Assembly of Layer-by-Layer Particles and Their Interactions with Biological Systems

Yan Yan, Mattias Björnmalm, and Frank Caruso*

Department of Chemical and Biomolecular Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

KEYWORDS: layer-by-layer, polyelectrolyte capsules, multilayered particles, drug delivery, endocytosis, tumor targeting

ABSTRACT: Multilayered polymer particles with a wide range of properties can be precisely engineered via the layer-by-layer technique using a multitude of templates and materials. Such multilayered particles are emerging as a powerful platform for biomedical applications. In this short review, we provide an overview of developments in the engineering of multilayered polymer particles, and describe recent progress in their utilization in biological systems. Emphasis is placed on engineering strategies that achieve different biological objectives, ranging from the triggered intracellular release of therapeutics to the induction of protective immunity in vivo through particle-enabled vaccination. Finally, we provide a critical outlook of the key issues associated with the continued development of multilayered particles for biological applications.

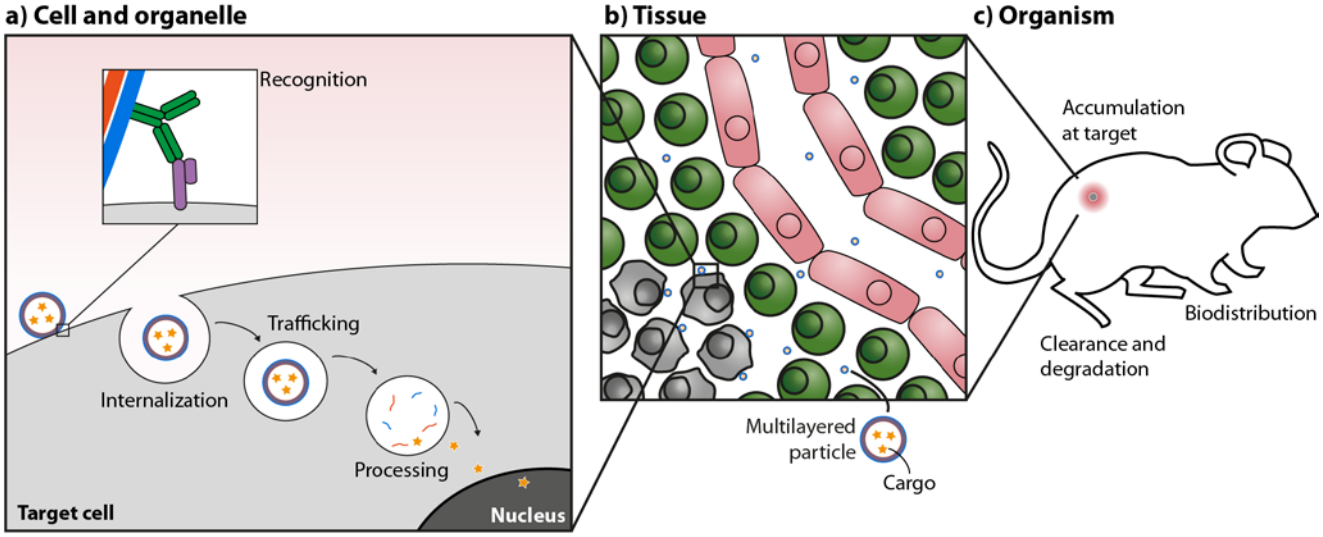
1. INTRODUCTION

Over the past three decades, the development of nanotechnology, which addresses the engineering of functional structures at the nanoscale, has led to the generation of a wide range of particle systems, such as gold nanoparticles (NPs), silica NPs, liposomes, and polymeric NPs.[1] These particulate systems have opened up exciting avenues to a broad range of biomedical applications, as they facilitate drug formulation and provide unprecedented sensitivity and specificity for diagnostic and therapeutic interventions.[2] One of the main goals of particle design for effective drug delivery is to improve drug specificity to the desired site of action, which can lead to higher efficacy and fewer adverse effects. This can be realized at three different levels: targeting tissue, targeting diseased cells within a tissue, and targeting intracellular compartments within a cell. The ability to precisely engineer particle properties that enable controlled particle mobility in biological systems holds the key to successful therapeutic delivery.

Layer-by-layer (LbL) assembly is a prominent method through which multilayered films with tailored properties can be fabricated on substrates.[3,4] This method can be used to prepare particles with multilayered shells through particle templating.[5,6] These surface-coated particles are referred to as core-shell particles, whereas the use of sacrificial templates results in the generation of hollow-shell particles (capsules) upon removal of the core particles.[5,6] Over the years, a range of physicochemical interactions, such as electrostatic interactions,[3] hydrogen bonding,[7] hydrophobic interactions,[8] and sequential covalent coupling,[9] have been exploited to prepare multilayered films. These developments have enabled the utilization of a broad spectrum of material building blocks, resulting in a suite of ultrathin LbL-assembled (multilayered) particles. Moreover, a range of templates with different morphologies, including inorganic particles (e.g., Au NPs and silica particles),[10] bacteria,[11] and red blood cells,[12] has been adapted to this technique. The diversity of templates and surface coating materials has given rise to an ever-expanding catalogue of LbL-assembled multilayered particles with various sizes, shapes, and chemical compositions. Owing to such versatility, multilayered particles have evolved to become a powerful platform for drug encapsulation, triggered drug release, and multicompartmentalized hierarchical assemblies.[13]

The utilization of LbL particles in biomedical applications is rapidly emerging with significant developments already achieved. For example, recent studies have demonstrated the use of multilayered particles for systemic tumor targeting[14] and for inducing protective immunity as a vaccine in animal models.[15] Furthermore, because a main feature of the LbL technique is precise control of individual properties of the final particles, a set of multilayered particles with well-defined physicochemical properties can be used for systematic studies on the influence of particle parameters on biological interactions. Multilayered particles can therefore offer an exciting paradigm for uncovering nano-bio interactions at multiple levels (Scheme 1), thus providing insights on specific biological effects linked to particle parameters, such as shape, size, composition and surface functionality. Such

knowledge is important to lay the foundation for the continued development of rationally designed LbL particles for biological applications.



Scheme 1. Interactions between multilayered particles with biological systems at various levels. a) Interactions with cells and subcellular organelles involve recognition of particles by target cells and subsequent intracellular processing for controlled release. b) Interactions with tissue involve accumulation and penetration of particles within a tissue to access the targeted cells. c) Interactions at the organism level involve particle pharmacokinetics and biodistribution, including particle clearance and degradation.

In this short review, we provide an overview of recent advances in developing and using multilayered polymer particles in biological systems. We focus on the assembly and biological interactions of multilayered particles, which include four key aspects: (i) evolution of the assembly methods used for preparing multilayered particles; (ii) cellular processing and intracellular drug delivery of multilayered particles; (iii) interactions between multilayered particles with targeted cells and tissues; and (iv) future challenges for multilayered particles in biological applications.

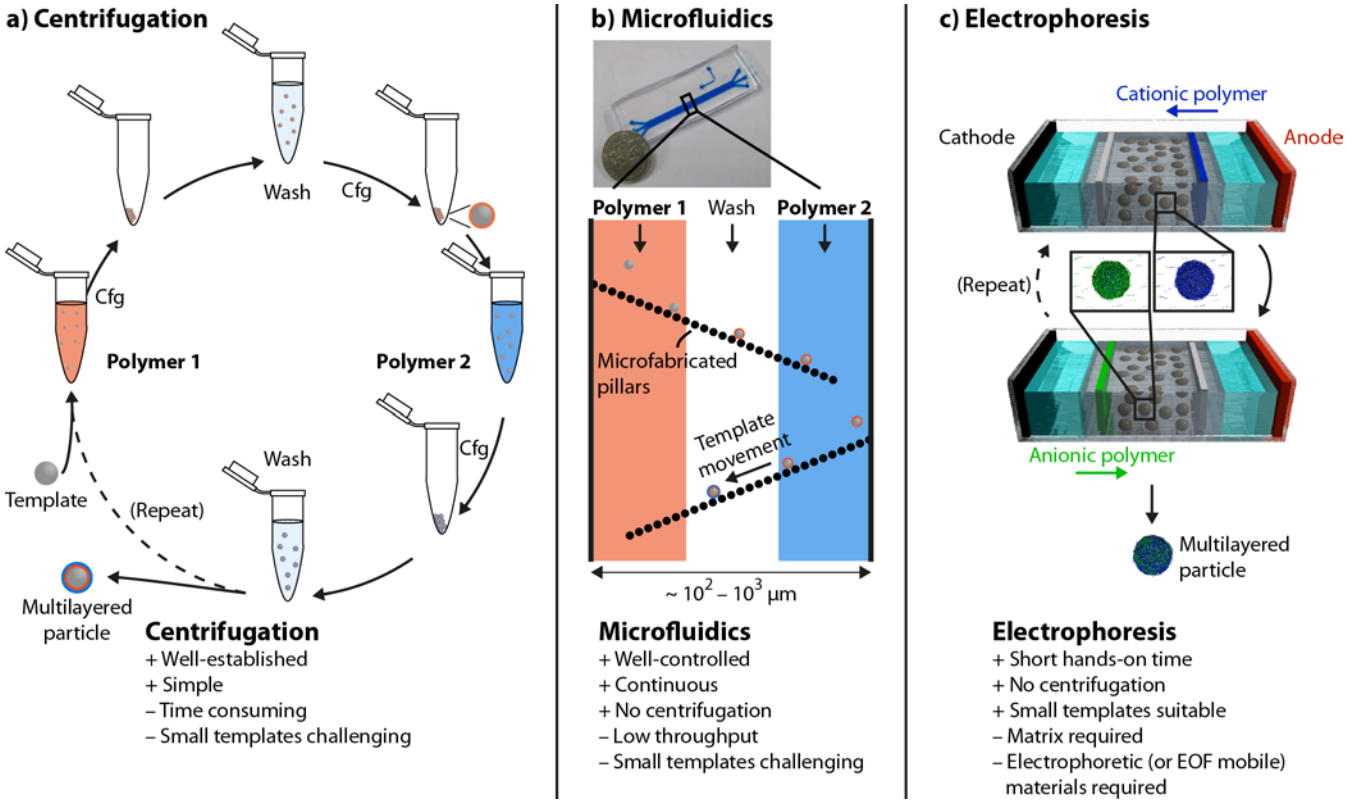
2. ASSEMBLY OF MULTILAYERED MICRO- AND NANOPARTICLES

Particles with polymer multilayers are typically assembled through the sequential deposition of materials via complementary interactions onto a template.[5,6] The main concept can be reduced to a series of steps involving mixing and separating templates with alternately deposited materials. The prototypical multilayered particle consists of nanometer-thick layers of alternating polyanions and polycations deposited onto a charged template.[5,6] Today, the layer-by-layer technology has expanded beyond its original scope allowing a diverse set of (i) templates, (ii) coating materials, and (iii) methods to be chosen from to engineer multilayered particles for specific applications. Herein, we provide a brief overview of the current state of these aspects in multilayered particle assembly. For a more in-depth discussion, several excellent recent reviews are available; for example by Tong et al.[16], De Koker et al.[17] and Such et al.[7]

The template onto which the layers are deposited may either be a sacrificial component that is dissolved to form hollow shell particles (capsules) or remain a part of the multilayered core-shell particles, e.g., as a drug reservoir for therapeutic applications or imaging agents. Two distinct classes exist; porous and non-porous templates.[10] An example of a non-porous template is silica particles, which are commercially available with a wide range of sizes (approximately 0.1 to 10 micrometers) and surface functionalities. To prepare capsules, the silica core can be dissolved using hydrofluoric acid. This approach has been successfully used to create capsules loaded with therapeutics, for example, functional DNA or protein.[18] Other well-established non-porous templates include gold particles, providing excellent contrast in electron microscopy and computerized tomography, and quantum dots, which are of interest because of their intrinsic fluorescence. Porous templates such as mesoporous silica and calcium carbonate particles have porous interiors that can be used to encapsulate compounds with a high loading capacity.[19-21] As the template governs the overall size and shape of the final particle, as well as affecting its internal structure and the way in which compounds can be loaded, the choice of template should be carefully considered together with the intended subsequent layering assembly, to yield the desired particles.

Many different materials have been used to form, or have been incorporated into, the multilayers on micro- and nanoparticles. Examples include: synthetic polymers;[6] natural polymers, such as DNA[22] and proteins;[23] metals, typically in the form of nanoparticles;[5] and lipids, as liposomes.[24] The first examples of multilayered particles using polyelectrolytes relied on electrostatic interactions to form the layers.[5,6] Subsequent studies have utilized complementary interactions such as hydrogen bonding,[7] hy-

drophobic interactions,[8] covalent bonding,[9] DNA hybridization,[25] van der Waals interactions,[26] and guest-host interactions.[27] Taken together, the plethora of available materials and driving forces enables the engineering of multilayered particles with a range of tunable properties, including stiffness, permeability, biofunctionalization (e.g., targeting antibodies[28]), and biodegradability.



Scheme 2. Several methods used for layer-by-layer assembly with benefits (+) and limitations (–) summarized. a) The centrifugation method involves centrifugation (cfg) to separate adsorbed and free polymer for each layer. b) Through microfluidics, polymer solutions can be confined in laminar flows and using microfabricated structures templates can be moved back and forth to achieve layering. Adapted with permission from ref 29. Copyright 2011 The Royal Society of Chemistry. c) 'Electrophoretic polymer assembly', where templates are immobilized inside an agarose matrix and layering can be accomplished by moving polymer using electrophoresis (or electro-osmotic flow (EOF)) through the matrix. Adapted with permission from ref 30. Copyright 2013 Wiley-VCH.

The conventional method for the assembly of multilayered particles uses centrifugation, which works well as long as the particles can be easily pelleted by centrifugal sedimentation (Scheme 2a). However, pelleting and resuspension are not easily automatable and require significant hands-on time, which can prove time-consuming if many layers are desired, especially since several washing steps are usually performed between each deposition cycle. Moreover, significant challenges are encountered when scaling down to smaller particle dimensions, especially in the sub-100 nm range, where effective centrifugation without aggregation becomes increasingly difficult. Although successful preparation of multilayered gold nanoparticles[31] and quantum dots[14] has been reported using the centrifugation method with extended centrifugation time, it is particularly challenging to generate multilayered particles with lower-density templates, such as silica or polymer nanoparticles.

To complement standard centrifugation and address some of these challenges, several alternative methods for the assembly of multilayered particles have been developed, including: surface acoustic wave atomization,[32] membrane filtration[33] and microfluidic methods.[29,34] In the atomization technique, LbL-particles are formed in a template-free manner, readily producing nanometer-sized multilayered particles, but the lack of template also puts some limits on the diversity of particles that can be synthesized.[32] Through membrane filtration, polymers can be sequentially and continuously added to, and removed from, a particle suspension to effect layer-by-layer build-up, but measures to minimize filter caking add some complexity.[33] Using microfluidics, the assembly process can be controlled with a high level of spatiotemporal precision.[35,36] For example, Kantak et al.[29] reported a microfluidic method, namely 'microfluidic pinball', to fabricate multilayered capsules. In that study, it was shown that using adjacent laminar flows of washing and polymer solutions in a microfluidic system, layer build-up can be achieved by physically displacing the templates back and forth between these solutions (Scheme 2b). In another example, Priest et al.[34] built a microfluidic system where layer build-up was achieved by sequential infusion and withdrawal of polymer solutions into a continuous flow of

templates. Both of these methods enabled the continuous production of particles with minimal hands-on time. However, the inherently small dimensions of microfluidic channels make scale-up for higher throughput challenging and small templates remain difficult to use. More recently, a natural immobilizing microfluidic system called 'electrophoretic polymer assembly' was developed by Richardson et al. (Scheme 2c).[30] After immobilizing nanometer- or micrometer-sized templates inside an agarose gel, polyelectrolytes could be injected and allowed to pass through the gel, analogous to the DNA gel electrophoresis technique. After several cycles of injecting polymers, the multilayered particles could be recovered by simply melting the agarose. Using this technology, stable LbL particles with 35 nm-diameter silica nanoparticles were generated, which is generally challenging to achieve using the centrifugation method. Even though the electrophoretic polymer assembly technique has some requirements, specifically (i) the need for a dissolvable porous immobilizing matrix, and (ii) that the materials used can be moved using electrophoresis or induced electroosmic flow,[30] advantages such as short hands-on time, amenability to automation, and simplified use of small templates, give rise to new possibilities in the fabrication of multilayered particles.

Taken together, the wide availability of templates and coating materials, combined with a growing number of assembly methods, enables the engineering of multilayered micro- and nanoparticles, thus allowing for systematic investigations, and ultimately control of, LbL particle-biological interactions.

3. CELLULAR PROCESSING OF AND INTRACELLULAR DRUG DELIVERY WITH MULTILAYERED PARTICLES

As many drug targets are localized to particular subcellular sites, it is essential to understand cellular processing of particles in order to achieve selective delivery at the molecular level. For this reason, in recent years there has been growing interest in investigating the cellular uptake and intracellular trafficking of multilayered particles.

Recently, it has been shown that multilayered particles and capsules with diameters ranging from submicrometers to a few micrometers, even without surface functionalization with targeting ligands, are readily internalized by a large variety of cells, including epithelial cells, macrophages, fibroblasts, monocytes and dendritic cells (DCs).[13,17] Many studies have also shown that flexible capsules are found to be deformed during the internalization process, whereas rigid LbL particles retained the original spherical shape.[13,17] Interestingly, Javier et al.[37] performed a detailed study on the deformation of capsules using poly(diallyldimethylammoniumchloride)/poly(styrene sulfonate) (PDADMAC/PSS) capsules made with a varied number of layers. Using transmission electron microscopy (TEM) and confocal microscopy, they revealed that the capsules with fewer layers tended to deform to a greater extent upon cellular uptake. The uptake mechanisms of multilayered capsules have been further investigated using TEM and pharmacological inhibitors. De Koker et al.[38] reported the formation of actin-rich plasma membrane extensions during the internalization of dextran sulfate/poly-L-arginine (DEXS/PARG) by DCs, and a decrease in uptake when macropinocytosis inhibitors were applied, suggesting that macropinocytosis is the major internalization route. Macropinocytosis is one of a number of endocytic mechanisms, which usually takes places in highly ruffled regions of the plasma membrane to form large endocytic vesicles (up to several micrometers) with extracellular fluid.[39] Consistent with the characteristics of macropinocytosis, cup-shaped cell membrane invaginations extending over submicrometer-sized thiolated poly(methacrylic acid) (PMA_{SH}) capsules upon internalization in human colorectal cancer cells was observed using TEM (Figure 1a, b).[40]

Following internalization, intracellular trafficking determines the subcellular location of multilayered particles. By co-localization with cellular organelle markers[40] or incorporation of a pH-sensitive fluorophore to the capsule walls,[37,41,42] the intracellular location of multilayered capsules have been elucidated (Figure 1c, d). Notably, regardless of the size and chemical composition of the capsules, as well as the cell lines investigated, persistent lysosomal accumulation is found in most cases.[37,40-42] Lysosomes are membrane-bound organelles that contain acid hydrolases to degrade macromolecules from various membrane trafficking, including endocytic, pathways.[43] Although several external triggers, such as near infrared light[44,45] and laser,[46] have led to a number of important advances, exploiting the cellular environment of endo-lysosomal trafficking for controlled degradation of multilayered capsules has become an area of active research.

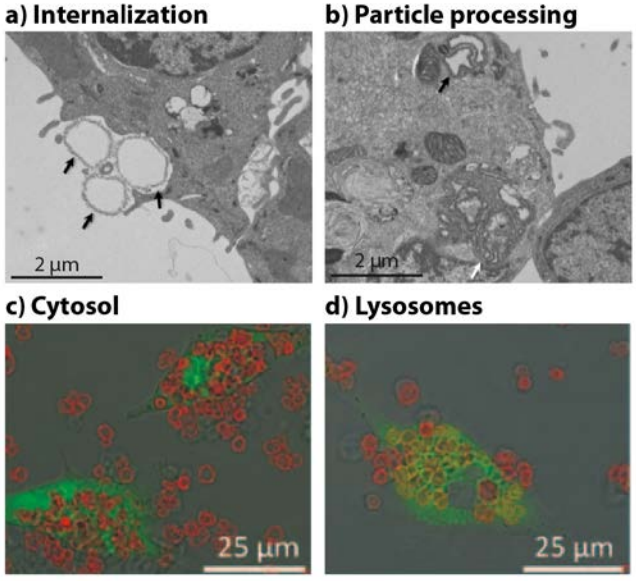


Figure 1. Internalization and cellular processing of multilayered particles. a,b) Transmission electron microscopy images of LIM1899 cells internalizing (a; black arrows) and processing (b; black and white arrows) PMA_{SH} capsules. Adapted with permission from ref 40. Copyright 2010 American Chemical Society. c,d) Colocalization confocal microscopy

images of bone-marrow derived mouse dendritic cells stained with either CellTracker (c; green) or LysoTracker (d; green) after incubation with RITC-labeled DEXS/PARG capsules (red). Green-red overlap indicates colocalization. Adapted with permission from ref 38. Copyright 2009 Wiley-VCH.

Herein, we focus on the endogenous physiological stimulators for triggered release. More specifically, (i) changes in pH, (ii) the presence of enzymes, and (iii) changes in redox potential are distinct physiological characteristics of the endo-lysosomal route, and mechanisms that respond to these stimuli have been engineered, alone or synergistically, into multilayered particles for triggered release.

pH-Responsive multilayered particles have been engineered through several routes. One approach is to incorporate pH-induced charge-shifting polymers in the multilayers, which significantly enhances the stability of capsules assembled from hydrolytically degradable polymers. The charge-shifting polymers undergo dynamic alterations in their net charge in response to pH, which can be exploited for cargo release in response to the acidification during endolysosomal trafficking. The use of such pH-triggered release has recently been shown for siRNA delivery in vitro. Elbakry et al.[47] originally investigated siRNA delivery using poly(ethyleneimine)/siRNA/poly-(ethyleneimine)-gold NPs (PEI/siRNA/PEI-Au NPs), and demonstrated knockdown of enhanced green fluorescent protein expression in stably transfected mammalian cells. However, random-assembled siRNA/PEI-agglomerates showed higher transfection efficiency than the multilayered nanoparticles. The most likely reason for the limited knockdown effect is that the high binding affinity between the gold nanoparticles and siRNA inhibited the effective release of siRNA. To improve the efficiency of siRNA release, Guo et al.[48] developed pH-responsive multilayered gold nanoparticles. These nanoparticles were comprised of PEI, charge-shifting cis-aconitic anhydride-functionalized poly(allylamine) (PAH-Cit), and 11-mercaptoundecanoic acid (MUA)-gold NPs. Compared to the commercial transfection agent lipofectamine and PEI, pH-responsive PEI/PAH-Cit/PEI/MUA-AuNPs demonstrated higher transfection efficiency for the same amount of siRNA.

Enzymatically responsive multilayered particles are generally made from biodegradable polyelectrolytes, such as polypeptides or polysaccharides. Rivera-Gil et al.[49] showed that after enzymatic degradation of internalized biodegradable DEXS/PARG microcapsules, the encapsulated proteins, DQ-ovalbumin (DQ-OVA), became available for proteolytic cleavage and were further degraded to form smaller vesicles inside the cytoplasm (**Figure 2**a). These proof-of-concept experiments demonstrate the ability to harness intracellular enzymes for activation and release of encapsulated cargo. In a separate study, the De Geest group further reported efficient antigen delivery to DCs using these DEXS/PARG microcapsules encapsulated with ovalbumin (OVA) in vitro.[38] By using TEM, a detailed intracellular degradation profile of the capsules was revealed, demonstrating that the microcapsules were ruptured in endolysosomal vesicles. As a result, the encapsulated antigen was processed into peptides, which were further presented to the cell surface, leading to activation of both CD_4 and CD8 T cells. Enzymatic degradation has also been used for intracellular delivery of chemotherapeutics. In a recent study, doxorubicin (DOX) was covalently conjugated to poly(L-glutamic acid) (PGA) to form multilayered DOX-loaded PGA microcapsules.[50] It was shown that DOX was effectively released from the capsules and further translocated to the nucleus in human colorectal cancer cells after cell internalization (Figure 2b), leading to a significant reduction of cell viability. Given the fact that multidrug resistance is an obstacle for effective treatment that is often associated with chemotherapeutic compounds, the cytotoxicity of drug-loaded PGA microcapsules was further examined in multidrug resistant human colorectal cancer cells.[51] It was demonstrated that drugs (DOX or paclitaxel (PTX)) encapsulated in PGA capsules exhibited significantly enhanced intracellular accumulation compared with the free drugs, as the cellular entry of the capsules was through endocytic routes, which bypassed multidrug resistant efflux pumps (e.g., P-glycoprotein) on the cell membrane. This study highlights the possibility of capsule-based delivery to facilitate restoration of drug sensitivity in resistant cancer cells.

Redox-responsive multilayered particles can be engineered to utilize the difference between the oxidizing extracellular milieu and the reducing environment of intracellular compartments (e.g., the cytoplasm). Due to the reversible nature, disulfide-thiol exchange is an attractive platform for controlled intracellular release. A prominent example of redox-responsive LbL capsules is disulfide-bonded PMA_{SH} capsules.[52] Through various loading strategies, a range of biomolecules has been successfully encapsulated in PMA_{SH} capsules, including siRNA,[53] peptides,[54] and chemotherapeutics.[40,55] Becker et al.[56] showed that the PMA_{SH} capsules loaded with siRNA targeting survivin are internalized by PC-3 prostate cancer cells and result in a reduced expression of survivin in vitro. In another study, De Rose et al.[54] incorporated model HIV vaccine peptides (KP9) into the PMA_{SH} capsules, and then assessed the KP9 antigen presentation ex vivo. It was found that KP9-loaded capsules stimulated CD8 T cells to simultaneously express both IFN-γ and TNF-α cytokines to elicit an immune response. Yan et al.[37] and Wang et al.[53] reported that PMA_{SH} capsules containing chemotherapeutics (i.e., DOX and PTX) significantly reduced the cell viability of human colorectal cancer cells in a dose-responsive manner while the empty capsules were well tolerated by the cells. Collectively, these studies suggest that encapsulated cargo with distinctively different physicochemical properties is effectively released from the redox-responsive capsules upon cellular uptake. A more recent study shed some light on the disulfide-thiol release mechanism occurring during the capsule-cell interactions.[57] In that study, lipophilic dye DiI (1,10-dioctadecyl-

3,3,30,30-tetramethylindo-carbocyanine perchlorate), which can cross lipid bilayers and diffuse to the cytoplasm, was used as a model cargo to evaluate the cargo release. Two types of DiI-loaded PMA_{SH} capsules, disulfide-bonded and non-cleavable thioether-crosslinked, were generated. It was shown that DiI was only released from the redox-responsive PMA_{SH} capsules following internalization. Interestingly, by blocking the thiols on the cell surface, namely exofacial thiols, the intracellular release of DiI from disulfide-bonded PMA_{SH} capsules was prevented. These exofacial thiols were found to promote the cellular interactions with the thiol/disulfide bond-containing capsules, leading to enhanced capsule internalization and redox-activated cargo release. This study suggests that redox changes within and outside the cells are dynamic, although the extracellular environment is generally viewed as redox-inert.

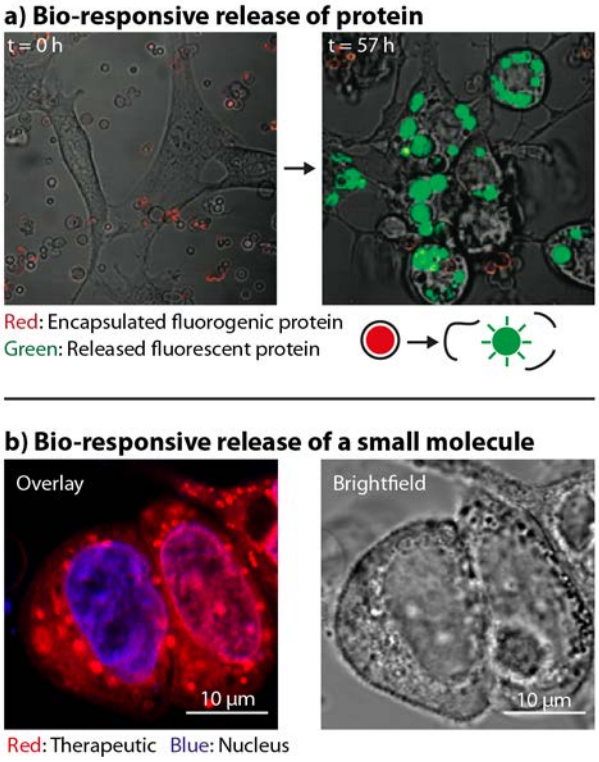


Figure 2. Confocal microscopy images of intracellular release of cargo by bioresponsive multilayered particles. a) Embryonic NIH/3T3 fibroblast incubated with DEXS/PARG capsules, filled with fluorogenic protein cargo, release the protein intracellularly, as indicated by the shift in fluorescence from weakly red (intact capsules) to bright green (released cargo). Adapted with permission from ref 49. Copyright 2009 American Chemical Society. b) LIM1899 cells treated with DOX-filled (red; therapeutic) PGA-capsules for 24 h. DOX was released from the capsules and translocated to the nucleus (blue). Adapted with permission from ref 50. Copyright 2010 American Chemical Society.

By engineering multi-responsive multilayered particles that respond to several physiological cues, the threshold for and the kinetics of cargo release, can be tuned. This is important because characteristics of biological environments often differ across different types of cells, and these variations could have a significant impact on the effectiveness of physiologically stimuli-triggered release. Recently, Liang et al.[58] developed dual responsive poly(2-diisopropylamino-ethyl methacrylate) (PDPA) capsules by combining a charge-shifting polymer, PDPA, with a cross-linker containing a central disulfide moiety, and demonstrated the synergistic effects of pH and redox potential for highly responsive cargo release. Moreover, the degree of the crosslinking provided an additional control for the degradation kinetics of the PDPA capsules.[59] In that study, by adjusting the amount of cross-linker used to stabilize the polymer films, the degree of crosslinking was tuned from 65% to 98%. This set of PDPA microcapsules was introduced to a dendritic cell line (JAWS II), and exhibited a gradually delayed intracellular degradation with an increasing degree of crosslinking, as observed by fluorescence microscopy.[59]

In summary, the understanding of how multilayered particles are internalized and processed in cells is steadily growing. Such knowledge has inspired the development of a collection of multilayered particles that are engineered to capitalize on the physiological changes of the endocytic pathways for intracellular delivery. It has been shown that multilayered particle-based delivery protects fragile cargo (e.g., peptides and siRNA) from degradation, enhances their intracellular concentration, and provides possibilities to tune their cellular pharmacokinetics en route towards optimal therapeutic benefits.

4. INTERACTIONS OF MULTILAYERED PARTICLES WITH TARGETED CELLS AND TISSUE

A century ago, Paul Ehrlich first envisaged the concept of a "magic bullet" where targeted therapy would specifically destroy diseased cells with minimal adverse effect.[60] To this end, multilayered particles utilizing both passive and active targeting approaches have recently been investigated. Here, we review these advances, including recent in vivo results from animal models where applications in both vaccination and tumor targeting have been evaluated.

The selective delivery of drug-loaded particles to solid tumors through systemic administration routes is often attributed to passive targeting via the 'enhanced permeability and retention' (EPR) effect.[61] The EPR effect is mainly caused by leaky tumor vasculature and poor lymphatic drainage, which enables particles to accumulate within the tumor tissue. Poon et al.[62] explored the applicability of multilayered particles for passive tumor targeting in vivo. In that study, carboxylated quantum dots (QDs) were sequentially coated with poly-L-lysine (PLL) and dextran sulfate (DXS), then terminated with an outer layer of either PLL, DXS or hyaluronic acid (HA). Using real-time intravital imaging to detect the core QDs fluorescence, the pharmacokinetics and biodistribution of these multilayered particles were examined. It was shown that surface composition significantly impacts nanoparticle biodistribution. A long blood elimination half-life (~9 h) and low liver accumulation (~10 – 15% recovered fluores-

cence/g) were observed in the HA-terminated multilayered nanoparticles. This long blood circulation time allowed a pronounced EPR effect for nanoparticle accumulation in subcutaneously induced solid KB tumors. However, the passive targeting was short-lived with a maximum accumulation at 24 h post injection, suggesting the need to further enhance the tumor interstitial retention of particles.

In a following study, the Hammond group reported a new targeting strategy that exploits the tumor microenvironment to expand upon the EPR effect.[14] The multilayered nanoparticles, comprised of QDs, iminobiotin-functionalized poly(L-lysine) (PLLib), neutravidin (Nav), and biotin-functionalized poly(ethylene glycol) (PEG), were shown to shed the PEG outer layer in the acid hypoxia tumor microenvironment, resulting in the exposure of the positively charged inner PLL layer. This switch of the particle surface charge promoted particle internalization by tumor cells, eliciting persistent tumor retention of multilayered particles for over 48 h (Figure 3a).

Active targeting by decorating the particle surface with high affinity ligands to promote specific interactions can be an effective approach to complement the EPR effect. Recent studies in this area have demonstrated that cell selectivity in vitro can be achieved by conjugating targeting ligands to multilayered particles. The targeted capsules can be prepared either through physical adsorption or chemical conjugation of the ligands with the outer surface of the capsules. Cortez et al.[63] demonstrated that electrostatic interactions can be exploited to functionalize LbL core-shell particles and capsules with the humanized A33 monoclonal antibody (huA33 mAb), which targets A33 antigen-expressing colorectal cancer cells. It was also shown that particles and capsules with a negatively charged outer surface, prior to biofunctionalization, reduce non-specific interaction with the cell plasma membrane, which, in general, is negatively charged.[64] Due to the high affinity of biotin and avidin, and high stability of the complex in a wide range of pH and ionic strengths, this non-covalent conjugation approach has also been used to prepare antibody-functionalized capsules.[65] In a recent study, two monoclonal antibodies, huA33 mAb and epidermal growth factor receptor monoclonal antibody (EGFR mAb) were biotinylated, complexed with neutravidin (Nav), and individually coupled to biotin-containing PMA_{SH} capsules.[65] The cellular binding and internalization of these antibody-functionalized capsules were significantly enhanced compared with control human immunoglobulin (IgG)-functionalized capsules in human colorectal cancer cells, which endogenously express both A33 antigen and EGFR on the cell surface. Given the complexity of biological environments, it is often more desirable to chemically conjugate targeting ligands to capsules because it can provide more precise control over the ligand density and stability. Kamphuis et al.[28] reported the functionalization of low-fouling poly(N-vinylpyrrolidone) (PVPON) capsules with huA33 mAb using a "click" (Cu(I)-catalyzed azide-alkyne cycloaddition) reaction (Figure 3b). The antibody-functionalized PVPON capsules exhibited a striking specificity to A33 antigen expressing colorectal cancer cells in vitro, even when the targeted cells comprised less than 0.1% of the total cell population. Besides these conventional coupling strategies, recent progress in capsule biofunctionalization has been made to achieve site-specific attachment to allow an optimal orientation of the targeting molecules.[66] This involved a Sortase A (Srt A)-based coupling approach, in which Srt A binds protein substrates that contain an LPETG peptide motif, cleaves between the threonine and glycine residues, and subsequently catalyzes conjugation between the carboxyl group of threonine with the N-terminal amino group of a polyglycine, forming an amide bond.[67] In that study, genetically engineered single-chain variable fragments (scFv) that contain a LPETG sequence were functionalized on alkyne-modified PVPON/alkyne-modified PEG ($PVPON_{Alk}/PEG_{Alk}$) microcapsules coated with polyglycine as surface moieties. As the scFv binds selectively to the activated glycoprotein (GP) IIb/IIIa receptor on platelets in thrombi, the scFv-functionalized capsules demonstrated a nine-fold increase in thrombi-binding compared to the mutated scFv-functionalized capsules, suggesting significantly enhanced specificity.[66]

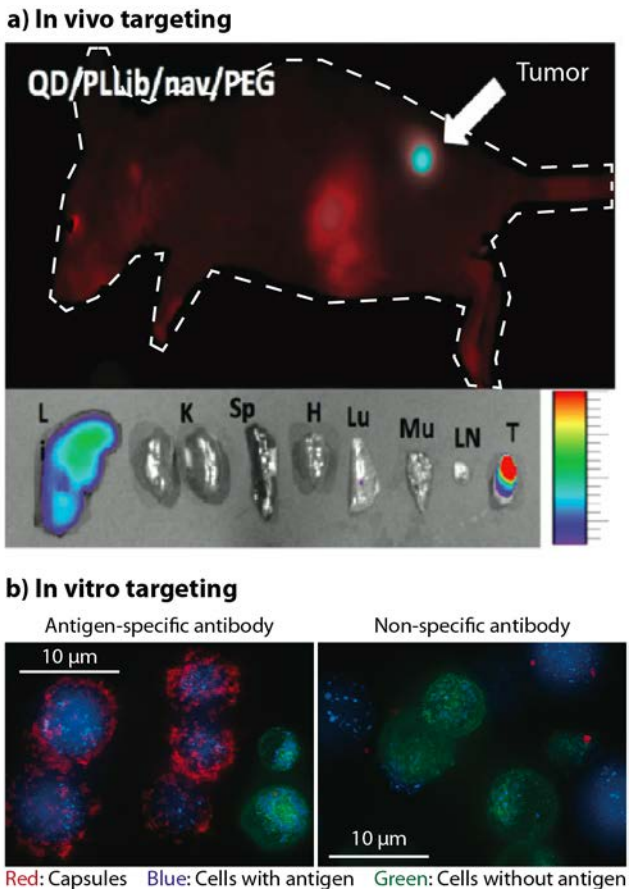


Figure 3. In vivo and in vitro targeting of multilayered particles to cells and tissues. a) Spectrally unmixed lateral scan of a MDA-MB-435 mouse showing biodistribution of PLLib/Nav/PEG functionalized quantum dots 48 h after administration. Harvested organs demonstrate the highest particle accumulation was in the tumor (T), followed by the liver (L), while other organs showed low or no accumulation.

Adapted with permission from ref 14. Copyright 2011 American Chemical Society. b) Fluorescence microscopy images of LIM2405+ (blue) and LIM2405– cells (green) incubated with PVPON capsules (red) functionalized with either an antigen-specific monoclonal antibody or a non-specific antibody (IgG). Adapted with permission from ref 28. Copyright 2010 American Chemical Society.

Vaccine delivery is another area where significant advances in targeted delivery using multilayered particles have been demonstrated. The integration of particles and immunity exploits the inherent capability of antigen presenting cells (APCs) to (i) vigorously recognize exogenous particles and (ii) induce effective protective immunity for vaccination purposes. Specifically, the primary goal of particle-based vaccines is to deliver antigen to APCs, in particular DCs, and to activate both innate and adaptive immunity through MHC class I and class II pathways.[68]

Antigen delivery, for vaccination, using multilayered particles in vivo has been investigated by several groups. The first study was performed by Sexton et al.[69] In that study, ovalbumin (OVA) and OVA-derived immunogenic peptides were encapsulated in PMA_{SH} capsules, which were subsequently intravenously injected into transgenic mice expressing two different T-cell receptors (OT-I and OT-II) to monitor the T-cell proliferation induced by OVA presentation. It was found that these capsules effectively stimulated the proliferation of OVA-specific CD_4 and CD8 T-cells in mice, resulting in at least a 6-fold greater proliferation of CD8 T-cells and a 70-fold greater proliferation of CD_4 T-cells compared to an equivalent amount of OVA protein alone (Figure 4a). Different to intravenous administration, the De Geest group investigated vaccine delivery through other immunization routes using biodegradable DEXS/PARG microcapsules as antigen carriers. It was shown that these microcapsules induce a moderate immune response with recruitment of macrophages and DCs to the site of administration following both mucosal and subcutaneous immunization.[70,71] The influx of APCs was found to actively transport the particles to the draining lymph nodes with a predominant localization in the T-cell zone.[15] Using OVA as a model vaccine, it was shown that the DEXS/PARG capsule-based immunization stimulated CD8 and CD_4 T-cell proliferation through both MHC class I and class II pathways, and strongly boosted the numbers of OVA-specific interferon gamma (IFN-γ) secreting CD_4 and CD8 T-cells compared to soluble OVA (Figure 4b). In addition, an increase in CD_4 follicular T-helper cell response was also observed, which plays an important role in supporting antibody responses. On the level of the B-cell immune response, it was consistently shown that the particle vaccine delivery promoted the formation of germinal centers and increased the number of plasma cells, leading to increased protective antibody titers. To enhance the immunogenicity of antigen for potent immune responses, De Geest et al.[72] decorated the OVA-loaded DEXS/PARG capsule surface with phophorothioate-stabilized oligodeoxynucleotides containing unmethylated CpG (CpG), which functions as an adjuvant by binding to the Toll-like receptor TLR9 in DCs. It was found that codelivery of antigen and adjuvant by the DEXS/PARG capsules elicited stronger IFN-γ secretion upon CD8 and CD_4 T-cell activation compared with capsules that only contained the antigen.

Taken together, these studies demonstrate an increasing capability to engineer targeting and specificity into multilayered particles, as well as their potential to induce enduring and effective immunity in vivo.

5. CONCLUSIONS AND FUTURE CHALLENGES

The past decade has witnessed the rapid and exciting development of multilayered particles with many sought after properties, such as cargo encapsulation, controlled degradation, and surface functionalization. It is now clear that highly versatile multilayered particles offer a range of unique opportunities as tailor-made delivery vehicles to enhance the efficacy and specificity of therapeutics. Many of the proof-of-concept studies reviewed here demonstrate the on-going expansion of biological investigations from in vitro to in vivo and provide a foundation from which the field of multilayered particles in biomedicine can advance.

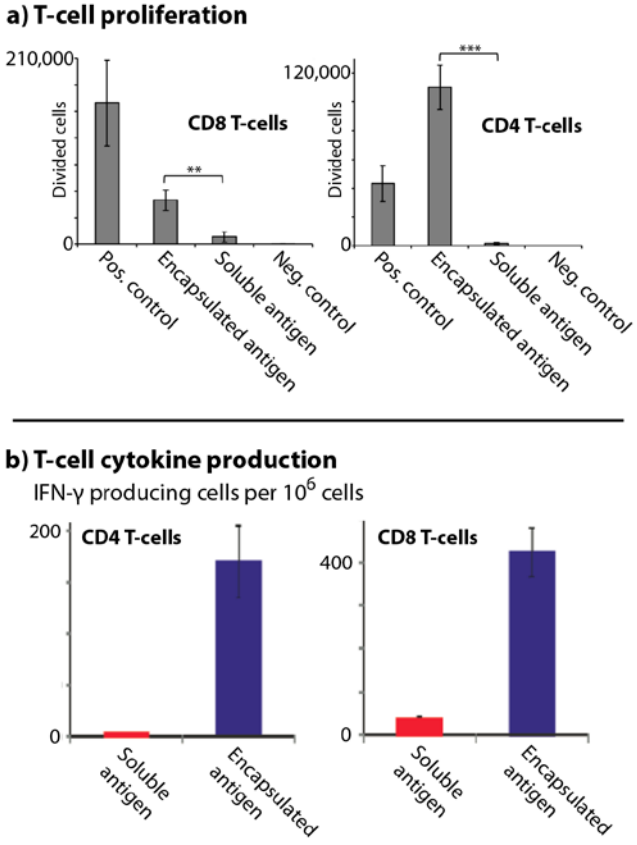


Figure 4. Enhanced T-cell response through multilayered particle vaccination. a) Proliferation of antigen-specific CD8 and CD_4 T-cells in vivo following intravenous injection of soluble or PMA_{SH}-encapsulated antigen. Vaccinations with endotoxin adjuvanted antigen-coated splenocytes or buffer were used as positive and negative controls, respectively. Adapted with permission from ref 69. Copyright 2009 American Chemical Society. b) Production of interferon gamma (IFN-γ) by antigen-specific CD_4 and CD8 T-cells in vivo after

subcutaneous vaccination with soluble or DEXS/PARG-encapsulated antigen, as determined by ELISPOT. Adapted with permission from ref 15. Copyright 2012 American Chemical Society.

However, to realize the full potential in biological applications, significant challenges still remain. The conventional technique of LbL assembly through centrifugation is conceptually simple and powerful, but practically time-consuming and labor-intensive, and small low-density particles remain challenging to engineer. Desired features for the next generation of assembly methods therefore include: (i) faster assembly time; (ii) amenability to automation, which would prove valuable for improving scalability of assembly, ultimately allowing translation from the bench; and (iii) easy production of submicron-sized particles, thus facilitating investigation of the full nanometer-micrometer spectrum of multilayered particles. Advances may come from methods such as microfluidics[29,34-36] or immobilizing matrices[30] for LbL, or by using different techniques, such as 'particle replication in non-wetting templates' (PRINT)[73] or 'continuous assembly of polymers' (CAP),[74] in combination with LbL.

While significant progress has been made in developing multilayered particles for drug and vaccine delivery, the ability to target particles to specific tissues, cells and intracellular compartments is still very limited. A precise knowledge of how physicochemical properties of multilayered particles impact their biological performance is generally lacking. This deficiency significantly hinders the rational design of particles. Thus, generating definitive correlations between particle parameters (e.g., size, shape, and surface functionality) and particle trajectories in vitro and pharmacokinetics in vivo will be priorities for future studies. As the LbL technique provides a high level of control over individual particle variables, multilayered particles present an exciting paradigm for these systematic investigations. This is exemplified by recent work on the shape-dependent cellular uptake of LbL capsules[75] and surface composition-dependent mucosal irritation.[76] Further, understanding the array of intrinsic biological mechanisms that mediate particle mobility at subcellular, cellular, and organismal levels using quantitative techniques, such as proteomics,[77] will inform the design of next-generation particles. The ability to harness such endogenous machinery will be imperative for highly lesion-specific therapeutic delivery. Addressing these questions through increased interdisciplinary collaborations will undoubtedly accelerate the development of multilayered particles for biological applications.

AUTHOR INFORMATION

Corresponding Author

* E-mail: fcaruso@unimelb.edu.au (F.C.)

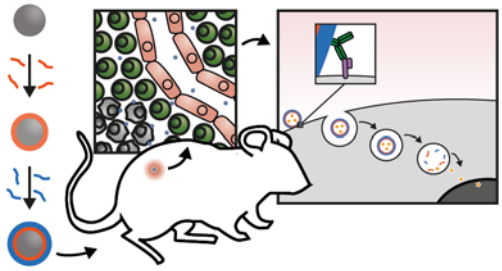
Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by the Australian Research Council under the Australian Laureate Fellowship (F.C., FL120100030) and Discovery Early Career Researcher Award (Y.Y., DE130100488) schemes. M.B. acknowledges funding from the Australian Government through an International Postgraduate Research Scholarship and an Australian Postgraduate Award.

Insert Table of Contents artwork here (max 3.6 cm x 8.4 cm)



REFERENCES

1. Albanese, A.; Tang, P. S.; Chan, W. C. W., *Annu. Rev. Biomed. Eng.* **2012**, *14*, 1.
2. Yan, Y.; Such, G. K.; Johnston, A. P. R.; Best, J. P.; Caruso, F., *ACS Nano* **2012**, *6*, 3663.
3. Decher, G.; Hong, J. D.; Schmitt, J., *Thin Solid Films* **1992**, *210*, 831.
4. Decher, G., *Science* **1997**, *277*, 1232.
5. Caruso, F.; Caruso, R. A.; Mohwald, H., *Science* **1998**, *282*, 1111.
6. Donath, E.; Sukhorukov, G. B.; Caruso, F.; Davis, S. A.; Mohwald, H., *Angew. Chem. Int. Ed.* **1998**, *37*, 2202.
7. Such, G. K.; Johnston, A. P. R.; Caruso, F., *Chem. Soc. Rev.* **2011**, *40*, 19.
8. Serizawa, T.; Kamimura, S.; Kawanishi, N.; Akashi, M., *Langmuir* **2002**, *18*, 8381.
9. Zhang, Y. J.; Yang, S. G.; Guan, Y.; Cao, W. X.; Xu, J., *Macromolecules* **2003**, *36*, 4238.
10. Wang, Y.; Angelatos, A. S.; Caruso, F., *Chem. Mater.* **2008**, *20*, 848.
11. Balkundi, S. S.; Veerabadran, N. G.; Eby, D. M.; Johnson, G. R.; Lvov, Y. M., *Langmuir* **2009**, *25*, 14011.
12. Kolesnikova, T. A.; Skirtach, A. G.; Mohwald, H., *Expert. Opin. Drug Deliv.* **2013**, *10*, 47.
13. Yan, Y.; Such, G. K.; Johnston, A. P. R.; Lomas, H.; Caruso, F., *ACS Nano* **2011**, *5*, 4252.
14. Poon, Z.; Chang, D.; Zhao, X. Y.; Hammond, P. T., *ACS Nano* **2011**, *5*, 4284.
15. De Geest, B. G.; Willart, M. A.; Hammad, H.; Lambrecht, B. N.; Pollard, C.; Bogaert, P.; De Filette, M.; Saelens, X.; Vervaet, C.; Remon, J. P.; Grooten, J.; De Koker, S., *ACS Nano* **2012**, *6*, 2136.
16. Tong, W. J.; Song, X. X.; Gao, C. Y., *Chem. Soc. Rev.* **2012**, *41*, 6103.
17. De Koker, S.; Hoogenboom, R.; De Geest, B. G., *Chem. Soc. Rev.* **2012**, *41*, 2867.
18. Becker, A. L.; Johnston, A. P. R.; Caruso, F., *Small* **2010**, *6*, 1836.
19. Dong, A. G.; Wang, Y. J.; Tang, Y.; Ren, N.; Zhang, Y. H.; Gao, Z., *Chem. Mater.* **2002**, *14*, 3217.
20. Sukhorukov, G. B.; Volodkin, D. V.; Gunther, A. M.; Petrov, A. I.; Shenoy, D. B.; Mohwald, H., *J. Mater. Chem.* **2004**, *14*, 2073.
21. Wang, Y. J.; Yu, A. M.; Caruso, F., *Angew. Chem. Int. Ed.* **2005**, *44*, 2888.
22. Cavalieri, F.; Postma, A.; Lee, L.; Caruso, F., *ACS Nano* **2009**, *3*, 234.
23. An, Z. H.; Tao, C.; Lu, G.; Mohwald, H.; Zheng, S. P.; Cui, Y.; Li, J. B., *Chem. Mater.* **2005**, *17*, 2514.
24. Stadler, B.; Chandrawati, R.; Goldie, K.; Caruso, F., *Langmuir* **2009**, *25*, 6725.
25. Johnston, A. P. R.; Read, E. S.; Caruso, F., *Nano Lett.* **2005**, *5*, 953.
26. Kida, T.; Mouri, M.; Akashi, M., *Angew. Chem. Int. Ed.* **2006**, *45*, 7534.
27. Wang, Z. P.; Feng, Z. Q.; Gao, C. Y., *Chem. Mater.* **2008**, *20*, 4194.
28. 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., *J. Am. Chem. Soc.* **2010**, *132*, 15881.
29. Kantak, C.; Beyer, S.; Yobas, L.; Bansal, T.; Trau, D., *Lab Chip* **2011**, *11*, 1030.
30. Richardson, J. J.; Ejima, H.; Lörcher, S. L.; Liang, K.; Senn, P.; Cui, J.; Caruso, F., *Angew. Chem. Int. Ed.* **2013**, *52*, 6455.
31. Schneider, G.; Decher, G., *Nano Lett.* **2004**, *4*, 1833.
32. Qi, A. S.; Chan, P.; Ho, J.; Rajapaksa, A.; Friend, J.; Yeo, L. L., *ACS Nano* **2011**, *5*, 9583.
33. Voigt, A.; Lichtenfeld, H.; Sukhorukov, G. B.; Zastrow, H.; Donath, E.; Baumler, H.; Mohwald, H., *Ind. Eng. Chem. Res.* **1999**, *38*, 4037.
34. Priest, C.; Quinn, A.; Postma, A.; Zelikin, A. N.; Ralston, J.; Caruso, F., *Lab Chip* **2008**, *8*, 2182.
35. Dendukuri, D.; Doyle, P. S., *Adv. Mater.* **2009**, *21*, 4071.
36. Valencia, P. M.; Farokhzad, O. C.; Karnik, R.; Langer, R., *Nat. Nanotechnol.* **2012**, *7*, 623.
37. Javier, A. M.; Kreft, O.; Semmling, M.; Kempter, S.; Skirtach, A. G.; Bruns, O. T.; del Pino, P.; Bedard, M. F.; Raedler, J.; Kaes, J.; Plank, C.; Sukhorukov, G. B.; Parak, W. J., *Adv. Mater.* **2008**, *20*, 4281.
38. 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., *Angew. Chem. Int. Ed.* **2009**, *48*, 8485.
39. Doherty, G. J.; McMahon, H. T., *Annu. Rev. Biochem.* **2009**, *78*, 857.
40. 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., *ACS Nano* **2010**, *4*, 2928.
41. Kreft, O.; Javier, A. M.; Sukhorukov, G. B.; Parak, W. J., *J. Mater. Chem.* **2007**, *17*, 4471.
42. Javier, A. M.; Kreft, O.; Alberola, A. P.; Kirchner, C.; Zebli, B.; Susha, A. S.; Horn, E.; Kempter, S.; Skirtach, A. G.; Rogach, A. L.; Radler, J.; Sukhorukov, G. B.; Benoit, M.; Parak, W. J., *Small* **2006**, *2*, 394.
43. Luzio, J. P.; Pryor, P. R.; Bright, N. A., *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 622.
44. Radt, B.; Smith, T. A.; Caruso, F., *Adv. Mater.* **2004**, *16*, 2184.
45. Ochs, M.; Carregal-Romero, S.; Rejman, J.; Braeckmans, K.; De Smedt, S. C.; Parak, W. J., *Angew. Chem. Int. Ed.* **2013**, *52*, 695.
46. Skirtach, A. G.; Dejugnat, C.; Braun, D.; Susha, A. S.; Rogach, A. L.; Parak, W. J.; Mohwald, H.; Sukhorukov, G. B., *Nano Lett.* **2005**, *5*, 1371.
47. Elbakry, A.; Zaky, A.; Liebkl, R.; Rachel, R.; Goepferich, A.; Breunig, M., *Nano Lett.* **2009**, *9*, 2059.
48. Guo, S. T.; Huang, Y. Y.; Jiang, Q. A.; Sun, Y.; Deng, L. D.; Liang, Z. C.; Du, Q. A.; Xing, J. F.; Zhao, Y. L.; Wang, P. C.; Dong, A. J.; Liang, X. J., *ACS Nano* **2010**, *4*, 5505.
49. Rivera-Gil, P.; De Koker, S.; De Geest, B. G.; Parak, W. J., *Nano Lett.* **2009**, *9*, 4398.
50. Ochs, C. J.; Such, G. K.; Yan, Y.; van Koeverden, M. P.; Caruso, F., *ACS Nano* **2010**, *4*, 1653.
51. Yan, Y.; Ochs, C. J.; Such, G. K.; Heath, J. K.; Nice, E. C.; Caruso, F., *Adv. Mater.* **2010**, *22*, 5398.
52. Zelikin, A. N.; Quinn, J. F.; Caruso, F., *Biomacromolecules* **2006**, *7*, 27.
53. Zelikin, A. N.; Becker, A. L.; Johnston, A. P. R.; Wark, K. L.; Turatti, F.; Caruso, F., *ACS Nano* **2007**, *1*, 63.
54. De Rose, R.; Zelikin, A. N.; Johnston, A. P. R.; Sexton, A.; Chong, S. F.; Cortez, C.; Mulholland, W.; Caruso, F.; Kent, S. J., *Adv. Mater.* **2008**, *20*, 4698.

55. Wang, Y. J.; Yan, Y.; Cui, J. W.; Hosta-Rigau, L.; Heath, J. K.; Nice, E. C.; Caruso, F., *Adv. Mater.* **2010**, *22*, 4293.
56. Becker, A. L.; Orlotti, N. I.; Folini, M.; Cavalieri, F.; Zelikin, A. N.; Johnston, A. P. R.; Zaffaroni, N.; Caruso, F., *ACS Nano* **2011**, *5*, 1335.
57. Yan, Y.; Wang, Y. J.; Heath, J. K.; Nice, E. C.; Caruso, F., *Adv. Mater.* **2011**, *23*, 3916.
58. Liang, K.; Such, G. K.; Zhu, Z. Y.; Yan, Y.; Lomas, H.; Caruso, F., *Adv. Mater.* **2011**, *23*, H273.
59. Liang, K.; Such, G. K.; Zhu, Z. Y.; Dodds, S. J.; Johnston, A. P. R.; Cui, J. W.; Ejima, H.; Caruso, F., *ACS Nano* **2012**, *6*, 10186.
60. Strebhardt, K.; Ullrich, A., *Nat. Rev. Cancer* **2008**, *8*, 473.
61. Cheng, Z. L.; Al Zaki, A.; Hui, J. Z.; Muzykantov, V. R.; Tsourkas, A., *Science* **2012**, *338*, 903.
62. Poon, Z.; Lee, J. B.; Morton, S. W.; Hammond, P. T., *Nano Lett.* **2011**, *11*, 2096.
63. Cortez, C.; Tomaskovic-Crook, E.; Johnston, A. P. R.; Radt, B.; Cody, S. H.; Scott, A. M.; Nice, E. C.; Heath, J. K.; Caruso, F., *Adv. Mater.* **2006**, *18*, 1998.
64. Cortez, C.; Tomaskovic-Crook, E.; Johnston, A. P. R.; Scott, A. M.; Nice, E. C.; Heath, J. K.; Caruso, F., *ACS Nano* **2007**, *1*, 93.
65. Shimoni, O.; Postma, A.; Yan, Y.; Scott, A. M.; Heath, J. K.; Nice, E. C.; Zelikin, A. N.; Caruso, F., *ACS Nano* **2012**, *6*, 1463.
66. Leung, M. K. M.; Hagemeyer, C. E.; Johnston, A. P. R.; Gonzales, C.; Kamphuis, M. M. J.; Ardipradja, K.; Such, G. K.; Peter, K.; Caruso, F., *Angew. Chem. Int. Ed.* **2012**, *51*, 7132.
67. Ton-That, H.; Mazmanian, S. K.; Faull, K. F.; Schneewind, O., *J. Biol. Chem.* **2000**, *275*, 9876.
68. Rice-Ficht, A. C.; Arenas-Gamboa, A. M.; Kahl-McDonagh, M. M.; Ficht, T. A., *Curr. Opin. Microbiol.* **2010**, *13*, 106.
69. 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., *ACS Nano* **2009**, *3*, 3391.
70. De Koker, S.; Naessens, T.; De Geest, B. G.; Bogaert, P.; Demeester, J.; De Smedt, S.; Grooten, J., *J. Immunol.* **2010**, *184*, 203.
71. De Koker, S.; De Geest, B. G.; Cuvelier, C.; Ferdinande, L.; Deckers, W.; Hennink, W. E.; De Smedt, S.; Mertens, N., *Adv. Funct. Mater.* **2007**, *17*, 3754.
72. De Geest, B. G.; Willart, M. A.; Lambrecht, B. N.; Pollard, C.; Vervaet, C.; Remon, J. P.; Grooten, J.; De Koker, S., *Angew. Chem. Int. Ed.* **2012**, *51*, 3862.
73. Morton, S. W.; Herlihy, K. P.; Shopsowitz, K. E.; Deng, Z. J.; Chu, K. S.; Bowerman, C. J.; DeSimone, J. M.; Hammond, P. T., *Adv. Mater.* **2013,** DOI: 10.1002/adma.201302025.
74. Goh, T. K.; Guntari, S. N.; Ochs, C. J.; Blencowe, A.; Mertz, D.; Connal, L. A.; Such, G. K.; Qiao, G. G.; Caruso, F., *Small* **2011**, *7*, 2863.
75. Shimoni, O.; Yan, Y.; Wang, Y. J.; Caruso, F., *ACS Nano* **2013**, *7*, 522.
76. De Cock, L. J.; Lenoir, J.; De Koker, S.; Vermeersch, V.; Skirtach, A. G.; Dubruel, P.; Adriaens, E.; Vervaet, C.; Remon, J. P.; De Geest, B. G., *Biomaterials* **2011**, *32*, 1967.
77. Lai, Z. W.; Yan, Y.; Caruso, F.; Nice, E. C., *ACS Nano* **2012**, *6*, 10438.