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Account

Understanding Cell Interactions Using Modular Nanoparticle Libraries*

Georgina K. Such[A,C] *and Angus P. R. Johnston*[B]

[A]Department of Chemistry, The University of Melbourne, Parkville, Vic. 3010, Australia.
[B]Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Vic. 3052, Australia.
[C]Corresponding author. Email: gsuch@unimelb.edu.au

Nanoparticle delivery systems have significant potential to facilitate the delivery of novel therapeutics, such as proteins, DNA or small molecules. However, there are multiple biological barriers that need to be overcome to deliver the cargo in an active form. These challenges include evading clearance by the reticuloendothelial system, minimising adverse immune responses, targeting specific cells and tissues, and trafficking into the right compartment of the cell. In this account, we will discuss how nanoparticle structure can be tuned to optimise biological interactions and thus improve the ability of nanoparticles to overcome these barriers. The focus of this article will be on controlling cell targeting and trafficking within a cell, e.g. endosomal escape.

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Introduction

There are many potential therapeutics that show promise in vitro yet fail in vivo because they have adverse properties that prevent their direct delivery into a biological environment. These characteristics include issues with toxicity, solubility, or degradation in the case of biological therapeutics such as DNA or proteins. Nanoparticle delivery systems have emerged as a potential strategy to overcome these limitations, owing to their ability to encapsulate a range of cargo and protect it until it reaches a desired site.[1,2] The design of nanoparticle delivery systems has been an area of intense research for several decades, with nanoparticles typically designed based on inorganic, biological or polymeric building blocks.[3] Nanoparticle-based therapies that are now used in the clinic include: Doxcil, a PEGylated liposome for the delivery of doxorubicin; Abraxane,[4] paclitaxel bound to albumin, and, most recently, Onpattro, a lipid nanoparticle for the delivery of small interfering ribonucleic acid (siRNA).[5] However, the number of nanoparticles in clinical trials remains very low compared with the number of new nanoparticles published in the literature. This is largely owing to the range of biological roadblocks that a nanoparticle must migrate through to achieve effective delivery. To be clinically relevant, nanoparticles need to evade clearance by the reticuloendothelial system, minimise adverse immune responses, accumulate in the desired organ, bind to a specific cell, and finally be trafficked to the correct compartment within the cell. For delivery of anticancer drugs, achieving efficient accumulation in tumours remains a significant challenge. Chan and coworkers performed a broad review of papers investigating nanoparticle targeting to mouse tumours, and showed the median delivery to the tumour was low (0.7 % of injected dose).[6] Another significant roadblock to efficient delivery is engineering the nanoparticle to deliver its therapeutic cargo to the right compartment within the cell.[7,8] It is well established that nanoparticles are internalised into endosomes, which gradually acidify and fuse to lysosomes. These compartments are not the site of action for most drugs, and in the case of biological therapeutics, are a major site for degradation. Thus, it is vital for nanoparticles to be engineered to transport their cargo out of the endosomes or lysosomes and into the cytosol or nucleus (a process termed endosomal escape). It has been shown that even effective nanoparticles have poor endosomal escape of 1–2 %.[9] It is clear that there is still much to be discovered about how to migrate through these biological roadblocks and how nanoparticle structure affects biological interactions. Enhancing this knowledge has potential to improve the design of nanoparticle delivery systems by designing a guide book for understanding the structure–property relationships needed for optimal biological performance.

In this account, we will discuss our recent work using libraries of polymer nanoparticles to probe nanoparticle interactions with cells. The aim of this work has been to understand how nanoparticle structure can be used to improve biological interactions and thus optimise therapeutic delivery (Fig. 1). The impact of nanoparticle properties on two important biological roadblocks fundamental to effective delivery are discussed: targeting and endosomal escape.

Design of Modular pH-Responsive Nanoparticles

There has been significant research interest in charge-shifting monomers that transition from hydrophobic to hydrophilic for

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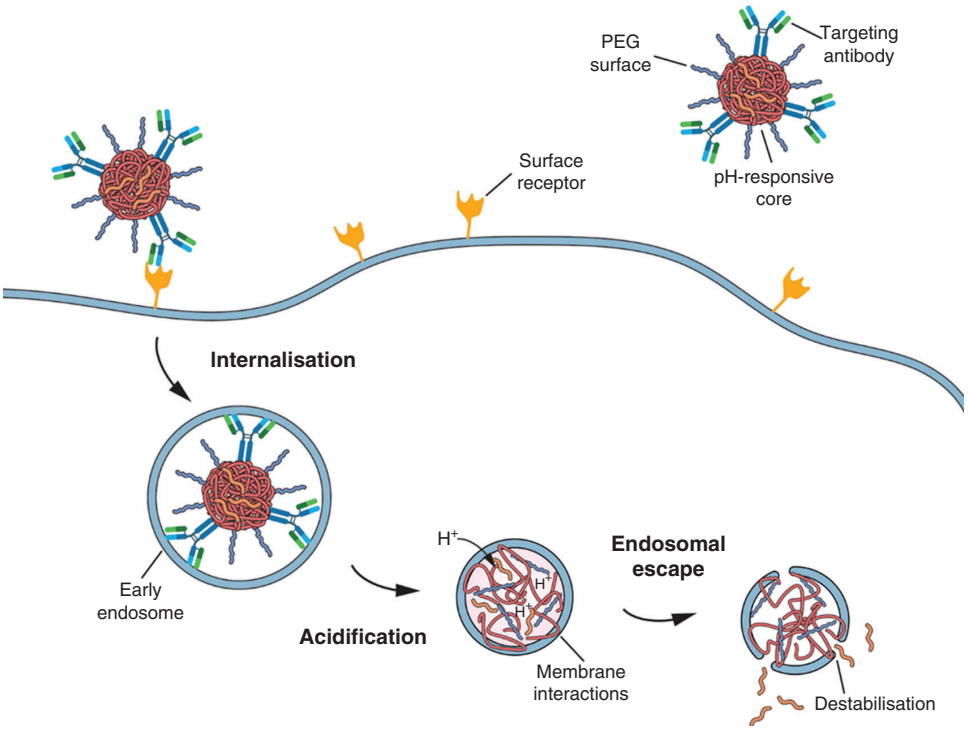

Fig. 1. Schematic demonstrating the targeting of a polymeric nanoparticle to a cell, internalisation, and then escape from the endosomal compartment. Endosomal escape occurs owing to nanoparticle disassembly and subsequent interaction with the endosomal membrane.

application in drug delivery systems. One of the most well studied families is 2-(diethylamino)ethyl methacrylate (DEAEMA) and 2-(diisopropylamino)ethyl methacrylate (DPAEMA). These monomers are of interest as they have a pH transition from hydrophobic to hydrophilic that occurs in the pH range of the endosomal or lysosomal compartments. This transition occurs owing to protonation of the tertiary amine groups in their side chain. The pK_a of this transition is tunable by having different functional groups on the amino group, and is pH 7.0 and pH 6.4 for DEAEMA and DPAEMA respectively. Nanoparticles can be formed from these materials by engineering an amphiphilic polymer with a hydrophilic block (e.g. polyethylene glycol (PEG)), with the pH-responsive component as the hydrophobic block. Several studies have used these materials to design therapeutic delivery systems.[10,11] When a DEAEMA/DPAEMA-*b*-PEG copolymer is at physiological pH (pH 7.4), nanoparticles are formed; however, the particles disassemble when the pH drops below the pK_a owing to protonation of the pH-responsive component. Gao and coworkers have done several elegant studies demonstrating charge-shifting micelles could be covalently modified with dyes and used as biosensors for both imaging the acidic tumour environment and internalisation into an endosome.[12] Charge-shifting materials are also of interest as many studies have demonstrated the capability for endosomal escape.

Investigating the Impact of Nanoparticle Structure on Endosomal Escape

To investigate the impact of nanoparticle structure on endosomal escape, we developed a nanoparticle based on two components, a homopolymer of poly(2-(diethylamino)ethyl methacrylate) (PDEAEMA) and a block copolymer of poly(ethylene glycol)-*b*-poly(2-(diethylamino)ethyl methacrylate) (PEG-*b*-PDEAEMA). We demonstrated these two polymers could self-assemble at physiological pH to form nanoparticles where the homopolymer formed the core hydrophobic region, and the hydrophilic shell polymer stabilised the structure.[13] The nanoparticles were stable at pH 7.4 but disassembled rapidly with a decrease in pH. Importantly, the nanoparticles were responsive to pH changes that occur during the acidification of endosomes. We demonstrated these nanoparticles had the capacity to disrupt the endosomal membrane by releasing calcein from the endosome into the cytosol. Calcein is a small fluorescent molecule that is membrane-impermeable. Therefore, when calcein is incubated with cells, it is macropinocytosed by the cell, but it remains trapped in endosomal or lysosomal compartments, which is visualised as punctate spots. Calcein fluorescence is quenched in the endosome through a combination of low pH and high concentrations of calcein, leading to self-quenching. If endosomal escape occurs, the fluorescence of calcein increases and is observed as bright diffuse fluorescence in the cytosol. In this study, although efficient endosomal escape of calcein was observed, the polymer remained trapped in the endosomes. This suggests that endosomal escape occurs through an interaction that destabilises the endosomal membrane, which allows the low-molecular-weight calcein to diffuse into the cytosol, but the higher-molecular-weight polymer remains trapped in the endosome. Our results suggest endosomal escape through membrane disruption is challenging for larger cargo; thus further work is required to enhance the extent of membrane disruption in order for larger therapeutics to escape effectively.

In a subsequent study, we developed a library of nanoparticles to test the effect of polymer molecular weight on membrane interaction and endosomal escape.[14] A range of PDEAEMA homopolymers were synthesised with molecular weights of 7, 27, 56 and 106 kDa using reversible addition–fragmentation

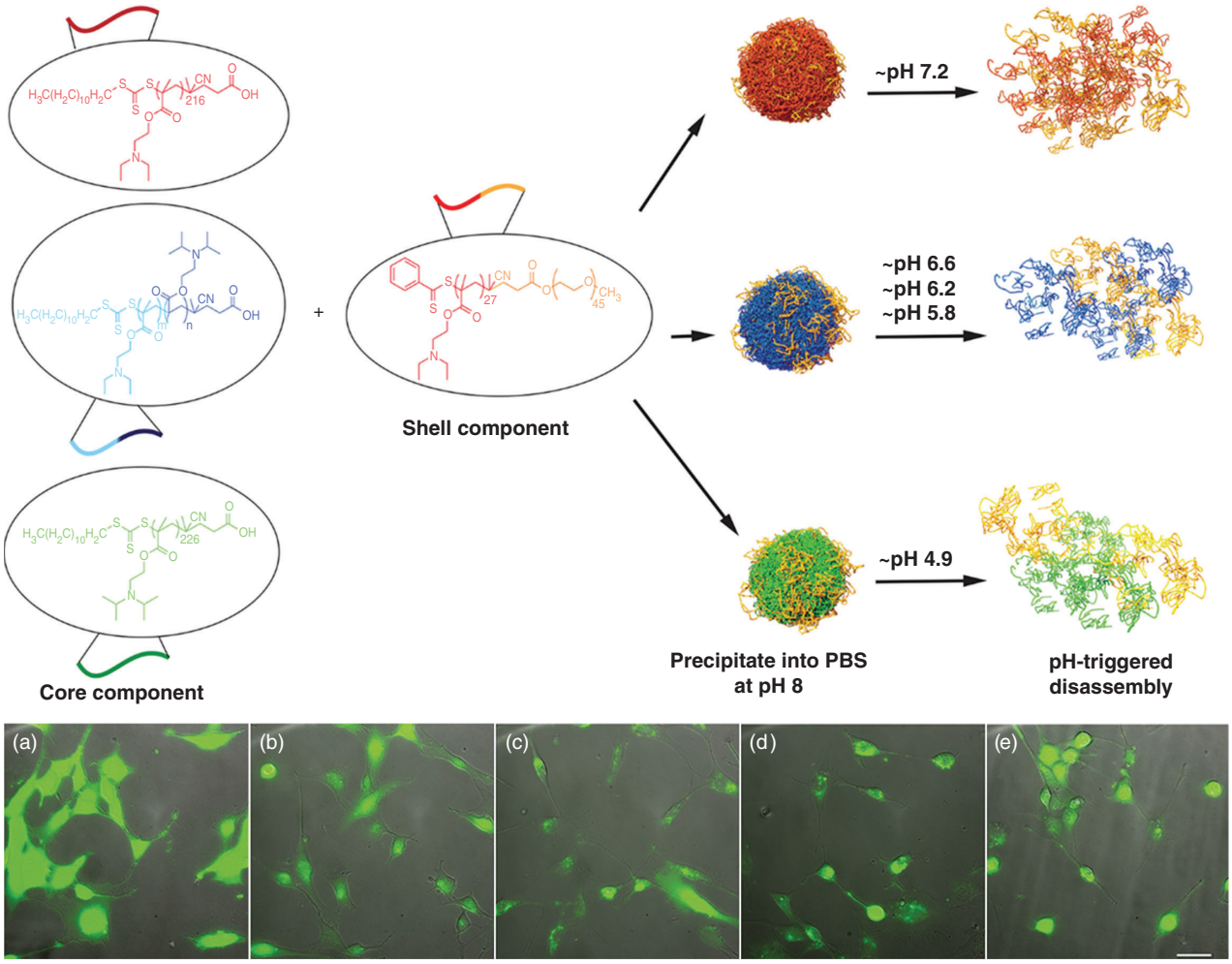

Fig. 2. Controlling endosomal escape of pH responsive nanoparticles by tuning the disassembly of the nanoparticles using polymer pK_a of the core component. Endosomal escape of the nanoparticle tested using the calcein assay using (a) core polymer of 2,2-(diethylamino)ethyl methacrylate, PDEAEMA; (b) core polymer with 3 : 1 ratio, 2,2-(diethylamino)ethyl methacrylate (PDEAEMA) : 2,2-(diisopropylamino)ethyl methacrylate (PDPAEMA); (c) 1 : 1 PDEAEMA : PDPAEMA; (d) 1 : 3 PDEAEMA : PDPAEMA; (e) PDPAEMA. Scale bar is 20 μm. Reprinted with permission from refs [15] and [16].

chain transfer (RAFT). These polymers were combined with PEG-*b*-PDEAEMA of 16 kDa to form nanoparticles using nanoprecipitation. We showed that the physicochemical properties of the nanoparticles were similar, regardless of the molecular weight of the core polymer, with all nanoparticles having similar sizes, pH-responsive disassembly, and cellular toxicity. In contrast, the cellular interactions of the different nanoparticles were significantly different. The 7-kDa core nanoparticles had significantly higher association with 3T3 cells than the high-molecular-weight core particles. In addition, minimal endosomal escape was observed for the 7-kDa core nanoparticles, even though more cell association was observed. Less than 1 % endosomal escape was observed for 7-kDa core nanoparticles, whereas 27- and 106-kDa nanoparticles had 38 and 42 % endosomal escape respectively. The particles were also loaded with a model protein cargo, ovalbumin (Ova). Microscopy images showed that Ova was colocalised with the endosomal or lysosomal compartments for all nanoparticles, indicating that the membrane disruption was only sufficient to allow the diffusion of the low-molecular-weight calcein from the endosome, and the 43-kDa Ova remained trapped.

These pH-responsive nanoparticles were also used to probe the impact of compositional variation on endosomal escape.[15] In this work, the core copolymer was a random copolymer of PDEAEMA and PDPAEMA at mol ratios of 1 : 0, 3 : 1, 1 : 1, 1 : 3 and 0 : 1. Particle disassembly could be tuned by changing the ratio of the two components, with a higher ratio of PDPAEMA leading to a lower disassembly point. The disassembly pH of the nanoparticles was tuned from pH 7.2 (100 % PDEAEMA) to 4.9 (100 % PDPAEMA). pH-dependent membrane disruption was demonstrated using red blood cell (RBC) lysis. Interestingly, maximal RBC lysis was observed ~0.5 pH above the pH at which the nanoparticles disassembled, indicating particle rearrangement and membrane interaction before the nanoparticle disassembled. Endosomal escape was probed using the calcein assay in NIH 3T3 cells at concentrations of 0.5×10^9, 1.5×10^9, 5×10^9 and 15×10^9 particles mL^{-1} (Fig. 2). It was shown particle concentration was an important determinant of endosomal escape. In addition, an interesting trend of endosomal escape was observed across the particle library. Highest escape (>60 %) was observed with nanoparticles that disassembled at either extreme of pH, e.g. PDEAEMA (pH 7.2) and PDPAEMA (pH 4.9). In contrast, there was minimal endosomal escape for 1 : 1 PDEAEMA : PDPAEMA (pH 6.2). The decrease in endosomal escape in the particles with the intermediate disassembly pH suggests two different mechanisms are playing a role in

endosomal escape. A control particle with a non-pH responsive core (but with pH-responsive PEG-*b*-PDEAEMA shell) was also investigated. This particle exhibited no membrane disruption (through RBC lysis) or endosomal escape. This suggests the core polymer is the driving force behind the endosomal escape in this system.

From our work in this area, we have demonstrated that endosomal escape can be tuned by controlling the nanoparticle structure and composition. The mechanism of endosomal escape is likely to be due to interactions of the polymer with the endosomal membrane. However, there is still much to be understood about the process of endosomal escape, including how disruption of the endosomal membrane can be tuned to release cargo with a higher molecular weight. Understanding this process is fundamental for achieving more efficient release of biological cargo such as proteins.[17] Other groups have also demonstrated the influence of nanoparticle structure on endosomal escape, with Stayton and coworkers recently showing polymer architecture can play a role in enhancing the delivery of proteins to the cytosol.[18] Although it is not clear why architecture affects endosomal escape, it is possibly due to enhancement in the scale of membrane interaction with more branched structures. There is also a clear need to develop quantitative tools to assess endosomal escape, as assays such as calcein are highly qualitative and subjective. Several more quantitative techniques such as split green fluorescent protein (GFP) are now in development.[17]

Targeting

The ability to target nanoparticles to specific organs and cells within the body is an important goal of nanomedicine. To achieve this, there needs to be an improved understanding of how the surface chemistry of nanoparticles can be tuned to optimise biological interactions. Recently, we probed the impact of protein density on the surface of nanoparticles for controlling cell interactions.[19] This study was also conducted using PDEAEMA and PDEAEMA-*r*-PDPAEMA polymers. Holo-transferrin-polymer conjugates were synthesised by conjugating holo-transferrin to either PDEAEMA or PDEAEMA-*r*-PDPAEMA using click chemistry. Nanoparticles were formed when the pH was raised above the pK_a of the polymer, with the pH-responsive polymer forming the core of the nanoparticles and the holo-transferrin on the surface. To investigate the impact of surface density of the nanoparticles, this conjugate was also combined at a 1 : 20 ratio with PEG-*b*-(PDEAEMA-*r*-PDPAEMA). The cell association of the 1 : 0 and 1 : 20 systems was then investigated in HEK cells transfected with the transferrin receptor (TfR). We demonstrated there was minimal targeting with the 1 : 0 system, with similar binding to cells expressing the TfR compared with wild-type cells that did not. In contrast, the 1 : 20 system showed specific binding to cells expressing the TfR, with ~4× higher binding compared with the wild type cells that lacked the TfR. In a related study, we demonstrated that PEG density and length play an important role in controlling the non-specific interactions of these nanoparticles with cells.[20] The orientation of the targeting group on the surface of the nanoparticles can also play an important role in the targeting efficiency to cells. Using amber codon reassignment to incorporate reactive, synthetic amino acids at different positions in a single-domain antibody (sdAb), we have shown that we can control the orientation of proteins on the surface of a quantum dot (Qdot). Qdots with optimally oriented sdAbs showed >5-fold improvement in cell targeting compared with randomly oriented sdAbs.[21]

To design an effective nanoparticle delivery system, many biological roadblocks must be overcome. However, very little is understood about how nanoparticle structure impacts on these roadblocks. This means that although many nanoparticles are developed, very few proceed to clinical trials. One strategy to improve this translation is to systematically investigate the impact of nanoparticle structure on different biological interactions, such as targeting and endosomal escape. It can be seen from our studies that small changes in nanoparticle structure can significantly impact these biological interactions. It is hoped that improving the understanding of nanoparticle biological interactions could lead to a framework for better nanoparticle design in the future.

Conflicts of Interest

The authors declare no conflicts of interest.

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