucose-H_{2-6}; 0.4 × 2H, $ArOCH_2$; 0.4 × 3H, $ArOCH_3$; 0.4 × 3H, acetal-OCH_3, 0.4 × 2H, $CH_2NHC{=}O$), 3.11 (br. app. s, 0.4 × 2H, CH_2N), 2.77 (br. app. d, 0.4 × 6H, $(CH_3)_2N$), 2.45 (br. app. s, 0.4 × 2H, $CH_2C{=}O$), 2.08 (br. app. s, 0.4 × 2H, $ArOCH_2CH_2$).

Compound **12**. Reaction of compound **2** (200 mg, 0.292 mmol ester) and amine $\mathbf{B_4}$ (629 μL, 2.92 mmol) in anhydrous DMSO (2 mL) gave compound **12** as a white powder (201 mg, 82% yield, 0.4 amine $\mathbf{B_4}$ per glucose unit). ^{1}H NMR (500 MHz, D_2O) δ 7.31−6.63 (m, 0.4 × 4H, Ar), 6.22−5.44 (m, 0.4 × 1H, acetal-H), 5.38−4.91 (m, 1H, glucose-H_1), 4.40−3.06 (m, 6H, glucose-H_{2-6}; 0.4 × 2H, $ArOCH_2$; 0.4 × 3H, $ArOCH_3$; 0.4 × 3H, acetal-OCH_3, 0.4 × 2H, $CH_2NHC{=}O$), 3.07−2.77 (m, 0.4 × 10H, CH_2NH, CH_2NH_2), 2.43 (br. app. s, 0.4 × 2H, $CH_2C{=}O$), 2.07 (br. app. s, 0.4 × 2H, $ArOCH_2CH_2$), 1.97−1.86 (m, 0.4 × 2H, $NHCH_2CH_2CH_2NHC{=}O$), 1.80 (br. app. s, 0.4 × 2H, $NHCH_2CH_2CH_2NH_2$), 1.74−1.45 (m, 0.4 × 4H, $NHCH_2CH_2CH_2CH_2NH_2$).

Compound **13**. Reaction of compound **2** (200 mg, 0.292 mmol ester) and amine $\mathbf{B_5}$ (601 μL, 2.92 mmol) in anhydrous DMSO (2 mL) gave compound **13** as a white powder (214 mg, 79% yield, 0.4 amine $\mathbf{B_5}$ per glucose unit). ^{1}H NMR (500 MHz, D_2O) δ 7.31−6.69 (m, 0.4 × 4H, Ar), 6.14−5.44 (m, 0.4 × 1H, acetal-H), 5.39−4.94 (m, 1H, glucose-H_1), 4.40−3.24 (m, 6H, glucose-H_{2-6}; 0.4 × 2H, $ArOCH_2$; 0.4 × 3H, $ArOCH_3$; 0.4 × 3H, acetal-OCH_3), 3.17 (0.4 × 2H, $CH_2NHC{=}O$), 3.03 (br. app. s, 0.4 × 2H, CH_2NH_2), 2.62−2.17 (m, 0.4 × 12H, CH_2N; 0.4 × 2H, $CH_2C{=}O$), 2.08 (br. app. s, 0.4 × 2H, $ArOCH_2CH_2$), 1.87 (br. app. s, 0.4 × 2H, $NCH_2CH_2CH_2NHC{=}O$), 1.68 (br. app. s, 0.4 × 2H, $NCH_2CH_2CH_2NH_2$).

Compound **14**. Reaction of compound **2** (200 mg, 0.292 mmol ester) and amine $\mathbf{B_6}$ (765 μL, 5.84 mmol) in anhydrous DMSO (2 mL) gave compound **14** as a white powder (205 mg, 91% yield, 0.4 amine $\mathbf{B_6}$ per glucose unit). ^{1}H NMR (500 MHz, D_2O) δ 7.35−6.70 (m, 0.4 × 4H, Ar), 6.23−5.43 (m, 0.4 × 1H, acetal-H), 5.23−4.92 (m, 1H, glucose-H_1), 4.37−2.98 (m, 6H, glucose-H_{2-6}; 0.4 × 2H, $ArOCH_2$; 0.4 × 3H, $ArOCH_3$; 0.4 × 3H, acetal-OCH_3, 0.4 × 2H, $CH_2NHC{=}O$), 2.89−2.21 (m, 0.4 × 10H, CH_2NH, CH_2N; 0.4 × 2H, $CH_2C{=}O$), 2.07 (br. app. s, 0.4 × 2H, $ArOCH_2CH_2$).

NMR Study of Cleavage of Amines from Amine-Dextran Conjugates. Amine-dextran (**3**−**14**, 2 mg) was dissolved in either deuterated phosphate buffer (700 μL, 30 mM, pH 7.4) or deuterated acetate buffer (700 μL, 30 mM, pH 5.0), the solution was kept at 37 °C, and ^{1}H NMR was acquired at desired time points. Ratio of integration of released small molecules and dextran-bound groups was compared at different incubation times and plotted using Excel.

Gel Electrophoresis for Binding and Release Studies. To study the binding of amine-dextran to short fragments of nucleic acids, a solution of firefly luciferase DNA (0.5 μg/μL, 10 μL) in PBS buffer (pH 7.4) was mixed well with a polymer solution (10 μL in PBS buffer) to give nitrogen (of polymer)/phosphate (of DNA) ratio of 100, 10, and 1. The solution was shaken for 20 min to allow the complex to form and loaded to a gel [0.5 g of agarose, 50 mL of tris-acetate-EDTA buffer, 1 drop of ethidium bromide (Gene Choice) solution (final concentration = 0.5 μg/mL)]. The gel was developed for 40 min (100 V, 3.0 A, 300 W) and visualized under UV at 254 nm, and digital images were collected with an EpiChemi II Darkroom unit fitted with a CCD camera (UVP, Upland, CA). Complexes at higher N/P ratios (10 and 100) moved further toward the negative electrode, indicating overall positive charge of the complexes. In the DNA release study, a solution of firefly luciferase DNA (1 μg/μL, 5 μL) in PBS buffer (pH 7.4) was mixed well with a polymer solution (5 μL in PBS buffer) to give nitrogen (of polymer)/phosphate (of DNA) ratio of 100, 10, and 1. The solution was shaken for 20 min, and pH was adjusted to 5.0 by addition of acetate buffer (10 μL, 150 mM, pH 4.98). The samples used in the release study at pH 7.4 were prepared as described above. The mixture was then incubated at 37 °C for 48 h before loading to the agarose gel.

Cell Lines and Culture. HeLa cell line stably expressing firefly luciferase (HeLa-*luc*) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% GlutaMAX, and 500 μg/mL Zeocin (all from Invitrogen except the serum, which was from Hyclone (Logan, UT)). Cell incubations were performed in a water-jacketed 37 °C/5% CO_2 incubator.

In Vitro siRNA Transfection Assay. HeLa-*luc* cells were seeded (15,000 cells/well) into each well of a 96-well clear tissue culture plate (Costar, Corning, NY) and allowed to attach overnight in growth medium. The medium was composed of DMEM (with phenol red), 10% FBS, and 1% GlutaMAX. The polymer/siRNA ratio was expressed as the nitrogen/phosphate (N/P) ratio, where N represents moles of amine on the polymer and P represents moles of phosphate on siRNA. Cells were transfected with 0.5 μg of siRNA complexed with polymer at various N/P ratios to determine the optimum for transfection efficiency. In order to exclude nonspecific gene silencing by the polyplexes themselves, the cells were also incubated with polyplexes prepared using a negative control siRNA (Silencer Negative Control #1 siRNA). Working dilutions of each polymer were prepared (at concentrations necessary to yield the different N/P ratios) in PBS (pH 7.4). Polymer solutions (50 μL) were mixed with siRNA solutions (50 μL of 100 μg/mL siRNA in PBS) and incubated for 20 min at rt to allow complex formation. The complex solutions were then diluted with 900 μL of medium (DMEM supplemented with 10% FBS-containing). Existing medium was replaced with 100 μL of each polymer/siRNA sample in triplicate wells. In the case of experiments without serum, the medium was replaced 4 h after polyplex addition with fresh serum-containing (10% FBS) growth medium. The cells were allowed to grow for a total of 48 h before being analyzed for gene expression. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and PEI (hyperbranched, 25 kDa, Sigma-Aldrich) were used as positive controls for siRNA delivery and was prepared according to the manufacturer's instructions. Complexes containing equivalent doses of siRNA to polyplexes were prepared by mixing Lipofectamine 2000 and siRNA (3.8:1 ratio). As negative controls, both equivalent doses of free siRNA in medium and medium alone were used.

After 48 h, the plate was centrifuged (1200 × g, 5 min) and the medium was replaced with DPBS (100 μL/well). The cells were centrifuged (1200 × g, 5 min) once more, the buffer was replaced with Glo Lysis Buffer (120 μL/well, Promega, Madison, WI), and the plate was vortexed at rt for 20 min. Samples from each well (100 μL) were transferred to the wells of a white 96-well tissue culture plate (Corning, Lowell, MA). Steady-Glo luciferase assay reagent (Promega) was reconstituted according to the manufacturer's instructions and injected into each well in series (100 μL/well) using a GloMax 96 microplate luminometer (Promega). After a 10 s postinjection delay, each well was read with a 2 s integration time.

Total Protein Assay. Cells treated identically and in parallel with transfection assays were tested on a second 96-well plate. After washing, the cells were lysed with Mammalian Protein Extraction Reagent (M-PER, 50 μL/well, Pierce, Rockford, IL) by incubating for 10 min at rt. PBS (50 μL/well) was then added, and the plate was briefly vortexed. Samples from each well (50 μL) were transferred to a black 96-well plate (Corning) already containing PBS (100 μL/well). A solution of 3 mg/mL fluorescamine in acetone (50 μL) was added to each well and mixed well using a multichannel pipet. After 5 min,

fluorescence was measured using a SpectraMax M3 multimode microplate reader (ex. 400 nm, em. 460 nm). Protein concentrations were determined using bovine serum albumin as a standard. Relative light units (RLU) from the luminometer were normalized to the total mass of cellular protein. The resulting data (RLU/mg of protein) are given as a mean ± standard deviation of three independent measurements. Percentage knockdown was calculated by comparison of treated cells to untreated cells. The data were compared to the knockdown of cells treated with particles loaded with control siRNA or control siRNA/Lipofectamine complexes.

Viability Assay. Cells treated identically and in parallel with transfection assays were tested on a third 96-well plate. A 3.0 mg/mL solution of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) in medium (40 μL) was added directly to each well, and the plate was incubated for an additional 30 min. The medium was then replaced with DMSO (200 μL/well), 100 μL of which was transferred to another clear-bottom 96-well assay plate (Pro-Bind, Falcon) containing 100 μL of DMSO and 25 μL of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) per well. The absorbances at 570 nm were measured using a SpectraMax M3 multimode microplate reader (Molecular Devices). Cell viability was normalized to the absorbance measured from untreated cells. Data are represented as a mean ± standard deviation of three measurements.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, NMR data, additional degradation experiments, and ethidium bromide exclusion assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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