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Reduction-Sensitive Polymer-Shell-Coated Nanogels for Intracellular Delivery of Antigens

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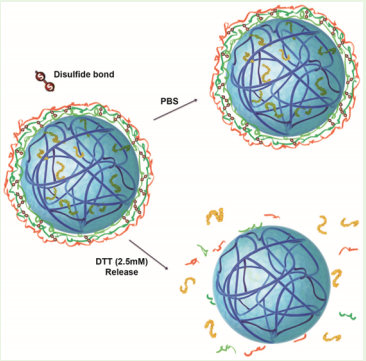
Supporting Information

ABSTRACT: Nowadays, layer-by-layer assembled microsized particles receive interest as drug delivery systems. In the present study, we report nanosized hydrogels loaded with a protein antigen that are coated with a disulfide cross-linked polymer shell. These disulfide bonds are stable in the nonreducing extracellular environment, but are reduced in the intracellular environment. This in turn leads to disintegration of the polymer shell and subsequent antigen release. Furthermore, we demonstrate the ability of these core−shell nanogels to boost the MHC class I antigen presentation by dendritic cells to $CD8^+$ T cells.



KEYWORDS: *layer by layer, ovalbumin, nanogel, disulfide, intracellular delivery*

Layer-by-Layer (LbL) technology has been used for the design of drug delivery systems with an appropriate coating for controlled release.[1,2] The major benefit of LbL assembly is its versatility in terms of materials that can be chosen as layer component to engineer colloidal objects with a wide variety of surface properties.[1−5] However, the clinical translation is still challenging because (1) the release rate of the encapsulated drugs in LbL-based drug delivery systems is mainly governed by the shell thicknesses and prone to burst release due to rapid swelling and/or disintegration under physiological conditions, and (2) LbL coatings require multistep procedures.[6−9] Many LbL assembled drug delivery systems developed so far have micrometer dimensions,[5,10−12] while nanoscale systems have been prepared by LbL coating of solid drugs or colloidal particles of silica, gold, metal oxides, etc., and removable core templates.[1,2,5,13,14] Also, LbL assembled particles have been developed to control the release of their payload in different manners,[15−17] and selective triggered release of encapsulated payloads at the site of action is preferred. For this purpose, the LbL shell should remain intact and impermeable under physiological conditions, but readily disassemble in response to a specific stimulus.[18−20] The reduction-sensitivity of disulfide bonds has received great attention for intracellular drug delivery because disulfides possess excellent stability in a nonreducing extracellular environment, but are prone to cleavage under reductive conditions in intracellular space.[21−24] This phenomenon has been exploited by a variety of drug carriers including disulfide-cross-linked LbL films and particles.[25−28]

Among medical interventions, vaccination has remarkable impact on global health. However, it remains a big challenge to elicit an effective cellular immune response to cancers. To generate antigen-specific cellular immune responses, the antigen needs to be processed and loaded into MHC class I molecule of dendritic cells (DCs) and presented to $CD8^+$ T cells.[29,30] Nowadays, many studies focus on subunit vaccines based on proteins, because these protein antigens are safer and more defined than live, attenuated vaccines. Unfortunately, these highly defined antigens are exogenous proteins with poor immunogenicity.[31] During the last decades, vaccine delivery systems based on nanoparticles have been developed to not only deliver antigens to DCs intracellularly but also facilitate antigen cross-presentation.[32−34] In the present paper, we report on a strategy that allows loading of protein antigens into nanogels followed by LbL coating with a reducible disulfide-cross-linked polymer shell. This shell keeps loaded antigen stably entrapped in nanogels in the nonreducing extracellular environment. In response to reductive conditions, this polymer shell can disassemble, resulting in the release of the protein payload (Scheme 1).

In the first step, cationic dextran nanogels with a diameter of approximately 200 nm and a zeta-potential of 22.6 mV were prepared from methacrylated dextran and trimethyl aminoethyl methacrylate (TMAEMA) by inverse mini-emulsion photo-

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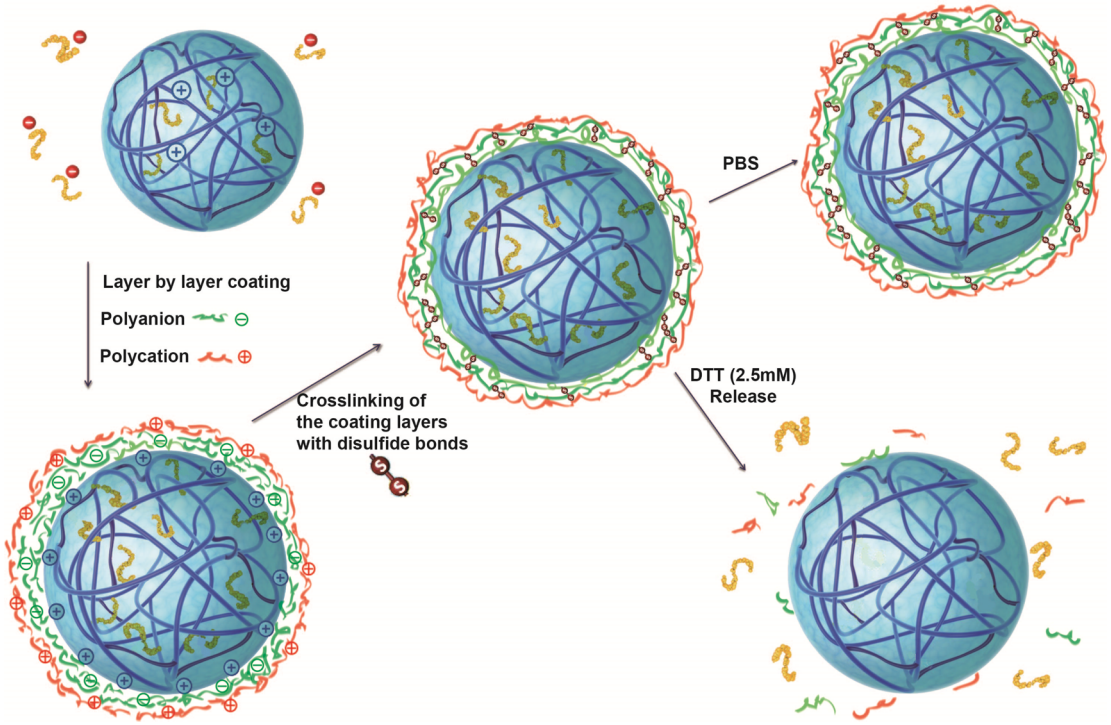
Scheme 1. Schematic Representation of the LbL Coating of Protein-Loaded Nanogels



polymerization.[35] The model antigen ovalbumin (OVA) was subsequently postloaded into the nanogels in a low ionic strength buffer (HEPES, 20 mM, pH 7.4) via electrostatic interaction as previously reported.[35] The nanogels maintained a positive surface charge of 21.3 mV and similar size of 200 nm after OVA loading.

Subsequently, oppositely charged polymers were used for LbL coating of the nanogels via electrostatic attraction.[36,37] For this purpose, first, the polymers used for the LbL coating should not penetrate into the nanogel core as this could result in desorption of the loaded protein due to competition for binding sites. Second, the LbL coating should be impermeable for the loaded protein after cross-linking and third, when non-cross-linked, the LbL coating should disassemble under physiological conditions. Therefore, polyanions and polycations with different molecular weights were prepared by free radical polymerization of respectively methacrylic acid (MAA) or TMAEMA, and a neutral hydrophilic comonomer, *N*-(2-hydroxypropyl) methacrylamide (HPMA) to tune the charge density (Scheme 2A, B, and Tables S1 and S2). The positively charged OVA loaded nanogels were alternately coated with the polyanion and polycation, and the zeta-potential and OVA loading during the coating process were monitored (Table S3). The nanogels incubated with the homopolymers of MAA and TMAEMA showed substantial release of OVA during the coating process, whereas the nanogels with copolymers containing pHPMA of high molecular weight and with reduced charge density retained most of the protein in HEPES buffer. PHPMA-*co*-MAA reversed the surface charge to −22.7 mV, and to +31.8 mV after coating with pHPMA-*co*-TMAEMA, indicating the successful coating of the charged polymers on the nanogels. When the nanogels with non-cross-linked shell were exposed to physiological ionic strength (PBS, 164 mM), more than 98% of the encapsulated OVA was released within 1 h (Table S3). In conclusion, pHPMA-*co*-MAA (molar ratio 1:1, M_n = 539kD, PDI = 1.8) and pHPMA-*co*-TMAEMA (molar ratio1:1, M_n = 302kD, PDI = 2.2) were found suitable for the LbL coating.

In the next step, pyridyldisulfide groups were introduced in both the polyanion and polycation to enable oxidative cross-linking of the LbL coating and consequently retain the OVA stably entrapped within the nanogels at physiological ionic strength, while also enabling to trigger dissociation of the polymer shell and subsequently release of the OVA antigen under reducing conditions. PHPMA-*co*-MAA-*co*-PDTEMA (*N*-[2-(2-pyridyldithio)] ethyl methacrylamide) with 11 mol % of pyridyldisulfide containing units and pHPMA-*co*-TMAEMA-*co*-PDTEMA with 15 mol % of pyridyldisulfide units were prepared (Scheme 2c, d) and alternating layers of these polymers were adsorbed onto the OVA loaded nanogels. Next, dithiothreitol (DTT) corresponding to 0.5 mol equiv of pyridyldisulfide groups of the coating polymers was added to cleave half of the pyridyldisulfide groups to yield thiol groups. Subsequently, these thiol groups reacted with the remaining 0.5 mol equiv of pyridyldisulfide groups by thiol disulfide exchange, resulting in a disulfide-cross-linked LbL coating. The cross-linking efficiency was checked by measuring the released 2-mercaptopyridine, which corresponded to 99% of reacted pyridyldisulfide groups. OVA loaded nanogels coated with 1, 2, and 3 disulfide-cross-linked polymer layers were prepared and nanogels with 2 non-cross-linked layers (with positive surface charge) were used as a control. The coating of each layer resulted in reversal of the ζ-potential (Table 1). The size of nanogels increased from 200 to 263 nm after coating of the first layer and no significant increase of size was found upon deposition of the second and third layer. This could be attributed to a larger mass of polymer that is adsorbed as the first layer (Table 1). As listed in Table 1, more than 90% of the loaded OVA remained in the nanogels after coating and cross-linking of the polymer shell.

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Scheme 2. Synthesis Route of Anionic and Cationic Polymers for LbL Assembly

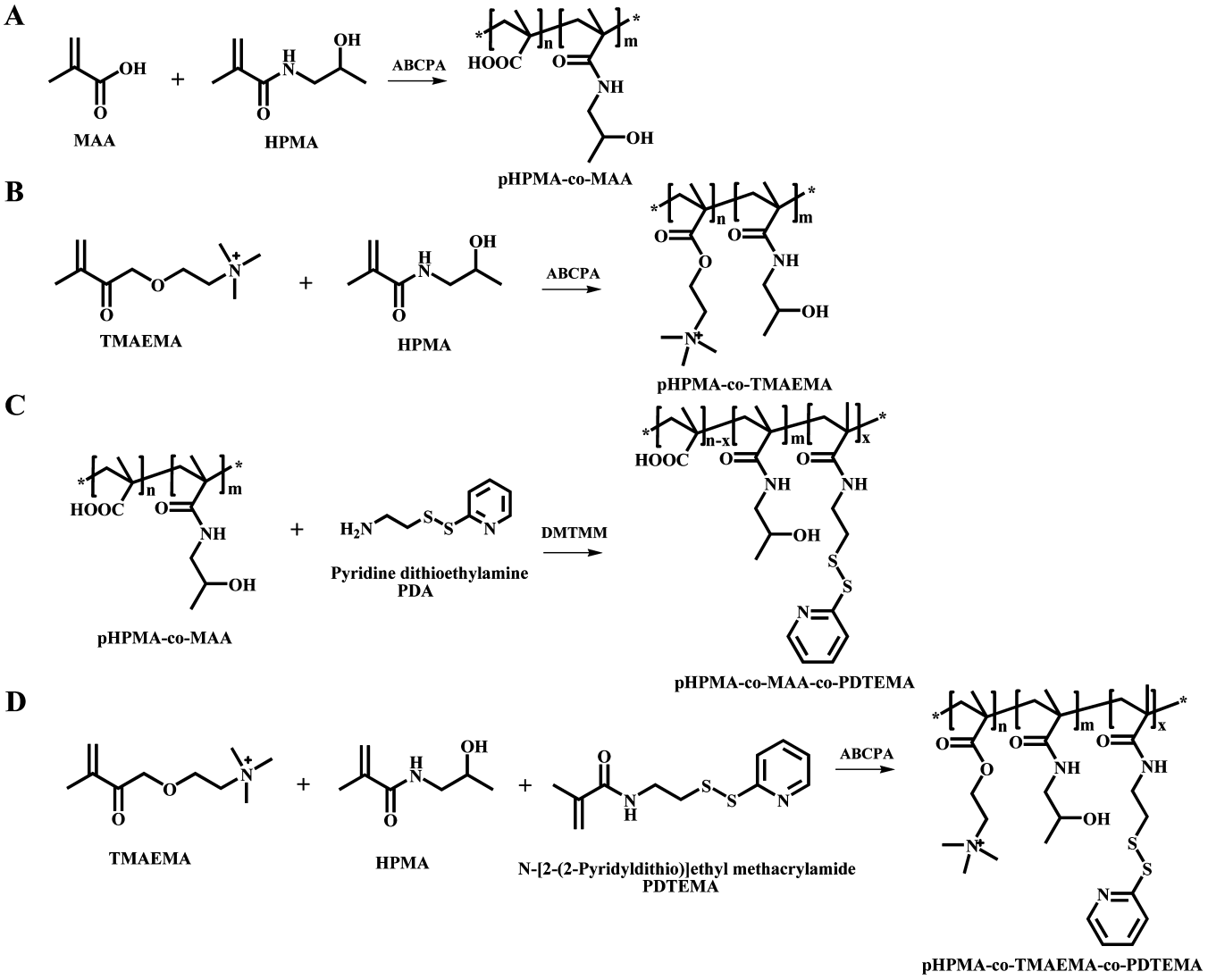

Table 1. *Z*-Average Hydrodynamic Diameter (Z_{ave}), Zeta Potential, Mass of Coated Polymers, Loading %, and Loading Efficiency (LE) of OVA-Loaded Nanogels (n = 3)

nanogels coated with	Z_{ave}[a] (nm)	ζ-potential (mV)	mass of polymer (mg/1 mg nanogels)[b]	LE (wt %)	loading (wt %)
1 disulfide-cross-linked layer	263 ± 5	−18.8 ± 0.5	0.35 ± 0.00	99.2 ± 0.1	22.6 ± 0.1
2 disulfide-cross-linked layers	271 ± 9	22.2 ± 0.9	0.38 ± 0.01	93.8 ± 0.6	21.2 ± 0.2
3 disulfide-cross-linked layers	278 ± 9	−27.0 ± 0.5	0.42 ± 0.01	92.2 ± 2.1	20.4 ± 0.1
2 noncross-linked layers	265 ± 8	27.0 ± 1.0	n.d.	83.3 ± 3.0	19.3 ± 0.1

[a]PDI for all formulations was <0.16. DLS showed particles with narrow PDI indicating that no significant aggregation occurred due to the coating procedure. [b]The method of quantification of the amount of polymers coated on nanogels is described in the Supporting Information.

The release of OVA from disulfide-cross-linked LbL coated nanogels was examined under physiological conditions (PBS, 164 mM, pH 7.4, 37 °C) and thereafter under reducing conditions (2.5 mM of DTT in PBS). Figure 1A shows that nanogels with a non-cross-linked shell release their payload rapidly within 1 h after incubation in PBS. In contrast, only ∼10% of the loaded OVA was released from nanogels with a disulfide-cross-linked LbL coating upon incubation in PBS for 8 h. This means that the cross-linked coating is indeed impermeable for OVA. Interestingly, these nanogels showed a triggered release of OVA upon incubation in PBS containing DTT (2.5 mM), due to the reduction of the disulfide bridges and desorption of the polymers. No significant difference of OVA release was found for nanogels coated with 1, 2, or 3 cross-linked layers. To visualize the coating layers on the cationic gels and the triggered release of OVA, we performed confocal laser scanning microscopy (CLSM) experiments using microgels rather than nanogels to avoid diffraction limitated resolution. Note that these microgels exhibit, apart from a larger size, similar properties as the nanogels.[35] The FITC-OVA loaded microgels were coated with either a 2 layer cross-linked or a 2 layer non-cross-linked LbL coating labeled with rhodamine B, followed by incubation in respectively HEPES, PBS with(out) DTT (2.5 mM in PBS). CLSM images (Figure

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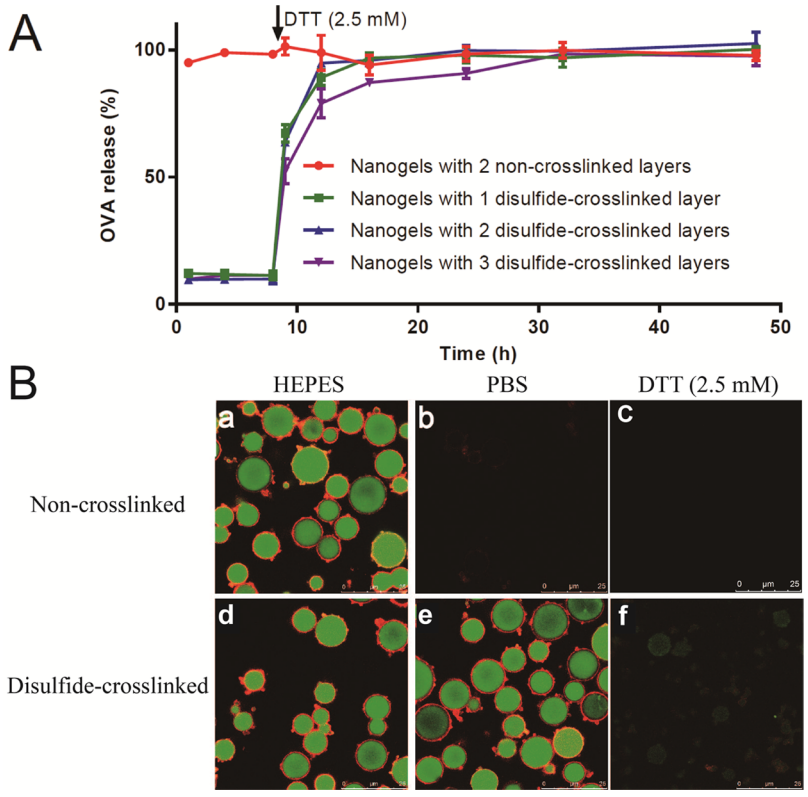

Figure 1. (A) OVA release from nanogels coated with polymer shell in PBS pH 7.4 at 37 °C; DTT was added to 2.5 mM final concentration at 8h. B) Confocal snapshots of FITC-OVA loaded microgels with 2 non-cross-linked layers and with 2 disulfide-cross-linked layers in HEPES, PBS, and DTT (2.5 mM).

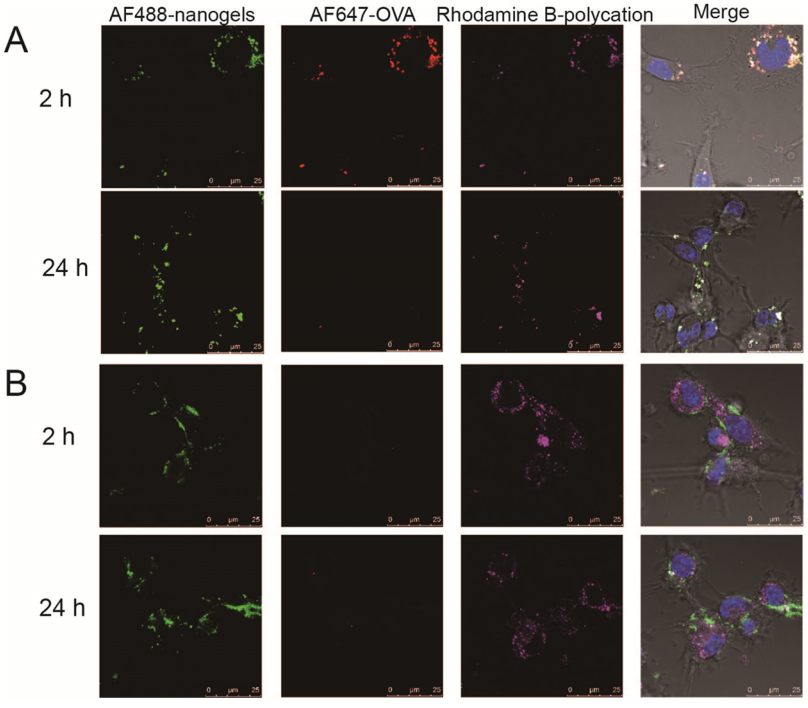

Figure 2. Intracellular release of OVA was studied by CLSM with AF647-OVA (red) loaded AF488-nanogels (green) coated with rhodamine B-polymer (magenta). The images were taken after D1 dendritic cells were incubated with nanogels coated with (A) 2 layers of disulfide-cross-linked shell, and (B) 2 layers of non-cross-linked shell for 2 and 24 h.

1Ba and Bd) demonstrate that microgels with both cross-linked and non-cross-linked shell show a clear ring of red fluorescence (originating from the rhodamine B-labeled polycation) surrounding the OVA (green fluorescence) loaded microgel, indicating a stable LbL coating onto the microgels in HEPES buffer of low ionic strength. When the particles were incubated

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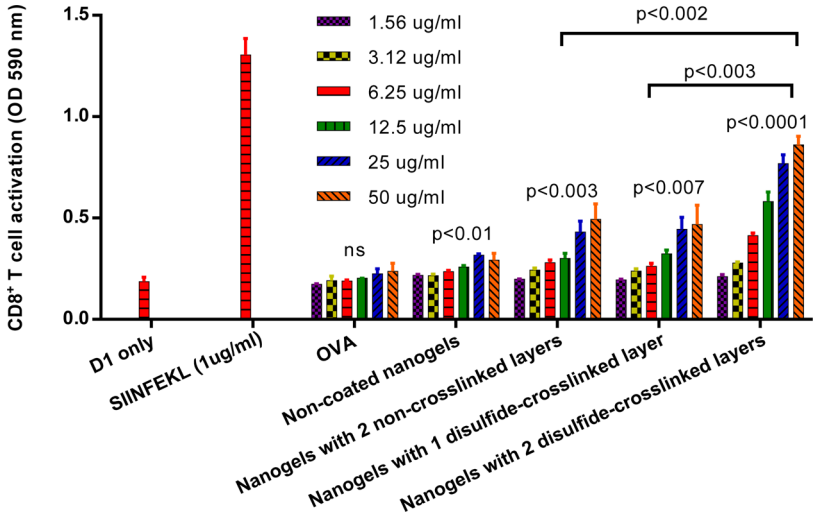

Figure 3. Activation of SIINFEKL-specific CD8$^+$ T cells (B3Z) after coculturing with DCs. DCs were incubated with various formulations for 24h with titrated amounts of OVA (n = 3). SIINFEKL (1 μg/mL) was used as positive control. Representative results from one out of three experiments are shown. Comparison between groups was conducted with Student's t test. Statistical significance was considered when $p < 0.05$. Statistical analyses were done by comparison with the D1 only group unless specified with markings. ns, not significant.

with PBS, the higher ionic strength of this medium resulted in destabilization of the non-cross-linked LbL coating and the release of OVA from the microgels (Figure 1Bb), whereas the cross-linked LbL coating remained intact upon exposure to PBS (Figure 1Be). Importantly, incubation of these particles in the presence of 2.5 mM DTT led to destabilization of the cross-linked LbL coating and triggered the release of OVA (Figure 1Bf).

To visualize the intracellular delivery of entrapped OVA, we loaded Alexa Fluor 647 (AF647) labeled OVA in nanogels that were labeled with Alexa Fluor 488 (AF488), and coated with rhodamine B labeled polymer. D1 is a well-defined immature murine dendritic cell line with proliferative capacity, high antigen uptake ability, and low T cell stimulatory efficiency. CLSM images were taken after incubation of the particles with D1 cells for 2 and 24 h (Figure 2). Images of cells incubated with nanogels coated with 2 disulfide-cross-linked layers having a positive surface charge showed colocalization of AF488-nanogels, AF647-OVA and rhodamine B-polycation after 2 h, indicating OVA was taken up with disulfide-cross-linked coated nanogels into the cells. After 24 h, limited signal of AF647-OVA was observed compared to the signal after 2 h incubation, while overlapping signals of AF488-nanogels and rhodamine B-polycation were still visible. Likely, the entrapped OVA was released from the nanogels in reductive cellular compartments and subsequently processed by DCs, which can be measured by antigen presentation assay.[38,39] On the other hand, limited fluorescence of OVA was observed in the pictures of nanogels with non-cross-linked shell after 2 and 24 h (Figure 2B), and the polycation was not colocalized with nanogels in the same compartments of cells, indicating that the coating polymers were disassociated from the nanogels (and subsequently taken up), while the protein was released before the nanogel particles were taken up by cells. These released free-OVA was not internalized as efficiently as those entrapped in nanogels, therefore barely any signal from free-OVA can be observed using the same experimental settings. Furthermore, when incubated free-OVA + empty nanogels coated with 2 disulfide-cross-linked layers with D1 cells (Figure S3), only nanogels and coating polymers were colocalized in the cell, whereas barely any signal of OVA was seen.

The ability of OVA loaded nanogels with disulfide-cross-linked shell to activate OVA-specific CD8$^+$ T cells (B3Z cells) was tested in vitro by an MHC class I antigen presentation assay (Figure 3, for cytotoxicity see Figure S4). Non-cross-linked and noncoated nanogels slightly enhanced T cell activation as compared to non treated cells, while the nanogels with cross-linked shell activated OVA-specific CD8$^+$ T cells to a significantly higher extent (factor of 2) than their non-cross-linked counterparts and bare nanogels. Noteworthy, nanogels with one cross-linked layer were less efficient in stimulating CD8$^+$ T cells presumably due to the negative surface charge resulting in low uptake by D1 cells.[40] The significantly improved MHC class I antigen presentation by nanogels with 2-layer cross-linked shell indicates that considerable quantities of OVA were delivered and processed in D1 cells by these cationic nanogels (as shown in Figure 2A).

To conclude, we have developed nanogels in which a protein antigen (OVA) was entrapped followed by deposition of a disulfide-cross-linked LbL coating. The antigen remained stably encapsulated under nonreducing conditions, but was readily released in response to a reductive environment. Moreover, these particles showed delivery of the encapsulated antigens to DCs, and facilitated the MHC class I antigen cross-presentation. Therefore, delivery of antigen to DCs exploiting reduction-sensitivity of disulfide systems can be an efficient way to boost the MHC class I antigen presentation. However, further research is required to study the fate of encapsulated antigen in DCs and the mechanism of cross-presentation facilitated by disulfide-cross-linked LbL coated nanogels. The application of these nanogels can be expanded to delivery of cationic biotherapeutics by replacing the TMAEMA monomers with methacrylic acid followed by LbL coating with cationic polymers as the first layer, which is currently under investigation in our lab. Hence, this technology can be applied as a general platform for encapsulating a wide variety of biotherapeutics.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.6b00651.

Materials and methods, supplementary figures, table describing polymer synthesis, characterization, LbL coating, cross-linking, and cell study (PDF)

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Notes

The authors declare no competing financial interest.

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