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Cell penetrating peptide-modified poly(lactic-co-glycolic acid) nanoparticles with enhanced cell internalization

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ABSTRACT

The surface modification of nanoparticles (NPs) can enhance the intracellular delivery of drugs, proteins, and genetic agents. Here we studied the effect of different surface ligands, including cell penetrating peptides (CPPs), on the cell binding and internalization of poly(lactic-co-glycolic) (PLGA) NPs. Relative to unmodified NPs, we observed that surface-modified NPs greatly enhanced cell internalization. Using one CPP, MPG (unabbreviated notation), that achieved the highest degree of internalization at both low and high surface modification densities, we evaluated the effect of two different NP surface chemistries on cell internalization. After 2 h, avidin-MPG NPs enhanced cellular internalization by 5 to 26-fold relative to DSPE-MPG NP formulations. Yet, despite a 5-fold increase in MPG density on DSPE compared to Avidin NPs, both formulations resulted in similar internalization levels (48 and 64-fold, respectively) after 24 h. Regardless of surface modification, all NPs were internalized through an energy-dependent, clathrin-mediated process, and became dispersed throughout the cell. Overall both Avidin- and DSPE-CPP modified NPs significantly increased internalization and offer promising delivery options for applications in which internalization presents challenges to efficacious delivery.

1. Introduction

Polymer nanoparticles (NPs) are attractive options for the delivery of drugs, proteins, and genetic agents. In particular, polymeric NPs can be especially useful for the delivery of biologics, as they can encapsulate and protect proteins and oligonucleotides (ONs) from degradation, while conferring the delivery advantages of: prolonged stability, controlled release, increased loading, targeting capabilities, and low toxicity in one modality [1–4]. When paired with the delivery of small-interfering RNA (siRNA) or other ONs to promote gene silencing, these carriers offer a viable technology to protect delicate cargo until intracellular localization has occurred, thereby enabling highly specific ONs to bind to or degrade complementary DNA or RNA target sequences.

Of the wide variety of polymeric vehicles utilized to tailor the delivery of biologics [5–9], poly(lactic-co-glycolic acid) (PLGA) NPs have proven markedly successful in combating a number of pathologies, including infectious diseases and cancer, due to their known biocompatibility and efficacy [8,10–14]. Furthermore, the degradation rate of PLGA – comprised of poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) – can be tailored based on the ratio of these comonomers [15–17]. Whereas higher ratios of hydrophilic PGA increase the degradation rate of PLGA; increased incorporation of the more hydrophobic PLA has the opposite effect [17,18]. Generally PLGA 50:50, consisting of a 50:50 ratio of PLA to PGA, provides a significantly faster degradation rate than other comonomer ratios, prompting degradation within days to weeks [19,20]. Additionally, by varying this ratio, PLGA blends may "optimally" tailor the delivery rates of encapsulated molecules. Relative to PLGA, the use of either PGA or PLA alone has been limited by hydrolytic instability (PGA) [21,22] and slow degradation rate (PLA) [10].

Previous work in our group has demonstrated the successful delivery of siRNA and other ONs from unmodified PLGA (50:50) NPs [3,4]. We have shown that unmodified siRNA-encapsulated PLGA (50:50) NPs elicit both mRNA knockdown and significant therapeutic effect when delivered intravaginally against HSV-2 infection in a murine model [3,4]. Similarly, we demonstrated therapeutic efficacy of siRNA (siSurvivin) PLGA (50:50) NPs in an *in vivo* bladder cancer model [23]. Although unmodified NPs successfully delivered siRNA, decreased gene expression *in vitro* and *in vivo*, and provided therapeutic efficacy [3,4], polymeric delivery vehicles – like other gene delivery platforms – must overcome the

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challenges associated with intracellular delivery to exert maximum effect [2,24–27]. Therefore we hypothesized that modifying the NP surface with ligands specific for cell uptake and endosomal escape may enhance cell binding and internalization. To address this hypothesis, we chose to quantify and compare the intracellular delivery of our previously established unmodified PLGA 50:50 NPs with surface-modified PLGA 50:50 NPs, to better understand the differences between, and the fate of NPs during cell association and internalization.

At the cellular level, the primary endocytotic mechanisms of NP internalization include phagocytosis, a process by which specialized cells transport carriers via phagosomes and phagolysosomes for degradation, and pinocytosis, in which the cellular uptake of fluids and molecules occurs within small vesicles. Four types of pinocytosis are currently known, including macropinocytosis, clathrin and caveolin-mediated endocytosis, and clathrin/caveolin independent endocytosis [28,29]. NPs internalized by the cell via one of these mechanisms may: be sorted into endosomes, and subsequently entrapped and degraded in lysosomal compartments; be released or escape directly into the cytoplasm; or undergo cellular recycling [28,29]. For NP-encapsulated agents to exert maximum effect in the cytosol, it is imperative that NP delivery be optimized to increase cell uptake and endosomal escape [24]. While NPs offer a promising way to deliver agents, NP internalization is impacted by several factors including size, material, surface modification, surface charge, and shape [30–33]. Furthermore, the rate and mechanism of internalization is cell-type dependent and varies based on these factors, making NP design for optimal cellular uptake a challenge [31,34].

To enhance the internalization of hydrophobic and negatively-charged PLGA NPs, a major focus is on the discovery and incorporation of ligands as surface modifications that improve target cell association and internalization. CPPs, comprised of short, polycationic or amphipathic peptides, have significantly improved the delivery of a variety of molecules, including siRNA, plasmid DNA, antisense ONs, PNA, proteins, and peptides [35–40]. CPPs have been designed to overcome both extracellular and intracellular barriers, by promoting movement of cargo across cell membranes and in some cases enabling the release of molecules trapped inside endosomes into the cytoplasm [41,42]. Due to the partly hydrophobic and cationic nature of some CPPs, they are able to penetrate the negatively-charged cell membrane at low micromolar concentrations without inflicting membrane damage [43].

Importantly, CPPs can promote the internalization of conjugated cargo such as NPs; however, the role of CPPs in the transport of NPs – which are much larger than single molecules or complexed oligonucleotides – across the cell membrane is not well understood [31,44]. Internalization of CPPs attached to cargo depends on both the physical properties of the CPP, together with factors including the nature of the cargo and the concentration used [45]. In some cases, the mechanism of uptake for a CPP alone (unconjugated CPP) can be quite different relative to its uptake when attached to cargo such as NPs [46,47], due to conformational changes in the CPP after conjugation.

Recent work by our group and others has focused on increasing the cellular internalization of drug delivery vehicles, by surface modification with these cell targeting and positively-charged ligands [1,2,23,48–50]. To conjugate CPPs to the surface of our NPs, we combined our siRNA PLGA approach with prior expertise in our lab that established two different surface-modification chemistries – avidin and DSPE-PEG [1,51]. These surface chemistries had been assessed *in vitro* and *in vivo* with different ligands to provide tumor targeting and mucosal diffusion [1,52–57]. Based on this previous work, we knew that avidin-modification alone enhances cell uptake and allows conjugation of a variety of peptides [1,53,54], and that DSPE-PEG, of different molecular weights, can alter transport and release characteristics (e.g. mucosal) of encapsulant [55]. In this previous work, utilization of PEG chain lengths greater than 2 kDa enabled similar or enhanced diffusion properties when conjugated to the NP surface, with the absolute diffusion coefficient dependent on DSPE-PEG modification density. Therefore for this study, we selected DSPE-PEG(2000) as the surface-modification chemistry to balance diffusive and encapsulant release properties that might be hindered by longer chain lengths in future applications.

In this work we produced NPs that were modified to include surface attachment of chitosan and several different CPPs: penetratin (AP), end-binding protein 1 (EB1), MPG, and MPGΔNLS. Chitosan, a polysaccharide, was included for comparison with CPPs because of its known benefit in cell/mucoadhesion and tight junction penetration. AP, a 16 amino acid sequence from the third helix of the antennapedia homeodomain, is one of the most studied CPPs with demonstrated success in achieving increased cell penetration [58]. EB1 is a derivative of AP with a triple helix believed to aid in endosomal escape [59]. MPG is a peptide that combines the fusion sequence of HIV (gp41) with the nuclear localization sequence (NLS) of the SV40 antigen. MPGΔNLS contains a single amino acid mutation in the NLS sequence to reduce nuclear localization [41,60].

In this study we: (1) tested these ligands, known to individually promote cell adhesion, uptake and/or endosomal escape, to determine those that provided the best cell binding and internalization after conjugation to the surface of PLGA NPs, (2) compared two different surface modification chemistries using the CPP that provided the highest cellular internalization, (3) evaluated the effect of surface ligand density on NP uptake, and (4) tracked the endocytotic pathway of CPP-NPs. To our knowledge this is the first study to test these two different surface-modified PLGA NPs with an assortment of ligands, and to demonstrate the predominant mechanism of action of the most readily internalized NP formulation.

2. Materials and methods

2.1. Materials

Poly(lactic-co-glycolic acid) with a carboxyl terminus (PLGA, 50:50 monomer ratio and 0.55–0.75 dL/g inherent viscosity) was purchased from LACTEL®. The lipid, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG), used for one of the surface modifications was purchased from Avanti Polar Lipids. The following peptides were synthesized with an N-terminus cysteine or biotin, followed by a serine-glycine spacer, and were RP-HPLC purified by the W.M. Keck Peptide Synthesis Facility at Yale University (New Haven, CT):

Penetratin (AP): SG-RQIKIWFQNRRMKWKK
End binding protein 1 (EB1): SG-LIRLWSHLIHIWFQNRRLKWKKK
MPG: SG-GALFLGFLGAAGSTMGAWSQPKKKRKV
MPGΔNLS: SG-GALFLGFLGAAGSTMGAWSQPKSKRKV

For uptake inhibition studies, the following chemicals were purchased: Dynasore (Enzo Life Sciences), Chlorpromazine (Sigma), Nystatin (Sigma) and LY294002 (Cell Signaling Technology). To determine intracellular localization, the following primary antibodies were ordered from Cell Signaling Technology and used to stain intracellular proteins: EEA1 (C45B10) rabbit mAb, Rab5 (C8B1) rabbit mAb, Rab7 (D95F2) XP™, clathrin heavy chain (CHC) (D3C6) XP® rabbit mAb, and Rab11 (D4F5) XP® rabbit mAb. LAMP-1, a mouse mAb, was ordered from Santa Cruz Biotechnology. Donkey anti-mouse and anti-rabbit rhodamine-conjugated secondary antibodies were purchased from Invitrogen.