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Tunable Reactivation of Nanoparticle-Inhibited β-Galactosidase by Glutathione at Intracellular Concentrations

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Abstract: Positively charged trimethylammonium-functionalized mixed monolayer protected clusters (MMPCs) of different chain lengths (C_8 and C_{11}) have been used to bind β-galactosidase through complementary electrostatic interactions, resulting in complete enzyme inhibition. This inhibition can be reversed in vitro by intracellular concentrations of glutathione (GSH), the main thiol component of the cell. The restoration of activity depends on the chain length of the monolayer. The activity of enzyme bound to particles with C_8 monolayer was completely restored by intracellular concentrations (1−10 mM) of GSH; however, little or no release was observed at extracellular GSH concentrations. In contrast, no restoration was observed for enzyme bound to the C_{11} particles at any of the concentrations studied. Taken together, these studies demonstrate that the GSH-mediated release of enzymes bound to MMPCs can be tuned through the structure of the monolayer, a significant tool for protein and drug delivery applications.

Introduction

Protein surface binding using artificial receptors has potential applications in controlling a wide array of cellular processes, including enzyme inhibition, signal transduction, and protein antigen/antibody interactions. Targeting protein surfaces for binding through fabrication of complementary surfaces provides an alternate strategy to traditional active site inhibition for controlling these processes,[1] with several recently reported scaffolds, including small molecule systems[2] and macromolecular receptors.[3] While enzyme inhibition and denaturation have been demonstrated at physiological concentrations using these receptors,[4,5] controlled reversal of enzyme inhibition mediated by intracellular factors has not been extensively studied. Modulation of inhibition by cellular factors has potential utility in designing synthetic enzyme inhibitors capable of carrying and releasing proteins in vivo; such trigger mechanisms exploiting intracellular conditions can be extended to protein and drug delivery strategies.[6]

In recent studies, we have demonstrated the utility of MMPCs for the regulation of enzyme activity[4,7] and control of DNA transcription.[8] Negatively charged MMPCs were shown to be very effective inhibitors of α-chymotrypsin.[4] However, the addition of positively charged surfactants could partially reverse the inhibition.[7] This controlled release of MMPC-bound chymotrypsin using synthetic molecules suggested the possibility that the nanoparticle-mediated inhibition could be tailored to facilitate enzyme reactivation by components found in vivo.

Here, we report the inhibition of β-galactosidase (β-gal) by trimethylammonium-functionalized mixed monolayer protected clusters (MMPCs) and demonstrate that the enzyme inhibition can be either maintained (using a longer monolayer) or reversed (using a shorter chain length) upon addition of glutathione (GSH) at intracellular concentrations (Figure 1). GSH is the most abundant low-molecular weight thiol present in cells[9] and is involved in many important functions in the body including the control of the redox environment in cells.[10] GSH is found at high intracellular concentrations (∼10 mM in liver cells)[11] and very low extracellular concentrations (<10 μM).[10] For example, blood plasma concentrations (2 μM)[12] are 1000-fold less than in erythrocytes (∼2 mM).[13] This vast difference in potential "releasing agent" inside cells as compared to outside

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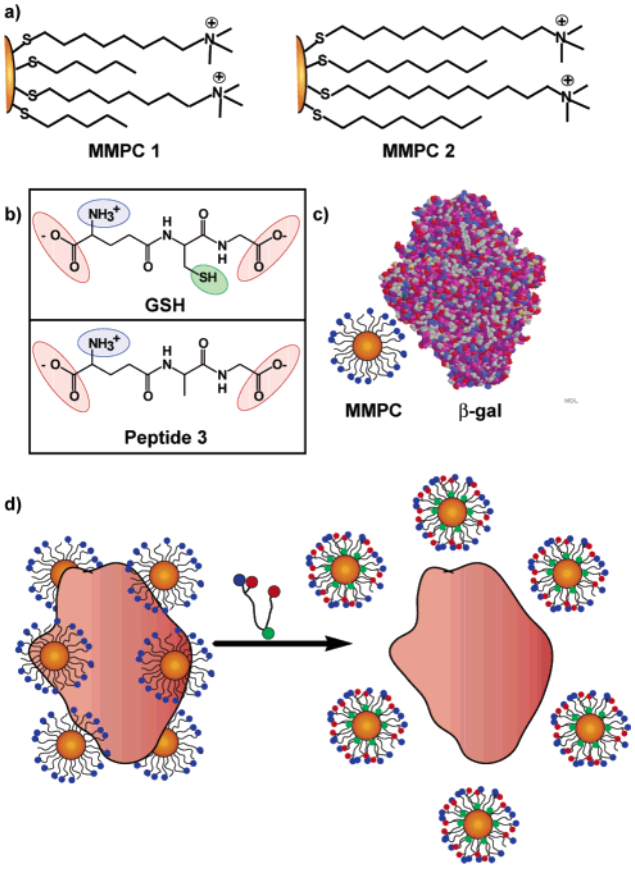

Figure 1. (a) Structures of MMPC scaffolds, (b) reduced glutathione and control peptide **3**, (c) relative sizes of MMPCs and β-gal, and (d) schematic representation of GSH-mediated disruption of MMPC-β-gal binding. Both MMPCs were ~50−60% functionalized with trimethylammonium end groups.

cells provides a potential mechanism for release and reactivation of MMPC-inhibited proteins in vivo.

Experimental Section

General. β-Galactosidase (grade VI from *E. coli*), and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Sigma-Aldrich Chemical Co. and were used without further purification. Agarose was purchased from EM Science. Sodium dodecyl sulfate was purchased from Fisher Scientific. Glycerol was purchased from Eastman Kodak Co. All activity assay experiments were done at 23 °C.

Activity Assays. β-Galactosidase (0.5 nM) was incubated with varying concentrations of MMPC **1** and MMPC **2** for 4.5 h for the inhibition studies. The final concentrations of MMPCs were 1, 3, 5, 10, 15, 20, 30, 40, and 50 nM. As a control, the enzyme inhibition was also studied with 30 nM of negatively charged carboxylate-functionalized nanoparticles after 4.5 h of incubation. For the enzyme reactivation studies, 0.5 nM of β-galactosidase was incubated with 30 nM of MMPC **1** and MMPC **2** for about 5 h, after which reduced glutathione (GSH) or peptide **3** was added from a 100 mM freshly prepared stock solution. The final concentration of sodium phosphate buffer (pH 7.4) for these experiments was 65 mM. For the ionic strength studies, 0.5 nM of β-galactosidase was incubated with 25 nM of MMPC **1** and MMPC **2** for about 5 h in varying sodium phosphate buffer (pH 7.4) concentrations (24, 50, 75 mM). The β-galactosidase stock concentration was 463 nM, while the MMPC stock concentration was 1 μM. The enzyme activity at predetermined time points was obtained by addition of the chromogenic substrate ONPG from a stock concentration of 4.4 mM to a final concentration of 0.44 mM. Composition of substrate stock solution: 200 mM sodium phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 1.33 mg/mL ONPG. The enzymatic activity for the inhibition assay was followed by monitoring product formation every 30 s for 10 min at 405 nm using a microplate reader (EL808 Bio-Tek Instruments, Inc.). The total reaction volume was 200 μL for each well. Samples were measured in triplicate. Enzymatic activity for the reactivation assay was followed using a UV-spectrophotometer (HP 8452A) at 410 nm. Data obtained from the plate reader were in the units of milli optical density/min, while data from the spectrophotometer were in absorbance/s, which directly reflected the rate of enzymatic activity for individual samples. Control experiments with β-galactosidase and GSH and peptide **3** were carried out along with other samples at identical time points.

Gel Electrophoresis. Agarose gels were prepared in TBE (Tris-borate EDTA) buffer (composition: 0.045 M Tris-borate and 0.001 M EDTA) at 1% final agarose concentration. Appropriately sized wells (40 μL) were formed by placing a comb in the center of the gel. The stock solution of the enzyme was 5 μM, and the MMPC stock concentration was kept at 80 μM. For the β-galactosidase−MMPC binding studies, samples were prepared with enzyme−MMPC molar ratios at 1:0, 1:3, 1:6, and 1:9, keeping the final concentration of the enzyme at 2 μM and varying the MMPC concentrations accordingly. The gels displaying enzyme−MMPC binding were placed in staining solution (0.5% Coomassie blue, 40% methanol, 10% acetic acid aqueous solution) for 1 h, followed by extensive destaining (40% methanol, 10% acetic acid aqueous solution) until protein bands were clear. For the study displaying interactions of GSH and peptide **3** with the MMPCs, the final concentrations of the nanoparticles and the peptides were 18 μM and 10 mM, respectively. To ensure the mobility of the positively charged MMPCs on the gel, SDS (sodium dodecyl sulfate) was added from a 10% stock solution to make a final concentration of 0.1% in the gel buffer and 1% in the samples for the experiment demonstrating interaction of the peptides with MMPCs. The samples for the enzyme binding experiments were incubated for ~5 h, whereas the samples for the latter experiment were incubated for ~4 h before loading 30 μL of samples into each well. All samples were incubated in sodium phosphate buffer (pH 7.4) with a final concentration of 65 mM. Next, 3 μL of 80% glycerol was added to each sample to ensure proper well loading, and a constant voltage (100 V) was applied for 30 min for sufficient separation.

Results and Discussion

For our studies, we targeted β-galactosidase, a large, tetrameric enzyme (17.5 × 13.5 × 9 nm),[14] with an overall negative surface charge at neutral pH (isoelectric point 4.6).[15] MMPCs featuring a 2 nm gold core and a trimethylammonium-functionalized monolayer were used to bind β-gal through electrostatic complementarity (Figure 1). The inhibition of β-gal was conducted with two different positively charged nanoparticles MMPCs **1** and **2** (C_8 and C_{11}, respectively) to control the GSH-mediated reversibility of the inhibition.

Inhibition of Enzyme Activity. The inhibition of β-gal activity on interaction with the nanoparticles was determined by incubation of 0.5 nM of β-gal with either MMPC **1** or **2** (concentrations ranging from 1 to 50 nM) in 65 mM sodium phosphate buffer (pH 7.4). After 4.5 h of incubation, the enzymatic activity was monitored by the hydrolysis of the chromogenic substrate (*o*-nitrophenyl-β-D-galactopyranoside (ONPG, 0.44 mM), demonstrating that both scaffolds are effective at inhibiting β-gal activity in a concentration-dependent manner (Figure 2). The enzymatic activity drops to ~20% as a

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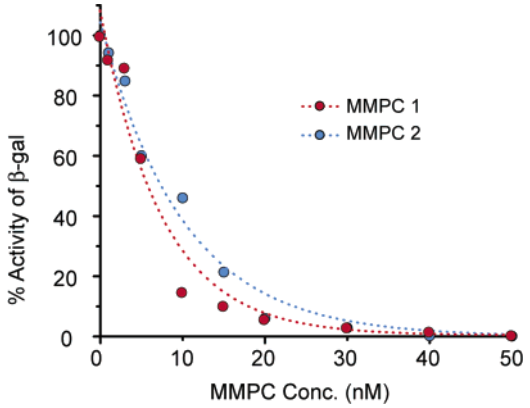

Figure 2. Activity of β-Gal (0.5 nM) 4.5 h after incubation with MMPC **1** and **2**. The plot has been normalized against native enzyme activity. At the concentrations where the blue dots (MMPC **2**) are not visible, they coincide with the red dots (MMPC **1**). The dotted lines indicate the trend in the data.

concentration of ~10−15 nM of nanoparticle is reached. Thereafter, the inhibition increases more slowly with the increase of MMPC concentration until ~50 nM, at which complete loss of enzymatic activity is observed.

The effect of increasing ionic strength on the binding of β-gal to MMPCs was investigated to confirm that this association was driven primarily by complementary electrostatic interactions,[16] as opposed to other protein particle interactions. In these studies, β-gal (0.5 nM) was incubated with MMPC **1** and **2** (25 nM) separately for 5 h in the presence of increasing concentrations of sodium phosphate buffer (pH 7.4). The increase in buffer concentration from 24 to 75 mM resulted in an increased β-gal activity from 0% to 25% for both MMPCs, consistent with a complementary electrostatic binding of the MMPCs to the enzyme (see Supporting Information).[17]

Binding of β-Gal to MMPCs. Agarose gel electrophoresis experiments were performed to demonstrate the formation of the β-gal−MMPC complex and determine the stoichiometry of association. The enzyme (2 μM) was incubated with nanoparticles at appropriate concentrations for 5 h in 65 mM sodium phosphate buffer (pH 7.4) before the loading of samples into wells. In the absence of MMPC, β-gal displayed a substantial migration toward the positive polarity, as expected due to its overall negative charge. However, upon addition of positively charged MMPCs **1** and **2**, a dramatic decrease in the mobility of the enzyme was observed (Figure 3), attributed to both an increase in size and the attenuation of surface charges on binding and complex formation.[18] At a 1:6 molar ratio of enzyme:

(16) As a control, negatively charged carboxylate-functionalized gold nanoparticles with a 2 nm core diameter were incubated with β-gal. The final concentrations for the MMPC and enzyme were kept at 30 and 0.5 nM, respectively, and the enzyme activity was monitored after 5 h of incubation. Minimal loss in enzyme activity (~5%) was observed, indicating the importance of electrostatic complementarity between the MMPC and the enzyme surfaces required for the inhibition process. Carboxylate-functionalized nanoparticles were prepared as previously reported: Simard, J.; Briggs, C.; Boal, A. K.; Rotello, V. M. *Chem. Commun*. **2000**, *19*, 1943−1944.

(17) For a detailed study on the effect of ionic strength on similar enzyme−MMPC interactions driven by complementary electrostatics, see: Verma, A.; Simard, J. M.; Rotello, V. M. *Langmuir* **2004**, *20*, 4178−4181. Ionic strength studies with NaCl displayed an irreproducible behavior with β-galactosidase; hence, the study was carried out by increasing the phosphate buffer concentration instead, which gave a reproducible platform for the studies.

(18) The trimethylammonium-functionalized MMPCs alone do not show any mobility on the agarose gel toward either polarities under the experimental conditions studied.

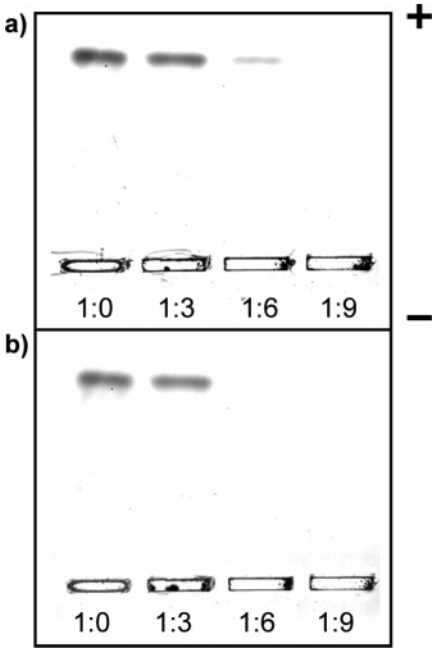

Figure 3. Gel electrophoresis of β-gal with varying molar ratios (enzyme: MMPC) of (a) MMPC **1** and (b) MMPC **2**. The concentration of the enzyme was 2 μM.

MMPCs, no residual protein band was observed for MMPC **2** (Figure 3b), while a very faint band was seen for MMPC **1** (Figure 3a), indicating an approximate 6:1 binding ratio of nanoparticles to enzyme molecule for these systems. The lower stoichiometry observed through gel electrophoresis as compared to the activity probably arises from the higher concentrations involved in the gel study relative to the activity assay, with concomitant change in the binding curves.

GSH-Mediated Reactivation of β-Gal. The GSH-mediated reactivation of β-gal−MMPC complexes was established by adding GSH to preincubated (~5 h) enzyme-nanoparticle solutions to give final GSH concentrations corresponding to extracellular and intracellular GSH levels. The enzymatic activity was monitored up to 4 h after GSH addition (Figure 4a). After 4 h, it was observed that there was minimal reactivation of β-gal on addition of GSH at blood plasma concentration (2 μM). Enzyme bound to MMPC **1** displayed approximately 10% reactivation, while no change in activity was observed from enzyme complexed with MMPC **2**. At intracellular levels of GSH, however, up to 95% of the native enzyme activity was rescued from MMPC **1** inhibition. Control experiments on the effect of GSH on β-gal alone showed a certain degree of inhibition, ranging from ~20% at 10 mM to ~10% at 5 mM of GSH concentrations, explaining the drop in recovery efficiency at these concentrations. Significantly, enzyme bound to MMPC **2** featuring the longer side chains did not display reactivation for any concentrations of GSH used, demonstrating that the reactivity of the MMPC can be readily tuned, which is an important issue for delivery applications.

Peptide **3** (Figure 1b) was synthesized to investigate the mechanism of GSH-mediated reactivation of β-gal. Peptide **3** is analogous to GSH except that it lacks a thiol group. Control experiments were carried out by addition of the peptide **3** to the 5 h preincubated enzyme−nanoparticle complex. After 4 h of peptide **3** addition, no restoration of β-gal activity under identical experimental conditions was observed from enzyme

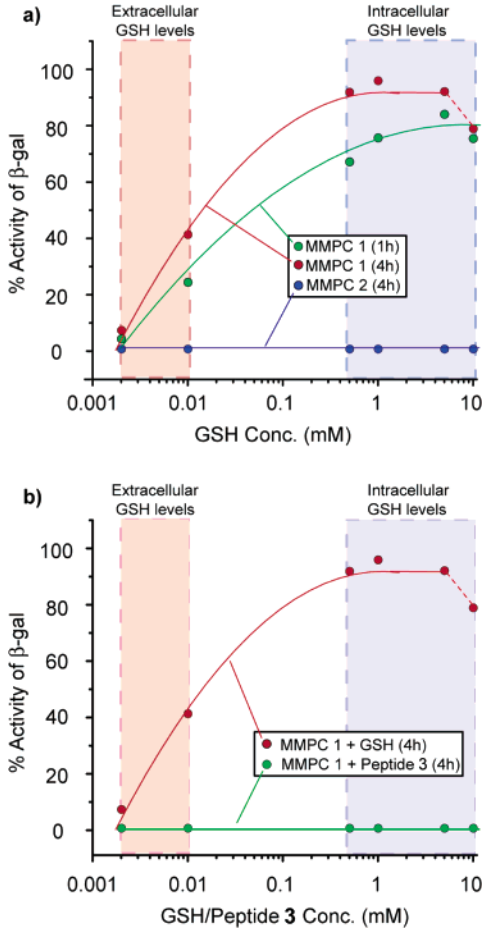

Figure 4. (a) Summary of percent activity of β-gal after addition of GSH. (b) Comparison of β-gal activity rescue by GSH and peptide **3**. The plots have been normalized against native enzyme activity. The lines indicate the trend in the data. Enzyme and MMPC concentrations are 0.5 and 30 nM, respectively. The hours indicate the time after either GSH or peptide **3** additions.

bound to either MMPCs (Figure 4b). As peptide **3** is analogous to GSH with the exception of the thiol group, this experiment demonstrates the importance of the thiol in mediating rescue of activity. The proposed mechanism for GSH-mediated reactivation of β-gal relies on the addition of GSH into the monolayer of MMPC **1**.[19,20] This results in mitigation of the positive charge on the trimethylammonium-functionalized nanoparticle monolayer due to the overall negative charge present on the GSH. This diminishes the electrostatic interaction between MMPC **1** and β-gal, leading to the dissociation and reactivation of the enzyme.[21] Enzyme bound to MMPC **2** did not display restoration of activity, indicating that GSH does not significantly interact with the longer C_{11} monolayer.[22]

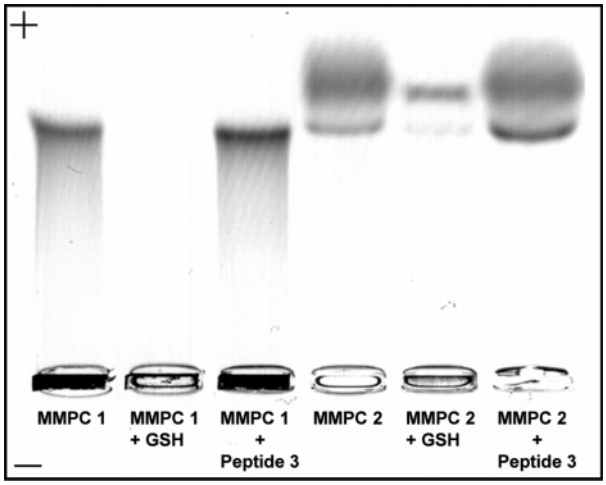

Figure 5. Gel electrophoresis displaying mobilities of the MMPC **1** and MMPC **2**. The final concentrations for MMPC and GSH/peptide **3** were kept at 18 μM and 10 mM, respectively.

Gel Electrophoresis Study of GSH−MMPC Interaction. Agarose gel electrophoresis experiments were performed to further investigate the monolayer composition upon interaction of nanoparticles with GSH (Figure 5). MMPCs **1** and **2** (18 μM) were incubated with 10 mM of GSH and peptide **3** separately for 4 h in 65 mM sodium phosphate buffer (pH 7.4). Gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) resulted in migration of the MMPCs toward the positive pole.[18] MMPC **1** alone and MMPC **1** with peptide **3** revealed similar mobility, demonstrating that peptide **3** does not interact with MMPC **1**. However, MMPC **1** incubated with GSH displayed no movement, indicating that the monolayer composition of MMPC **1** is altered by GSH via sulfur−gold interaction, resulting in the charge attenuation of the nanoparticle monolayer. All of the samples containing MMPC **2** displayed similar mobilities, suggesting little or no alteration of monolayer composition for MMPC **2** in the presence of either GSH or peptide **3**.[23]

Conclusion

In summary, we have demonstrated that the positively charged MMPCs of varying chain lengths effectively inhibit β-galactosidase. The inhibition due to short-chain (C_8) MMPC **1** can be completely reversed by glutathione at intracellular concentrations. However, a longer chain length (C_{11}) of the MMPC **2** monolayer provides effective protection against GSH-mediated reactivation of the enzyme. This study has implications in the design of nanoparticle monolayer for release of proteins in vivo mediated by GSH, as cationic MMPCs have been shown to internalize in cells in earlier studies.[24] The specificity of MMPCs toward biomacromolecules is not a requirement for protein/drug delivery applications, which is the scope of the present study.

(19) Previous precedents have shown that displacement of incoming thiol ligands on gold nanoparticles proceeds via an associative mechanism as opposed to a dissociative one. For example, see: (a) Hostetler, M. J.; Templeton, A. C.; Murray, R. W. *Langmuir* **1999**, *15*, 3782−3789. (b) Hostetler, M. J.; Green, S. J.; Stokes, J. J.; Murray, R. W. *J. Am. Chem. Soc.* **1996**, *118*, 4212−4213.

(20) It is known that GSH can form stable monolayer protected clusters (MPCs) by attachment to the gold surface through the sulfhydryl linkage. For examples of GSH MPCs, see: (a) Schaaff, T. G.; Knight, G.; Shafigullin, M. N.; Borkman, R. F.; Whetten, R. L. *J. Phys. Chem. B* **1998**, *102*, 10643−10646. (b) Schaaff, T. G.; Whetten, R. L. *J. Phys. Chem. B* **2000**, *104*, 2630−2641.

(21) The release of MMPC-bound protein and its subsequent regain of activity due to attenuation of nanoparticle surface charge have been observed in our earlier studies also. See ref 7.

(22) It is known that a longer chain effectively displaces a shorter chain ligand on the nanoparticle surface; however, the incorporation of a shorter chain on a nanoparticle featuring a longer chain ligand is not favorable. See: Ingram, R. S.; Hostetler, M. J.; Murray, R. W. *J. Am. Chem. Soc.* **1997**, *119*, 9175−9178.

(23) Slight aggregation was observed with MMPC **2** samples containing GSH, resulting in a thinner gel band. This might be due to some interaction of GSH with MMPC **2** at the higher gel concentrations; however, this interaction is not significant for enzyme reactivation at the lower MMPC concentrations used in the activity assay studies.

(24) Sandhu, K. K.; McIntosh, C. M.; Simard, J. M.; Smith, S. W.; Rotello, V. M. *Bioconjugate Chem.* **2002**, *13*, 3−6.

However, the specificity of MMPCs is required in other applications such as in vivo targeting of proteins, which is currently being investigated and will be reported in due course.

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Supporting Information Available: Synthesis and NMR of MMPC **1**, effect of GSH on β-galactosidase activity, and the ionic strength study. This material is available free of charge via the Internet at http://pubs.acs.org.

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