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Surface functionalisation of PLGA nanoparticles for gene silencing

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\textbf{A B S T R A C T}

This work presents a method for decorating the surface of poly (lactide-co-glycolide) (PLGA) nanoparticles with polyethyleneimine (PEI) utilising a cetyl derivative to improve surface functionalisation and siRNA delivery. Sub-micron particles were produced by an emulsion-diffusion method using benzyl alcohol. We demonstrate by x-ray photoelectron spectroscopy (XPS), 2.6 times higher surface presentation of amines using the cetyl derivative compared to non-cetylated-PEI formulations (6.5 and 2.5% surface nitrogen, respectively). The modified particles were shown by spectroscopy, fluorescent microscopy and flow cytometry to bind and mediate siRNA delivery into the human osteosarcoma cell line U2OS and the murine macrophage cell line J774.1. Specific reduction in the anti-apoptotic oncopogene BCL-w in U2OS cells was achieved with particles containing cetylated-PEI (53%) with no cellular toxicity. In addition, particles containing cetylated-PEI achieved 64% silencing of TNFa in J774.1 cells. This rapid method for surface modification of PLGA nanoparticles promotes its application for alternative cetylated functional derivatives as a strategy to control specific biological properties of nanoparticles.

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1. Introduction

Nanoparticle-based delivery is an established method to improve the therapeutic efficiency of drugs. The ability to overcome extracellular and intracellular barriers requires specific properties controlled by functional modifications of the nanoparticle. Surface engineered nanoparticles modified with hydrophilic polymers [1] and cell-specific ligands [2] have been used to reduce phagocytic-mediated blood clearance and improve cellular targeting, whilst, incorporation of functional polycations such as polyethylenimine (PEI) is a strategy to bind nucleotide based-drugs and facilitate endosomal escape [3]. Polymeric nanoparticles that contain nucleic acids can be classified into either polycation complex systems [1] and cell-specific ligands [2] have been used to reduce phagocytic-mediated blood clearance and improve cellular targeting, whilst, incorporation of functional polycations such as polyethylenimine (PEI) is a strategy to bind nucleotide based-drugs and facilitate endosomal escape [3]. Polymeric nanoparticles that contain nucleic acids can be classified into either polycation complex systems (polypelexes) formed through electrostatic self-assembly, or nanoparticles composed of a solid polymer matrix such as poly (lactide-co-glycolide) (PLGA) with the polycation encapsulated or attached to the particle surface [4]. An attractive class of nucleotide drugs are microspheres composed of PLGA are attractive for silencing applications because of their high stability, low toxicity and the possibility of controlled release of the cargo, unfortunately the knockdown efficiency is not as high as that observed with polypelex systems [10].

Hybrid systems formed by the inclusion of polycations into polymeric nanopospheres is a strategy to promote nucleic acid binding capacity and endosomal escape of solid nanoparticles whilst improving stability and lowering the toxicity associated with polycations. Controlling the level and location of functional PEI in PLGA nanoparticles to optimise delivery of siRNA is the focus of this work.

2. Materials and methods

2.1. Materials

PLGA (Medisorb 2A, 50:50) was obtained from Alkermes (UK). Prolong® Gold antifade reagent with DAPI, Trypan Blue, Phosphate Buffered Saline (PBS), RPMI media (RPMI), foetal bovine serum (FBS), penicillin and streptomycin (P/S). Trizol, Moloney Murine Leukemia Virus (MMLV) Reverse Transcription Kit and SYBR Green Kit were purchased from Invitrogen (USA). The Duoset Mouse TNFa ELISA kit was...
obtained from R&D systems (USA). TransIT-TKO was from MirusBio (USA). Oligo dT18 reverse transcription primer and qPCR primers were from DNA Technology (Denmark). Branched PEI (25 kDa), cetyl bromide, benzyl alcohol (BA), polyvinyl alcohol (PVA) and lipopolyaccharides from E. coli O111:B4 (IPS) was from Sigma (USA). Coverslips were from Menzel Gläser (Germany). Paraformaldehyde was from Electron Microscopy Sciences (USA). The unlabelled eGFP siRNA and the Cy3 labelled eGFP siRNA were from Dharmacon (USA), the BCL-w specific siRNA was from DNA Technology (Denmark) and the TNFα match and mismatched siRNAs were a gift from Mark Behlke (IDT; USA). The siRNA sequences used were: eGFP siRNA (with and without Cy3): sense: 5’-CAGGUAAGGCCCCAGACAUUUCGT-3’, antisense: 5’-GAA-CUUGGGCGGCUUAAUGUCG-3’TdT. BCL-w siRNA sequence: 5’-CCCAUGUCGCGAUGAC-3’, antitense: 5’-AGUUCUACGGCAGACGGUC-3’TNFα match siRNA sense: 5’-GCGAAGGAGGAACGCGACGCAU-3’, antisense: 5’-AGGCGGAGAAGACGACGCGACGAC-3’TNFα mismatched siRNA sequence: 5’-CGUCUAGGCUAAUUACGGACGAU-3’, antitense: 5’-AUACUGGGUUAACAGCCGGAUACG-3’. The BCL-w forward primer was: 5’-GCTGACCGGACAGTGGTGA-3’ and the reverse primer was: 5’-ATAGACGCTGATACCTCGGC-3’, the B2M forward primer was: 5’-CTCACTGCTCACG-CACA-3’ and the reverse primer was: 5’-TCTCCTTTCGTCGGGCTGTC-3’, the GAPDH forwards primer was: 5’-CAAGGTTGACGGTGATTGTT-3’ and the reverse primer was: 5’-CAACATGGTAACTGATGATTT-3’.

2.2. Cetyl-polyethyleneimine synthesis

Cetylated PEI (Cet-PEI) was synthesized by cetyl bromide alkylation of PEI (25 kDa) according to the method of Yamazaki et al. [11]. Cetyl bromide (2.2 g, 7.2 mmol) was added to chloroform solution containing PEI (3 g, 69.8 mmol ethyleneimine units) and triethylamine (0.729 g, 7.2 mmol) and the solution was refluxed overnight. Chloroform was removed under vacuum and the mixture was dissolved in ethanol, dialyzed against water using 3500 MWCO membrane to remove low molecular weight impurities, and freeze-dried. Degree of PEI substitution with cetyl groups was determined by 1H NMR. For initial XPS analysis 4% Cet-PEI (4% cetyl substitution) was used, 10% Cet-PEI was used for all other experiments.

2.3. Particle preparation

The modified PLGA particles were prepared by a double emulsion solvent-diffusion method. PLGA was dissolved in benzyl alcohol (BA) by overnight stirring at a concentration of 50 mg/ml and was afterwards filtered (0.2 μm filter). One hundred microlitres of BA containing either: 0.3, 6 or 9 mg non-cetylated-PEI or Cet-PEI (for producing pure, 1+, 2+ and 3+ PEI particles respectively) was added to 1 ml of PLGA solution. The two solutions were mixed and the resulting solution stirred at 1600 rpm while 2.2 ml of 12.5% of PLGA solution. The two solutions were mixed and the resulting solution stirred at 1600 rpm and was afterwards filtered (0.2 μm filter). One hundred microlitres of BA containing either: 0.3, 6 or 9 mg non-cetylated-PEI or Cet-PEI (for producing pure, 1+, 2+ and 3+ PEI particles respectively) was added to 1 ml of PLGA solution. The two solutions were mixed and the resulting solution stirred at 1600 rpm and was afterwards filtered (0.2 μm filter). One hundred microlitres of BA containing either: 0.3, 6 or 9 mg non-cetylated-PEI or Cet-PEI (for producing pure, 1+, 2+ and 3+ PEI particles respectively) was added to 1 ml of PLGA solution. The two solutions were mixed and the resulting solution stirred at 1600 rpm and was afterwards filtered (0.2 μm filter).

2.4. Scanning electron microscopy & zeta potential determination

Twenty five μg of particles suspended in 50 μl ddH2O were placed onto an aluminium SEM stub and allowed to dry at RT. The samples were visualised with a NOVA/ASEM at 60 mP H2O with a low vacuum detector. The zeta potential of 5 μg particles resuspended in 1 ml 20 m NaAc (pH 4.2) was determined on a Zetasizer 80 mT.

5 ± 10⁻⁹ mbar. The analysis was performed at 90° (relative to the analyser), providing a maximum probe depth of approximately 10 nm. PLGA nanoparticles were fixed on the SiO2 slides by repeatedly drying a drop of suspended nanoparticles to form a thick and stable film of nanoparticles on the slide. Data were analyzed by Vision software. The elemental composition of samples surfaces was obtained from survey spectra collected at a pass energy of 160 eV. High-resolution spectra were recorded at a pass energy of 20 eV. Binding energies were referenced to the aliphatic carbon peak at 285 eV.

Elemental analysis of nanoparticles performed after subtracting the oxygen and carbon portion related to the background to the total values of signal detected for those elements based on elemental composition of a bare SiO2 surface.

2.7. Cell maintenance and transfection

The human osteosarcoma U2OS and the murine macrophage-like J774.1 cell lines were kept at 37°C with 5% CO2 in RPMI media supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin and split every 2 or 3 days. Cells were seeded at concentrations of 2.5 × 10⁵ cells/cm² 24 h prior to transfection. On the day of transfection a positive knockdown control using the commercial PEI/cationic lipid-based TransIT-TKO was used according to the manufacturer’s protocol at an effective well concentration of 50 μm siRNA.

U2OS cells were transfected by adding 118.25 μl/cm² serum containing media and 6.25 μl/cm² particle/siRNA solution containing 31.25 μg/cm² particles with 6.25 pmol/cm² siRNA giving an effective well concentration of 50 μm siRNA. After 24 h the media was replaced.

J774.1 cells were transfected by adding 270 μl/cm² serum free media and 14 μl/cm² particle/siRNA solution containing 70 μg/cm² particles with 14 pmol/cm² siRNA giving an effective well concentration of 100 μm siRNA. After 4 h the media was replaced with serum containing media. In the fluorescence microscopy experiment serum containing media was used from the start and the media was not replaced during the 24 culture period.

2.8. Viability

Forty eight h after transfection in a 96 well plate, cells were evaluated for cellular viability using a tetrazolium-based viability assay. Twenty microtubes of aqueous cytotoxicity solution was added to 100 μl serum containing media and was then pipetted into each well. After 1.5 h the absorbance was measured at 562 nm. The absorbances of the blank wells were subtracted from the absorbance of the samples. Four replicate samples were performed.

2.9. Fluorescence microscopy

For microscopy, J774.1 cells were seeded on coverslips (8: 13 mm). Twenty eight hours after transfection in 24 well plates with Cy3 labelled siRNA the cells were washed 3 times in PBS and fixed for 20 min at RT with a 4% paraformaldehyde solution in PBS. After a further wash in PBS, extracellular fluorescent particles were quenched by 20 min incubation with 0.04% Trypan Blue in PBS at RT, then further washed 3 times in PBS and mounted with ProLong® Gold antifade reagent with DAPI as mounting media. Fluorescence was visualised at 40× magnification on a Zeiss Axiovert 200 m microscope fitted with a CoolSnap HQ CCD camera (Photometrics, AZ, USA), a 50 W Xenon Arc lamp (PerkinElmer, CA, USA), and MetaMorph Software (Molecular Devices Corporation, PA, USA). Exposure times were set to 30 ms for brightfield images, 100 ms for DAPI images and 200 ms for Cy3 images in all samples, overlay images were created in MetaMorph with 20% contribution from brightfield images and 100% contribution from the fluorescence images.

2.10. Flow cytometry

U2OS cells were transfected in 24 well plates with Cy3 labelled siRNA formulations and harvested 48 h after transfection by trypsinisation. The cell suspension was centrifuged for 5 min at 1500 rpm, washed with PBS, and incubated with 0.04% Trypan Blue for 15 min, washed with PBS again and analyzed on a FACScanBier. Cells were selected using forward and side scatter and Cy3 fluorescence was quantified using the geometric mean value of a histogram of the FL2 channel. Three replicates were performed and the averages normalized to the average of non-transfected cells (auto fluorescence).

2.11. Quantitative polymerase chain reaction

Cells were transfected with different formulations in 24 well plates. After 48 h they were washed with PBS and the RNA isolated using 1 ml Trizol. cDNA was synthesized using a MMLV reverse transcription kit with a dTT primer. Quantitative PCR was performed on an mpxro3005 machine using a SYBR green assay. A standard SYBR green program of 40 cycles was performed with each cycle consisting of 1 min annealing at 60 °C, 1 min extension at 72 °C and 30 s dissociation at 95 °C for all primer sets. Thresholds for finding CT values were set using the dR method in the mpxro program (version 4.10). Efficiencies for all primer sets were determined to be close to 100% using standard curves and BCL-w mRNA quantities normalized to the geometric mean of the housekeeping genes B2 M and GAPDH, calculated according to the following formula:

\[
\text{Efficiency} = \frac{10^{\frac{1}{m-1}} - 1}{} 
\]
mRNA = 2 geometric mean(CT(B2M) & CT(GAPDH))/CT(BCL/C0)

The experiment was performed in 4 biological replicates (except for the TransIT-TKO samples which were performed in 3 replicates) and qPCR was performed in technical duplicates.

2.12. Enzyme-linked immunosorbent assay

J774.1 cells were transfected in 96 well plates for 72 h. Selected samples were stimulated with 200 ng/ml LPS 16 h before analysis. At the end of the culture period cellular supernatants were saved for ELISA. The wells were then incubated with 500 μg/ml MTT in 100 μl serum containing media, after 30 min the MTT solution was removed and 100 μl DMSO was added to each well. Absorbance was read at 570 nm and corrected for scattering using the absorbance at 700 nm. The saved supernatants were then assayed for TNFα content using a TNFα Duoset ELISA kit according to the manufacturer’s protocol, absorbance was read at 450 nm and corrected for scattering using the absorbance at 570 nm. The TNFα concentrations were then determined using a standard curve. Finally the TNFα/MTT ratio was found as a measure of TNFα production pr cell.

3. Results

3.1. Particle size and morphology

PLGA nanoparticles were prepared without PEI or with 2× PEI or Cet-PEI (6 mg PEI/50 mg PLGA) and visualised using SEM (Fig. 1). The particles had a smooth, spherical morphology with a dispersed size ranging from 250 nm to 1 μm. Inclusion of PEI or Cet-PEI in the particles did not appear to alter shape or size distribution in comparison to pure PLGA nanoparticles.

3.2. Surface analysis

A precise measure of surface PEI was determined by analysing the elemental composition at the particle surface using XPS (Table 1). XPS revealed an incremental increase in nitrogen content dependent on the amount Cet-PEI, peaking at 2× Cet-PEI (0.0% at pure PLGA, 4.4% at 1× Cet-PEI, 6.5% at 2× Cet-PEI and 5.7% at 3× Cet-PEI). At that condition Cet-PEI appeared to be incorporated in 2.6 times higher amounts at the particle surface than non-cetylated-PEI (6.5% and 2.5%, respectively), which supported our hypothesis that the hydrophobic cetyl groups attached to the hydrophilic PEI molecules interacted more strongly with the PLGA particles core resulting in increased PEI surface coverage.

The surface charge of the particles was measured by zeta potential analysis to investigate whether the cetyl component increased the surface charge as would be expected (Table 1). The pure PLGA particles had a slightly negative surface charge (−3.16 mV), whilst, the particles including 2× Cet-PEI, were positively charged (43.2 mV) confirming the presence of positively charged amines within an outer PEI shell of the particles. Particles produced with 2× non-cetylated-PEI were less positively charged (27.9 mV) than particles made with the same amount of Cet-PEI, which suggested that the cetyl component increased the surface presentation of the PEI.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Atom % − N</th>
<th>Atom % − O</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure PLGA</td>
<td>0% (±0)</td>
<td>34.6% (±0.68)</td>
<td>−3.16 (±0.4)</td>
</tr>
<tr>
<td>PLGA + 1× Cet-PEI</td>
<td>4.4% (±0.17)</td>
<td>32.3% (±0.25)</td>
<td>Not Measured</td>
</tr>
<tr>
<td>PLGA + 2× Cet-PEI</td>
<td>6.5% (±0.71)</td>
<td>28.9% (±0.24)</td>
<td>−43.2 (±0.4)</td>
</tr>
<tr>
<td>PLGA + 3× Cet-PEI</td>
<td>5.7% (±0.72)</td>
<td>28.7% (±0.78)</td>
<td>Not Measured</td>
</tr>
<tr>
<td>PLGA + 2× PEI</td>
<td>2.5% (±0.14)</td>
<td>35.2% (±0.24)</td>
<td>+27.9 (±0.7)</td>
</tr>
</tbody>
</table>

Fig. 1. Scanning Electron Microscopy of nanoparticles. Particles were resuspended and dried onto aluminium stubs after which they were visualised with low vacuum scanning electron microscopy. Shown are pure PLGA particles, 2× Cet-PEI/PLGA particles and 2× non-cetylated-PEI/PLGA particles.
Electrostatic attachment of polyanionic nucleic acids onto the cationic surface of Cet-PEI nanoparticles was investigated using a spectrophotometric method (Sup. Fig. S1). When siRNA was added at 100 pmol siRNA/500 µg particles, pure PLGA particles did not bind any siRNA. In contrast, the 1× Cet-PEI, 2× Cet-PEI and 2× non-cetylated-PEI containing particles bound 78%, 93% and 63% of the added siRNA, respectively. When the PEI containing particles were added at 500 pmol siRNA/500 µg particles (maximum amount of siRNA investigated) the 1× Cet-PEI, 2× Cet-PEI and 2× non-cetylated-PEI containing particles bound 84%, 93% and 95% of the added siRNA, respectively. We speculate that as higher amounts of siRNA are adsorbed onto the particles the equilibrium between bound and non-bound siRNA is shifted towards the non-bound state meaning less and less of the additional siRNA is adsorbed. In contrast non-cetylated-PEI containing particles appear to adsorb all of the siRNA even when added 5 times the amount used for transfections. Binding of plasmid DNA onto the particle surface was also possible after particle incorporation of Cet-PEI or non-cetylated-PEI (Sup. Fig. S2).

3.3. In vitro studies

PEI-based particles, particularly polyplexes, have been shown previously to exhibit some degree of cellular toxicity [8]. In contrast, none of the PLGA based particles in this work, with or without 2× PEI or 2× Cet-PEI, before or after siRNA addition, showed any significant cellular toxicity in U2OS cells after 48 h as compared to the non-transfected control group (Fig. 2).

The cellular uptake and localisation in J774.1 cells of particles with adsorbed Cy3 labelled siRNA, was evaluated using fluorescence microscopy after 24 h (Fig. 3). Extracellular fluorescence was quenched using Trypan Blue. High intracellular fluorescence was observed in cells transfected with 2× Cet-PEI/PLGA and TransIT-TKO suggesting intracellular delivery of the siRNA. In contrast, intracellular fluorescence was less widespread when cells were transfected with non-cetylated-PEI/PLGA particles and no fluorescence was detected in non-transfected cells and cells transfected with pure PLGA particles.

We quantified siRNA uptake in U2OS cells after 4 h by analysing Cy3 siRNA transfected cells by flow cytometry (Fig. 4). PLGA particles incorporating Cet-PEI or non-cetylated-PEI increased the fluorescence of the U2OS cells (133% and 267% of background, respectively), whereas pure PLGA and TransIT-TKO did not increase fluorescence above that of non-transfected cells.

To investigate the siRNA activity of nanoparticles, we assessed the gene silencing of the therapeutic target BCL-w in U2OS cells, 48 h post-transfection using quantitative polymerase chain reaction (qPCR) analysis (Fig. 5). Compared to non-treated cells, the BCL-w mRNA level was specifically reduced by the 2× Cet-PEI/PLGA particles, 2× non-cetylated-PEI/PLGA particles and TransIT-TKO (by...
41%, 62% and 71%, respectively) but not by pure PLGA particles loaded with the BCL-w siRNA. The non-cetylated-PEI/PLGA particles and TransIT-TKO EGFP siRNA control, however, reduced the BCL-w level unspecifically (by 15% and 12%, respectively) while Cet-PEI/PLGA particles increased it slightly (by 11%). Accounting for this, the siRNA specific knockdown was 53% for Cet-PEI/PLGA particles, 46% for non-cetylated-PEI/PLGA particles and 59% for commercial TransIT-TKO.

Knockdown at the protein level was determined by transfection of J774.1 cells with TNFα siRNA. After 48 h selected wells, were stimulated with LPS and after 72 h post-transfection TNFα protein in the supernatants was determined using ELISA normalized to MTT (Fig. 6). We find that pure PLGA, 2 × Cet-PEI/PLGA and non-cetylated-PEI/PLGA all increase TNFα release in non-stimulated cells as compared to a non-transfected control (between 18% and 96% increase). LPS stimulation, however, resulted in a higher release of TNFα (278% increase). When TNFα siRNA was used, knockdown using Cet-PEI/PLGA particles was superior to that of pure PLGA particles and non-cetylated-PEI particles. Cet-PEI/PLGA particles showed 64% TNFα silencing after LPS stimulation compared to the non-transfected control. Compared to the mismatched controls, Cet-PEI/PLGA particles reduced TNFα levels by 39% if cells were non-stimulated and 46% if cells were LPS stimulated. TransIT-TKO was able to reduce TNFα in LPS stimulated cells by 67% compared to the non-transfected control and by 86% as compared to the mismatched control although TransIT-TKO with mismatched siRNA greatly increased TNFα release of LPS stimulated cells by 130%.

4. Discussion

This work provides a method to functionalise the surface of PLGA nanoparticles during an emulsification-diffusion production method by using a cetyl conjugate of PEI. Cetylation of PEI has previously been used as a strategy to incorporate hydrophilic PEI onto hydrophobic liposomal delivery vehicles in order to enhance their transfection efficiency [11]. Cet-PEI has also been used to form reverse micelles in hydrophobic solvents because of the differential partitioning of the cetyl and the PEI components which stems from their hydrophobic/hydrophilic nature, respectively [12]. Based upon these findings we sought to use a cetyl group to anchor PEI on hydrophobic PLGA cores. The technique is based on partitioning of the hydrophobic cetyl component into the organic phase during the single emulsification process to create an anchorage point within the nanoparticle matrix onto which PEI is tethered. The rapid diffusion of benzyl alcohol due to its miscibility at high dilutions results in nanoscale particles and does not necessitate high shear force homogenisation or the use of chlorinated solvents common to alternative emulsification-solvent evaporation methods. The mechanism of incorporation is supported by a previous finding that fatty acids were able to anchor avidin onto microparticles produced with a traditional double emulsification process [13].

The advantage provided by PLGA nanoparticles in comparison to polycation-based polyplexes is increased serum stability, lower toxicity [10] and the potential to control nucleic acid release based on the rate of PLGA degradation, dependent on the co-polymer ratio [14]. PLGA encapsulation has previously been used for delivery of DNA plasmids and siRNA [14–16]; however, intraparticle acidification has limited this approach [17]. Furthermore the relatively slow release [18] may be undesirable in many applications. The incorporation of polycationic PEI into the system has been used to bind nucleic acids onto the surface of PLGA particles and utilise the endosomolytic property of PEI to improve cytoplasmic localisation. It has been shown that this installs into the solid particle the properties of a polyplex without the associated toxicity [10]. A simple approach has been to adsorb alternating layers of DNA and PEI onto the surface of PLGA microparticles formed by a single emulsion/solvent evaporation process [19]. Alternatively, PEI can be incorporated into a PLGA particle matrix using a single emulsion/solvent diffusion method yielding a positively charged particle onto which siRNA is adsorbed [20]. Furthermore, siRNA/PEI polyplexes can be encapsulated into PLGA particles using a double emulsion/solvent evaporation technique [21]. Common scheme for all three approaches is that the assembly is driven by the electrostatic interactions between cationic PEI and the anionic PLGA. We

![Fig. 4. Quantitative Cellular Uptake.](image-url)

Fig. 4. Quantitative Cellular Uptake. To measure uptake, 62.5 μg particles were added with or without 12.5 pmol adsorbed Cy3 labelled siRNA to 100 000 U2OS cells in 250 μl media, after 24 h the media was replaced and after an additional 24 h the cells were harvested by trypsinization and analyzed with flow cytometry. Pure PLGA particles (pure), 2 × Cet-PEI/PLGA particles (2 × C), 2 × non-cetylated-PEI/PLGA particles (2 × P) and TransIT-TKO lipopolyplexes (TKO) are shown. The average fluorescence of three biological replicates normalized to the non-transfected control is displayed, error bars show standard deviation. “+” indicates Cy3 Labelled siRNA while “0” indicates that no siRNA was adsorbed.

![Fig. 5. Silencing of BCL-w in U2OS Cells.](image-url)

Fig. 5. Silencing of BCL-w in U2OS Cells. To analyse silencing efficiency, 62.5 μg particles were added with or without 12.5 pmol adsorbed mismatched or BCL-w specific siRNA to 10 000 U2OS cells in 250 μl media. After 24 h the media was replaced and after an additional 24 h the cells were harvested. The RNA was isolated using Trizol, cDNA was synthesized and the mRNA levels of B2 M, GAPDH and BCL-w from these were determined using qPCR. Pure PLGA particles (Pure), 2 × Cet-PEI/PLGA particles (2 × C), 2 × non-cetylated-PEI/PLGA particles (2 × P) and TransIT-TKO lipopolyplexes (TKO) are shown. The average ratio of BCL-w to the geometric mean of B2 M and GAPDH of three or four biological replicates normalized to the non-transfected control is displayed, error bars show standard deviation. “+” indicates BCL-w targeted siRNA while “-“ indicates EGFP targeted siRNA.
expect interaction stability to be compromised in vivo as a consequence of the polyanionic displacement of the PEI in the presence of serum.

To stabilize the interaction between the matrix and a surface-bound molecule such as PEI, particles can be surface modified chemically after particle synthesis to include covalently bound reactive groups that can be used to attach ligands [22,23]. This method, however, has the drawbacks of time-consuming steps and use of chemicals that can have detrimental effects on the particle matrix, the attached molecule or on cells and tissues.

The present work describes an approach utilising a cetyl derivative to anchor PEI into the matrix of PLGA particles. Previous studies with Cet-PEI [11,12] supports that binding in our system is mediated by the physical entrapment of the cetyl group in the matrix as well as by hydrophobic interactions between the cetyl group and the PLGA. This anchoring increased the zeta potential, siRNA loading and more than doubled the surface content of PEI measured by the nitrogen content in comparison with non-cetylated-PEI. We observed a specific knockdown of 53% using Cet-PEI/PLGA particles and 46% using non-cetylated-PEI/PLGA particles of the oncogene BCL-w in osteosarcoma cells and a 64% knockdown using Cet-PEI/PLGA particles of the inflammatory cytokine TNFα in J774.1 cells. These knockdown efficiencies are less potent than those observed by Katas et al. [20] and Patil et al. [21] who both used non-cetylated-PEI/PLGA particles, however, it is difficult to compare due to different cell lines, target genes, siRNA sequences and the compositions and sizes of the particles used. Also, the higher stability of the Cet-PEI/PLGA particles may prove their real advantage when tested under in vivo conditions. Similar to the studies by Katas et al. [20] and Patil et al. [21], our study did not indicate any cytotoxicity induced by the particles after PEI was incorporated into a PLGA matrix, in contrast to previous cytotoxicity reported for PEI polyplexes. We observed a slight increase in TNFα when J774.1 cells were added PLGA particles with or without Cet-PEI or non-cetylated-PEI. We have previously observed such increases in TNFα release for other transfection systems such as chitosan/siRNA complexes [24] but these complexes did not give any adverse immune reactions when they were used in vivo [25]. Silencing of BCL-w, demonstrated in this study, has in vivo potential in anti-cancer treatment [26–28] while silencing TNFα has in vivo potential in anti-rheumatoid arthritis treatment [25]. In addition, we have previously demonstrated that PLGA particles can be incorporated into cell support scaffolds [29], this raises the possibility for using our particle system in tissue engineering implant applications since BCL-w silencing induces osteogenic stem cell differentiation [30] and TNFα is involved in host immune responses. In applications requiring prolonged circulation and site-specific delivery, stealth and targeting properties may be installed by the use of cetylated PEG or cetyl targeting moieties. We can thus envisage, a tool box of cetylated compounds conferring different delivery properties when combined and anchored onto PLGA particles through the simple particle production method described here.

5. Conclusion

We have developed a simple preparation method to incorporate and enhance the surface presentation of hydrophilic polymers on solid PLGA particles using a cetyl derivative. Attachment of PEI onto the surface of PLGA particles binds and mediates siRNA delivery and silencing of therapeutically relevant targets in osteosarcoma and macrophage cell lines. This work presents a potential generic approach to modulate the biological properties such as silencing, stability and targeting of PLGA nanoparticles dependent on type of functional group conjugated to the cetyl component.

Acknowledgements

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2010.03.069.
Appendix

Figure with essential colour discrimination. Fig. 3 in this article has parts that are difficult to interpret in black and white. The full colour image can be found in the online version, at doi:10.1016/j.biomaterials.2010.03.069.

References