# Toward Therapeutic Delivery with Layer-by-Layer Engineered Particles

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**ABSTRACT** Layer-by-layer (LbL)-engineered particles have recently emerged as a promising class of materials for applications in biomedicine, with studies progressing from *in vitro* to *in vivo*. The versatility of LbL assembly coupled with particle templating has led to engineered particles with specific properties (*e.g.*, stimuli-responsive, high cargo encapsulation efficiency, targeting), thus offering new opportunities in targeted and triggered therapeutic release. This Perspective highlights an important development by Poon *et al.* on tumor targeting *in vivo* using LbL-engineered nanoparticles containing a pH-responsive poly(ethylene glycol) (PEG) surface layer. Further, we summarize recent progress in the application of LbL particles in the fields of drug, gene, and vaccine delivery and cancer imaging. Finally, we explore future directions in this field, focusing on the biological processing of LbL-assembled particles.

The convergence of nanotechnology and biomedicine underpins the development of novel materials with unique properties that enable highly specific therapeutic intervention at the cellular and patient level.[1] Particle-based therapeutics offer distinct advantages over traditional therapies, including targeted delivery of high payloads of multiple drugs, controlled drug release, and bypassing cellular surface multi-drug-resistance mechanisms.[2] For example, liposomes have been extensively studied and applied for improved treatment of cancer and infectious diseases.[3] Dendrimers, polymer micelles, and polymersomes are some further examples of particles being examined for delivery applications.[4,5] In recent years, there has also been growing interest in developing particles engineered through the highly versatile layer-by-layer (LbL) assembly method for biomedical applications (Table 1).[6] Typically, LbL particles are formed by the consecutive deposition of interacting polymers (*e.g.*, through electrostatic interactions and/or hydrogen bonding) onto particle templates, resulting in the formation of ultrathin, multilayered polymer coatings on particles of different size (from *ca.* 10 nm to several micrometers), shape, and composition.[7,8] These coated particles are referred to as core–shell particles. The use of a sacrificial particle template, however, allows the generation of capsules upon selective removal of the core particles. Recent significant advances in the development of LbL particles have enabled efficient cargo encapsulation, triggered release, and antibody-mediated targeting.[9] In addition, LbL capsules have recently been combined with liposomes to form multicomponent, triggerable delivery and microreactor systems.[10] Collectively, these results exemplify the versatility of LbL-engineered particles for integration with diverse polymeric systems, offering significant potential for multistaged controlled release.

In this issue of *ACS Nano*, Poon *et al.* report the generation and use of LbL-engineered, pH-responsive nanoparticles for systemic targeting of hypoxic tumors *in vivo*, highlighting the potential of the multilayered nanoparticles for targeted cancer therapy (Figure 1).[11] Carboxylated quantum dots (QDs) were sequentially coated with iminobiotin-functionalized poly(L-lysine) (PLLib), neutravidin (nav), and biotin-functionalized poly(ethylene glycol) (PEG), resulting in pH-responsive QD/PLLib/nav/PEG nanoparticles. In addition to increasing the circulation time, the

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**TABLE 1. Summary of the LbL-Engineered Particles Discussed in This Perspective, Including the Core Template, Composition of the Assembled Layers, Surface Functionalization, Particle Size, Biological System Investigated, and Application[a]**

| template | assembled layers | surface modification | diameter | biological system | application |
|---|---|---|---|---|---|
| | | | | core−shell particles | |
| QD | PLLib/nav | PEG | ∼75 nm | mouse tumor, *in vivo*[11] | tumor targeting |
| QD | PLL/DS | HA | ∼50 nm | mouse tumor, *in vivo*[25] | |
| Au | PAH/PSS | F-HPMA | ∼20 nm | human monocytic cells, *in vitro*[8] | chemotherapeutic delivery |
| Au | PEI/siRNA | | 27 nm | Chinese hamster ovary cells, *in vitro*[20] | siRNA delivery |
| | | | | capsules[b] | |
| $SiO_2$ | $PVPON_{Alk}$ | huA33 mAb | 800 nm | human colorectal cancer cells, *in vitro*[16] | targeting |
| $CaCO_3$ | DS/pAGR | | 2−3 $\mu$m | mouse embryonic fibroblasts, *in vitro*[22] | prodrug delivery |
| | | | | mouse bone marrow-derived dendritic cells, *ex vivo*[15] | vaccine delivery |
| | | | | mice, *in vivo*[23] | |
| $SiO_2$ | $PMA_{SH}$ | | 800 nm to 2 $\mu$m | human colorectal cancer cells, *in vitro*[12] | chemotherapeutic delivery |
| | | | | human prostate cancer cells, *in vitro*[17] | siRNA delivery |
| | | | | human blood cells, *ex vivo*[14] | vaccine delivery |
| | | | | transgenic mice, *in vivo*[21] | |
| $SiO_2$ | $PGA_{Alk}$ | | 3 $\mu$m | human colorectal cancer cells, *in vitro*[18] | chemotherapeutic delivery |
| | | | | multi-drug-resistant human colorectal cancer cells, *in vitro*[19] | |

[a] QD = quantum dots; PLLib = poly(L-lysine) functionalized with iminobiotin; nav = neutravidin; PEG = poly(ethylene glycol) functionalized with biotin; PLL = poly(L-lysine); DS = dextran sulfate; PAH = poly(allylamine); PSS = poly(styrenesulfonate); PEI = poly(ethylenimine); siRNA = short interfering RNA; $PVPON_{Alk}$ = alkyne-functionalized poly(*N*-vinylpyrrolidone); pAGR = poly(L-arginine); $PMA_{SH}$ = thiolated poly(methacrylic acid); $PGA_{Alk}$ = alkyne-functionalized poly(L-glutamic acid); HA = hyaluronic acid; F-HPMA = *N*-(2-hydroxypropyl)methacrylamide, functional terpolymer; huA33 mAb = humanized A33 monoclonal antibody. [b] The particle templates are removed to form capsules.

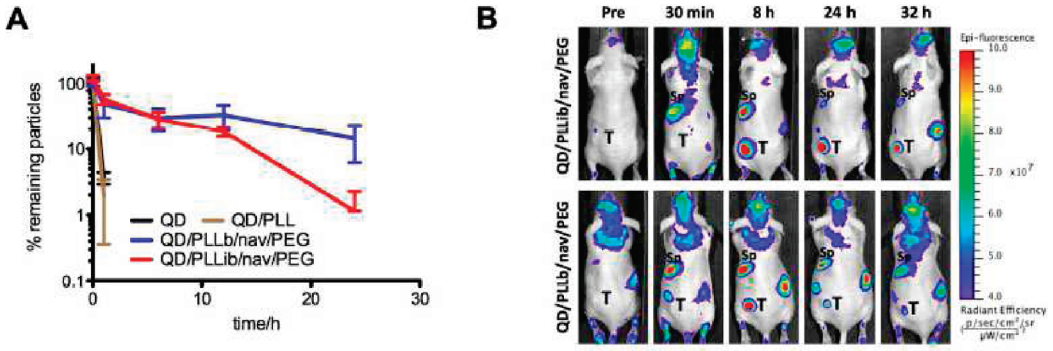
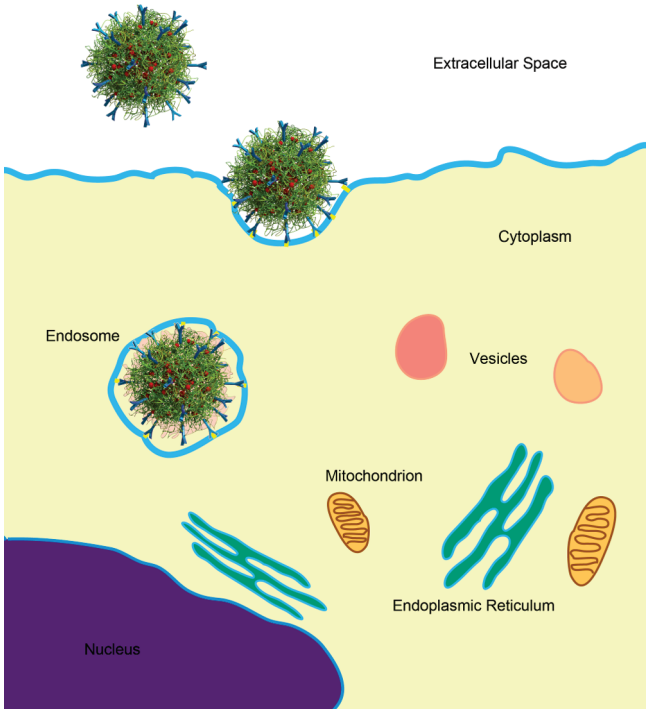



**Figure 1. (A) Blood circulation profiles of QD, QD/PLL, QD/PLLib/nav/PEG, and QD/PLLb/nav/PEG LbL-engineered nanoparticles. (B) Dorsal scans of representative mice bearing MDA-MB-435 tumors (left hind flank) by intravital imaging, showing the biodistribution of LbL nanoparticles. *T* = tumor; Sp = spleen. Reproduced from ref 11. Copyright 2011 American Chemical Society.**

PEG layer is selectively eroded by the acidity in the hypoxic tumor microenvironment. Consequently, exposure of positively charged layers beneath the PEG layer leads to enhanced tumor retention of the nanoparticles. These results underscore the importance of the surface properties of LbL particles on exploiting the native environment in biological systems to achieve desirable cellular responses.

In this Perspective, we highlight recent progress made in developing and using LbL-engineered particles for therapeutic delivery, focusing on three keys aspects: (i) engineering LbL particles to interact with specific classes of cells; (ii) cellular processing of LbL particles for drug, gene, and vaccine delivery to cells and animal models; and (iii) key challenges associated with further development of LbL particles to achieve significant outcomes in biomedicine.

Controlling specific interactions between LbL-engineered particles and only targeted cells is of paramount importance for targeted therapies and represents an immense challenge. Recent studies in this area have demonstrated that LbL particles with diameters ranging from ∼20 nm up to several micrometers are readily internalized by diverse types of cells, including epithelial cells,[12,13] monocytes,[8,14] and dendritic cells (DCs),[15] through endocytosis. Cell selectivity can be achieved by conjugating specific antibodies to LbL capsules. For example, Kamphuis *et al.* reported the coupling of azide-functionalized antibodies to alkyne-containing poly(*N*-vinylpyrrolidone) (PVPON) capsules




**Scheme 1. Cellular uptake of LbL-engineered particles. The particles are internalized by cells through endocytosis into endosomes, leading to high intracellular concentrations of therapeutic cargo.**

through click chemistry.[16] The antibody-functionalized PVPON capsules were found to specifically bind to colorectal cancer cells *in vitro*, even when the targeted cells comprised less than 0.1% of the total cell population. The presence of targeting antibodies,[16] combined with control over surface properties such as surface charge as demonstrated by Poon *et al.*,[11] is likely to enhance specific cellular interaction of LbL particles within a subset of cells.

The complex endocytic processing pathways that "guide" particles (and encapsulated therapeutics) to specific intracellular environments and target locations are integral to the delivery of therapeutics. As a result, there has been growing interest in better understanding the internalization and intracellular trafficking of LbL-engineered particles. Several reports have focused on the cellular uptake of LbL polymer capsules.[12,13,15,17] De Koker *et al.* demonstrated that, during the association of dextran sulfate/poly-L-arginine (DS/pARG) LbL capsules with DCs, the cell membrane extends over the capsules, forming cup-shaped invaginations.[15] Similarly, Yan *et al.* observed the formation of plasma membrane protrusions in human colorectal cancer cells when interacting with thiolated poly(methacrylic acid) ($PMA_{SH}$) LbL capsules, suggesting that macropinocytosis and phagocytosis are the predominant routes for cellular uptake of capsules.[12] It has also been shown that the internalized LbL capsules are deformed and located in membrane-enclosed compartments, which further mature to late endosomes or lysosomes.[12,13] In a more recent study, Becker *et al.* investigated the intracellular responses at the protein level following internalization of $PMA_{SH}$ LbL capsules.[17] It was shown that the cellular uptake of $PMA_{SH}$ capsules in human prostate cancer cells resulted in a concomitant decrease in the expression of antiapoptotic proteins (*e.g.*, survivin) and the activation of autophagy (a process for degrading cellular compartments). Given the close relationship between lysosomes and autophagosomes, the inherent endocytic network will have a significant impact on the internalization, transportation, degradation, and cargo release of LbL-assembled capsules; hence, further studies are required to elucidate the factors that govern cellular uptake and trafficking of LbL-engineered particles.

Delivering drugs encapsulated within LbL-engineered particles has significant potential for improving the bioavailability of a range of therapeutics, including chemotherapeutic compounds and short interfering RNA (siRNA) (Scheme 1). A number of LbL particles have been developed for the delivery of therapeutics, such as doxorubicin (DOX) and siRNA.[8,12,17−20] Recently, Ochs *et al.* reported biodegradable, click LbL capsules based on poly(L-glutamic acid) (PGA) that provide a high level of control over drug loading and release.[18] In that study, it was shown that DOX-loaded PGA capsules are internalized by human colorectal cancer cells, and following endocytosis, DOX was effectively released from the capsules and further translocated to the nucleus. These drug-loaded capsules resulted in a significant decrease in cell viability. By exploiting the versatility of LbL assembly, these particles can be engineered to incorporate a range of polymer−drug conjugates for targeted applications. The entry of particles *via* endocytosis can also be used to overcome multi-drug resistance in cancer cells, which often limits the effectiveness of chemotherapeutics. This was demonstrated for drugs encapsulated in LbL-assembled PGA capsules delivered *via* endocytic routes, which bypass multi-drug-resistant efflux pumps (*e.g.*, P-glycoprotein) located on the cell membrane surface, leading to sensitization in resistant cancer cells.[19] As an additional requirement for bioavailability, particle-based delivery systems should also direct and release therapeutics to appropriate intracellular sites of action for optimum activity. The aforementioned lysosomal intracellular fate is unsuited for the delivery of, for example, siRNA, which is rapidly degraded in lysosomes. In such cases, endosomal escape of the siRNA and trafficking to the

cytoplasm is required. Elbakry *et al.* prepared LbL-engineered gold nanoparticles coated with siRNA and poly(ethylenimine) (PEI).[20] The internalized nanoparticles were detected in caveolae-like structures and demonstrated effective knockdown of enhanced green fluorescent protein (EGFP) expression in stably transfected Chinese hamster ovary cells (CHO-K1), indicating the presence of siRNA in the cytoplasm. In another study, Becker *et al.* reported that $PMA_{SH}$ capsules containing siRNA that target survivin inhibited the expression of survivin protein in PC3 prostate cancer cells *in vitro*, suggesting that siRNA was released into the cytoplasm.[17] However, as noted earlier, empty $PMA_{SH}$ capsules also caused a decrease in the expression of survivin. This emphasizes the need to unravel the complex interactions that can occur between particles and cells.

Understanding the cellular trafficking of LbL particles is also of importance for vaccine development. The effective delivery of proteins and peptides to antigen-presenting cells (APCs) is a major barrier to the development of vaccines, as proteins and peptides are subject to degradation in serum and are too large to cross cellular membranes. To address these issues, De Rose *et al.* pioneered the use of LbL capsules for antigen delivery.[14] It was shown that the surface properties predominantly govern the binding of LbL capsules to the APCs in human blood. On the basis of this analysis, model HIV vaccine peptides (KP9) were encapsulated into redox-responsive ($PMA_{SH}$) capsules, and effective activation of CD8 T cells *ex vivo* was demonstrated. In a subsequent study, the immunostimulatory capability of $PMA_{SH}$ capsules was further demonstrated as a vaccine vector for proteins and peptides in transgenic mice.[21] Using ovalbumin (OVA) and OVA-derived immunogenic peptides as model vaccines, it was further shown that OVA-containing $PMA_{SH}$ capsules successfully activate OVA-specific CD4 and CD8 T cells following cellular uptake of the capsules in APCs *in vitro*. These capsules were also found to effectively stimulate the proliferation of OVA-specific CD4 and CD8 T cells in mice following intravenous administration. The capsules resulted in at least a 6-fold greater proliferation of CD8 T cells and a 70-fold greater proliferation of CD4 T cells (in comparison to the OVA protein alone), highlighting the potential of LbL capsules for the induction of durable and functional immune responses *in vivo*. The greater activation of CD4 T cells compared to CD8 T cells shown in this *in vivo* study is in agreement with the primary lysosomal internalization pathway demonstrated by other studies *in vitro*, suggesting that there could be important correlations between *in vitro* and *in vivo* behavior of LbL particles and should be explored in future studies. In a similar study, De Koker *et al.* demonstrated the applicability of DS/pARG LbL capsules for vaccine delivery in DCs.[15] Following endolysosomal uptake, the capsule wall was ruptured, probably due to the enzymatic degradation by lysosomal proteases, and both the endoplasmic reticulum (ER) and mitochondria appeared to interact indirectly with the ruptured capsules. Using OVA as a model antigen, it was demonstrated that OVA is processed by proteases into peptides, which resulted in both MHC Class I and II presentation to CD8 and CD4 T cells, respectively. Rivera-Gil *et al.* further examined enzymatic degradation of DQ-OVA delivered by DS/pARG LbL capsules in mouse fibroblasts, suggesting that the intracellular processing can be controlled by using different degradable polymers.[22] More recently, DS/pARG capsules were explored as antigen-delivery vectors for pulmonary immunization.[23] It was shown that these capsules were taken up by APCs after pulmonary vaccination and activated the adaptive (T cell) immune responses.

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In addition to biological processing at the cellular level, effective systemic delivery also requires selective tissue distribution and improved pharmacokinetic properties. This is important for developing nanotechnology-based therapeutics, and the Food and Drug Administration (FDA) and the Alliance for NanoHealth (ANH) have listed the biodistribution of nanoparticles as a top priority.[24] The circulation time and biodistribution of LbL-engineered particles following systemic administration have been evaluated in several recent studies. It was shown by intravital fluorescence imaging over a period of 48 h that the enhanced permeability and retention (EPR) effect from the leaky tumor tissue produces the initial targeting of QD/PLLib/nav/PEG nanoparticles.[11] The subsequent change in the surface of the nanoparticles, caused by the acidity in the tumor tissue, enhances retention of the particles in the tumor. Poon *et al.* also explored alternative approaches to control the pharmacokinetic characteristics of nanoparticles by exploiting the modular nature of LbL assembly.[25] On the basis of the electrostatic interactions between DS and PLL, LbL-coated QD nanoparticles were formed. The pharmacokinetic characteristics of

these LbL nanoparticles *in vivo* were evaluated, and it was shown that the number of layers and the surface chemistry are key factors for layer stability and nanoparticle biodistribution. Furthermore, it was demonstrated that the circulation time of DS/PLL LbL nanoparticles could be extended by incorporating hyaluronic acid (HA) as the outer layer. These results are promising and suggest that LbL particles can be further engineered to improve pharmacokinetics, tumor accumulation, and tumor penetration.

## OUTLOOK AND FUTURE CHALLENGES

Although early successes in generating and developing LbL-engineered particles for biomedical applications are promising, they also point to important future challenges. Understanding the dynamic interactions between LbL particles and biological systems is fundamental for developing safe and effective delivery systems. An understanding of the complex mechanisms of cellular uptake and processing of LbL-engineered particles is still in its infancy. Identification of the molecular assemblies and signaling pathways involving endocytosis of these particles will provide important insights into key questions, such as: How do particle–cell interactions influence intracellular trafficking and cargo release? How do cells orchestrate the spatial assembly of intracellular molecules to respond to the presence of particles? Detailed studies on the interactions and downstream cellular responses between particles and cells should lead to greater understanding of the intricate regulatory networks inside cells. Besides the pharmacokinetics in disease models, the biocompatibility and toxicity *in vivo* following various administration routes is yet to be determined. In addition, further research needs to be conducted on linking the biological responses to the intrinsic physicochemical properties of LbL particles. Since LbL assembly allows a high level of control over individual specific properties of the final particles, this approach represents an exciting paradigm to attain detailed knowledge of independent and coupled effects that material variables have on biological responses. Identification of specific attributes of the particles will ultimately allow the design and construction of particles for controlled biological interactions. Addressing these questions is likely to accelerate progress in developing LbL-engineered particles for biomedicine.

**Detailed studies on the interactions and downstream cellular responses between particles and cells should lead to greater understanding of the intricate regulatory networks inside cells.**

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## REFERENCES AND NOTES

1. Duncan, R. Polymer Conjugates as Anticancer Nanomedicines. *Nat. Rev. Cancer* **2006**, *6*, 688–701.
2. Heath, J. R.; Davis, M. E. Nanotechnology and Cancer. *Annu. Rev. Med.* **2008**, *59*, 251–265.
3. Petros, R. A.; DeSimone, J. M. Strategies in the Design of Nanoparticles for Therapeutic Applications. *Nat. Rev. Drug Discovery* **2010**, *9*, 615–627.
4. van Dongen, S. F.; De Hoog, H. P.; Peters, R. J.; Nallani, M.; Nolte, R. J.; van Hest, J. C. Biohybrid Polymer Capsules. *Chem. Rev.* **2009**, *109*, 6212–6274.
5. Sanson, C.; Diou, O.; Thévenot, J.; Ibarboure, E.; Soum, A.; Brûlet, A.; Miraux, S.; Thiaudière, E.; Tan, S.; Brisson, A.; *et al.* Doxorubicin Loaded Magnetic Polymersomes: Theranostic Nanocarriers for MR Imaging and Magneto-Chemotherapy. *ACS Nano* **2011**, *5*, 1122–1140.
6. Becker, A. L.; Johnston, A. P. R.; Caruso, F. Layer-by-Layer Assembled Capsules and Films for Therapeutic Delivery. *Small* **2010**, *6*, 1836–1852.
7. Caruso, F.; Caruso, R. A.; Möhwald, H. Nanoengineering of Inorganic and Hybrid Hollow Spheres by Colloidal Templating. *Science* **1998**, *282*, 1111–1114.
8. Schneider, G. F.; Subr, V.; Ulbrich, K.; Decher, G. Multifunctional Cytotoxic Stealth Nanoparticles. A Model Approach with Potential for Cancer Therapy. *Nano Lett.* **2009**, *9*, 636–642.
9. Such, G. K.; Johnston, A. P. R.; Caruso, F. Engineered Hydrogen-Bonded Polymer Multilayers: From Assembly to Biomedical Applications. *Chem. Soc. Rev.* **2011**, *40*, 19–29.
10. Städler, B.; Chandrawati, R.; Price, A. D.; Chong, S.-F.; Breheney, K.; Postma, A.; Connal, L. A.; Zelikin, A. N.; Caruso, F. A Microreactor with Thousands of Subcompartments: Enzyme-Loaded Liposomes within Polymer Capsules. *Angew. Chem., Int. Ed.* **2009**, *48*, 4359–4362.
11. Poon, Z.; Chang, D.; Zhao, X.; Hammond, P. T. Layer-by-Layer Nanoparticles with a pH Sheddable Layer for *In Vivo* Targeting of Tumor Hypoxia. *ACS Nano* **2011**, DOI: 10.1021/nn200876f.
12. Yan, Y.; Johnston, A. P. R.; Dodds, S. J.; Kamphuis, M. M. J.; Ferguson, C.; Parton, R. G.; Nice, E. C.; Heath, J. K.; Caruso, F. Uptake and Intracellular Fate of Disulfide-Bonded Polymer Hydrogel Capsules for Doxorubicin Delivery to Colorectal Cancer Cells. *ACS Nano* **2010**, *4*, 2928–2936.
13. Javier, A. M.; Kreft, O.; Semmling, M.; Kempter, S.; Skirtach, A. G.; Bruns, O. T.; Del Pino, P.; Bedard, M. F.; Rädler, J.; Kas, J.; *et al.* Uptake of Colloidal Polyelectrolyte-Coated Particles and Polyelectrolyte Multilayer Capsules by Living Cells. *Adv. Mater.* **2008**, *20*, 4281–4287.
14. De Rose, R.; Zelikin, A. N.; Johnston, A. P. R.; Sexton, A.; Chong, S.-F.; Cortez, C.; Mulholland, W.; Caruso, F.; Kent, S. J. Binding, Internalization, and Antigen Presentation of Vaccine-Loaded Nanoengineered Capsules in Blood. *Adv. Mater.* **2008**, *20*, 4698–4703.
15. De Koker, S.; De Geest, B. G.; Singh, S. K.; De Rycke, R.; Naessens, T.; Van Kooyk, Y.; Demeester, J.; De Smedt, S. C.; Grooten, J. Polyelectrolyte Microcapsules as Antigen Delivery Vehicles to Dendritic Cells: Uptake, Processing and Cross-Penetration of Encapsulated Antigens. *Angew. Chem., Int. Ed.* **2009**, *48*, 8485–8489.
16. Kamphuis, M. M. J.; Johnston, A. P. R.; Such, G. K.; Dam, H. H.; Evans, R. A.; Scott, A. M.; Nice, E. C.; Heath, J. K.; Caruso, F. Targeting of Cancer Cells Using Click-Functionalized Polymer Capsules. *J. Am. Chem. Soc.* **2010**, *132*, 15881–15883.
17. Becker, A. L.; Ivan Orlotti, N.; Folini, M.; Cavalieri, F.; Zelikin, A. N.; Johnston, A. P. R.; Zaffaroni, N.; Caruso, F. Redox-Active Polymer Microcapsules

for the Delivery of a Survivin-Specific siRNA in Prostate Cancer Cells. *ACS Nano* **2011**, *5*, 1335–1344.
18. Ochs, C. J.; Such, G. K.; Yan, Y.; van Koeverden, M. P.; Caruso, F. Biodegradable Click Capsules with Engineered Drug-Loaded Multilayers. *ACS Nano* **2010**, *4*, 1653–1663.
19. Yan, Y.; Ochs, C. J.; Such, G. K.; Heath, J. K.; Nice, E. C.; Caruso, F. Bypassing Multidrug Resistance in Cancer Cells with Biodegradable Polymer Capsules. *Adv. Mater.* **2010**, *22*, 5398–5403.
20. Elbakry, A.; Zaky, A.; Liebl, R.; Rachel, R.; Goepferich, A.; Breunig, M. Layer-by-Layer Assembled Gold Nanoparticles for siRNA Delivery. *Nano Lett.* **2009**, *9*, 2059–2064.
21. Sexton, A.; Whitney, P. G.; Chong, S.-F.; Zelikin, A. N.; Johnston, A. P. R.; De Rose, R.; Brooks, A. G.; Caruso, F.; Kent, S. J. A Protective Vaccine Delivery System for *In Vivo* T Cell Stimulation Using Nanoengineered Polymer Hydrogel Capsules. *ACS Nano* **2009**, *3*, 3391–3400.
22. Rivera-Gil, P.; De Koker, S.; De Geest, B. G.; Parak, W. J. Intracellular Processing of Proteins Mediated by Biodegradable Polyelectrolyte Capsules. *Nano Lett.* **2009**, *9*, 4398–4402.
23. De Koker, S.; Naessens, T.; De Geest, B. G.; Bogaert, P.; Demeester, J.; De Smedt, S.; Grooten, J. Biodegradable Polyelectrolyte Microcapsules: Antigen Delivery Tools with Th17 Skewing Activity after Pulmonary Delivery. *J. Immunol.* **2010**, *184*, 203–211.
24. Sanhai, W. R.; Sakamoto, J. H.; Canady, R.; Ferrari, M. Seven Challenges for Nanomedicine. *Nat. Nanotechnol.* **2008**, *3*, 242–244.
25. Poon, Z.; Lee, J. B.; Morton, S. W.; Hammond, P. T. Controlling *In Vivo* Stability and Biodistribution in Electrostatically Assembled Nanoparticles for Systemic Delivery. *Nano Lett.* **2011**, *11*, 2096–2103.