HA-decorated nanoparticles as tumour-targeting nucleic acids carriers

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ABSTRACT

In the context of nucleic acids delivery, an ideal carrier should not only provide the means for the transport of such payloads, but also protect their activity by limiting their exposure to harmful agents leading to either physical or enzymatic degradation. We here report on the development of nanoparticles designed to target CD44; which is a membrane protein overexpressed in many tumours, while also being the major receptor of hyaluronic acid (HA). The herein described particles present a chitosan (CS) core for nucleic acids complexation and a surface rich in HA.

Nanoparticles CD44-HA mediated endocytic process, and the intracellular localisation of nucleic acids and their effects were observed using fluorescently labeled nanoparticle components. Sections and 3D reconstructions of the target (HT-29, colorectal cancer cells) and off-target (THP-1 macrophages) CD44-expressing cells were registered at different time points.

Keywords: hyaluronic acid, tumour-targeting carriers, gene therapy, siRNA

1 INTRODUCTION

In designing a nucleic acid carrier for therapeutic purposes, it is necessary to identify a strategy for both stabilisation and protection of the payload, and selective targeting [1]. Viral carriers provide both aspects, but are often considered with suspicion due to the potential dangers associated (i.e. mutagenic and immunogenic responses) [2]. In turn, the use of synthetic vectors, typically cationic lipids and polymers, is typically marred by their high toxicity [3] and low efficiency in overcoming biological barriers [4]. The decoration of cationic complexes with targeting ligands is a strategy that helps overcome the above mentioned problems, as long as they also mask the typical membrane-disrupting and ultimately toxic action of positively charged materials.

We design nanoparticles that achieve targeting through the interactions between hyaluronic acid (HA) and CD44; this approach has been explored for cancer targeting, due to the overexpression of CD44 in tumours [5, 6, 7], and also employing the CD44 behaviour as an endocytic ligand [8].

Confocal microscopy is a valuable tool for analyzing the delivery of nucleic acids to cultured cells at the resolution of a single cell. In recent years, the design of labeled nanoparticle was used to study the endocytotic mechanism and their intracellular localisation in living cells [9, 10], quantification of uptaken is also performed but no observation of delivered cargos are measured [11]. The interest to observe the delivery mechanism of nucleic acids carriers intracellularly over time, from the membrane, through endocytosis, to the entry and delivery into the cytosol, and finally determining the induced effect still proves challenging. To our knowledge, there are no studies reporting the tracking of both nanoparticles and the biological effect of the cargo.While a number of reports describe the reported effect.

Chitosan is first complexed with nucleic acids, adding then HA to produce nanoparticles with an HA-displaying surface; depending on the molecular weight of chitosan, HA can be complexed with a different strength, allowing for a different surface presentation of HA and thus also for a modulation of HA/CD44 interactions [12, 13].

In this study we have evaluated the loading capacities of the nanoparticles and then focused on the internalization of CS//HA nanoparticles in two CD44-positive cellular models: a human colorectal-cancer cell line (HT-29) and a human macrophage cell model (THP-1-M, as a model for off-target interactions). Fluorescently labeled CS//HA nanoparticles were then used to track the nanoparticle-fate from the internalisation to the intracellular delivery, and finally to monitor the transfection efficiency.

2 MATERIALS AND METHODS

2.1 Nanoparticle preparation and characterisation

Chitosan (CS) obtained from crab shells, viscosimetric average molecular weight of 656 kDa (#51009219, Lot: WE44069811; Sigma Aldrich, Basingstoke, UK) was

degraded via nitrite-mediated degradation obtaining also a low average molecular weight chitosan (MW 35 kDa). Hyaluronic acid (HA) with average molecular weight of 305 kDa (GPC with triple detection) was purchased from Novozymes Biopharma A/S (Bagsvaerd, Denmark). Salmon sperm DNA (≤ 2000 bp, 10 mg/mL) was purchased from Sigma-Aldrich (UK). Silencing RNA (siRNA, 23 nt, 5 mg/mL, Standard A4 purification) was purchased from GE Healthcare (Dharmacon, UK). Please note that all the solutions were prepared with RNase-free reagents and sterilised using appropriate filters.

Nanoparticles were prepared as described by Almalik et al. [12]. Briefly, a 0.069 wt% CS solution was prepared dissolving the polymer (low and high MW) in 4.6 mM HCl RNA-free solution. After complete dissolution, the pH was adjusted to 5 by the addition of appropriate volumes of NaOH 0.1 M. A 3 mg/mL HA solution was prepared in water and adjusted to pH 5 by addition of 0.1 M HCl solution. For the nanoparticle preparation, a first complexation was obtained by mixing the CS solution with a nucleic acid solution (at different concentrations, accordingly to the target payload). A volume of the HA solution was placed under vigorous magnetic stirring; the same volume of CS/nuclei acid complex was pipetted in the vial and left stirring for 30 min at room temperature. All the procedures were performed in sterile and RNA-free conditions.

The Z-average size and the ζ-potential of nanoparticles were both measured at room temperature with a Zetasizer Nano ZS (Model ZEN3600; Malvern Instruments Ltd., UK) equipped with a solid state HeNe laser (λ=633 nm) at a scattering angle of 173°. Nanoparticles were suspended in deionised water at a concentration of 1 mg/mL for all the measurement performed. The encapsulation efficiency was determined by measuring the concentration of non-encapsulated nucleic acids with the PicoGreen or RiboGreen assay (Life Technologies, UK) for DNA and RNA, respectively. DNase I was used to investigate the protection of encapsulated nucleic acids. Recovered supernatant was assayed usign the BioAnalyzer (DNA capillary electrophoresis, Agilent Technologies).

2.2 *In vitro* models of CD44-mediated cell uptake

THP-1 monocytes were differentiated towards macrophages by addition of 50 ng/mL PMA in cell culture medium (THP-1-M). HT-29 cells were cultured in complete medium. The total CD44 content was quantified through western blotting for both the cell lines (data not shown). For equal loading and normalisation of protein levels, ACTB (β-actin) was chosen as a housekeeping gene for it has been described as one of the most stable genes during THP-1 differentiation. Membrane-bound CD44 was also observed staining cells with Alexa594-conjugated anti-human CD44 (BioLegend, UK). Cells were stained on ice and then fixed with 4% PFA.

Nanoparticles were prepared with fluoresceinamine-labeled HA or CS, in case of uptake or live imaging for nanoparticle tracking respectively. siGLO Cyclophilin B Control siRNA and siGLO Green Transfection Indicator (Dharmacon, UK) were used to track nucleic acid fate and transfection efficiency. Cell nuclei were stained with Hoechst, cell membrane was stained with Alexa633-ConcanavalinA or CellMask Deep Red, while Alexa488-Phalloidin was used to stain actin on fixed cells (Invitrogen, UK). Confocal acquisitions were performed using a Leica TCS SP5 AOBS inverted confocal using a 63x/0.60-1.40/HCX PL Apo objective. The confocal settings were as follows, section thickness 0.571 µm, scan speed 200 Hz, pixel size 125 nm, optimal Z section number determined by the confocal software. To eliminate any possible cross-talk between channels, images were collected with a sequential scan, usign the following laser lines and mirror settings: 405(30%) 460-475nm; 488(30%) 495-580nm; 594(50%) 615-665nm; 633(40%) 650-700nm.

3 RESULTS

We have monitored the Z-average size and ζ-potential of the particles (Figure 1) as a function of the amount of nucleic acids, in order to highlight any sign of decrease of stability due to neutralisation of surface charge and resulting agglomeration at high loads. Independently on their size, nucleic acids (siRNA, plasmids, genomic DNA) were successfully encapsulated in the nanoparticles up to a load of 25% in relation to the chitosan content, with an encapsulation efficiency >95% and negligible effects on the nanoparticle physical properties.

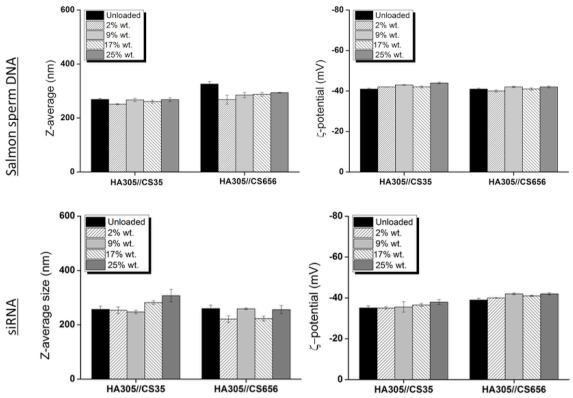

Figure 1: Z-average size (left handside) and ζ-potential (right handside) of low and high MW CS nanoparticles decorated with HA. Nucleic acids were loaded with different weigh ratios against CS (from 2 to 25%, grey shades) and un-loaded nanoparticles (black) were used as a reference value. No influence of nucleic acid size was observed, as shown in CS//HA loaded with salmon sperm (top row) and siRNA (bottom row).

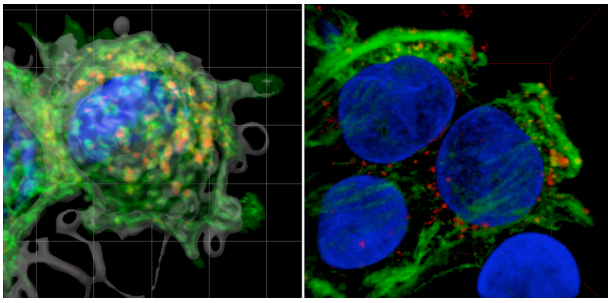

Figure 2: 3D rendering of membrane-bound CD44 on THP-1-M (left) and HT-29 (right) cells. Nuclei (blue), actin (green) and CD44 (red) are highlighted; macrophages (left hand side) were also stained with CellMask^{TM} to detect cell membrane (white). Volumes of about (30×30×15)μm^3 are rendered.

The nanoparticles were also prepared by separately labelling HA, CS, and nucleic acids with different fluorescent tags, which allowed to separately follow: i) the CD44-mediated internalisation, ii) the intracellular trafficking and iii) their individual components fate in a colorectal cancer (HT-29) and a macrophage (THP-1M) model. Sections showing the CD44 expression in colorectal cancer cells and macrophages are shown in Figure 2. The volumetric reconstruction of the living cells via confocal microscopy allows to follow in real time the cell-nanoparticle interactions. An example of HA/CD44 interaction in HT-29 after 2 hours treatment with nanoparticles is shown in Figure 3. After treatment cells were fixed and then sections were acquired using confocal microscope. As shown, both low and high MW CS nanoparticles are uptaken after 2 hours, about 0.1 μg NPs/ μg protein and 0.2 μg NPs/ μg protein respectively.

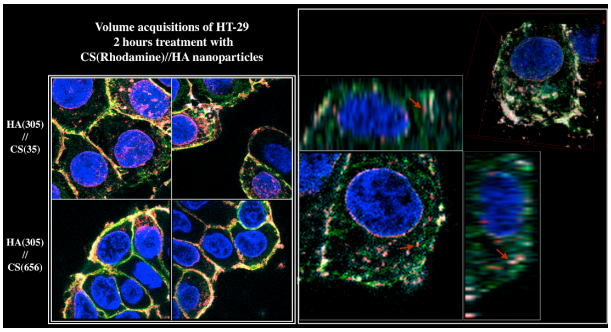

Figure 3: HT-29 treated for 2 hours with fluorescently labeled and unloaded CS//HA nanoparticles (red). Maximum projections of (40×40)μm^2 and 1 μm thich of low and high MW CS (lef hand side); orthogonal projection and 3D rendering of a single cell exposed to low MW CS nanoparticles for 2 hours. Cells were fixed after the treatment and nuclei (blue), actin (green) and glycosaminoglycans (white) were stained to locate nanoparticles within the cells.

4 CONCLUSION

In summary, the study demonstrated that CS//HA nanoparticle are a suitable cargo for nucleic acid in the CD44-mediated endocytic process. It is believed that nanoparticles enter cells through the endosomal pathway, eventually escaping into the cytoplasm, allowing for the release of the loaded content. Moreover, nucleic acid with different size can be successfully encapsulated and then delivered to CD44 expressing cells. We observed that the uptake of CS//HA nanoparticles by THP-1 macrophages is about 10% lower compare to that of HT-29. Human macrophages were also evaluated as a potential off-target; however an increased uptake in HT-29 was observed.

5 ACKNOLEDGEMENTS

Financial support from the Technology Strategy Board (TSB) under the grant agreement 33362-239223. The Bioimaging Facility microscopes used in this study were purchased with grants from BBSRC, Wellcome and the University of Manchester Strategic Fund.

REFERENCES

[1] Ding Y, Jiang ZW, Saha K, Kim CS, Kim ST, Landis RF, et al. Gold Nanoparticles for Nucleic Acid Delivery. Mol Ther 2014;22(6):1075-83.

[2] Glover DJ, Lipps HJ, Jans DA. Towards safe, non-viral therapeutic gene expression in humans. Nat Rev Genet 2005;6(4):299-U29.

[3] Lv HT, Zhang SB, Wang B, Cui SH, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. J Control Release 2006 10;114(1):100-9.

[4] Juliano R, Bauman J, Kang H, Ming X. Biological Barriers to Therapy with Antisense and siRNA Oligonucleotides. Mol Pharmaceut 2009;6(3):686-95.

[5] Misra S, Heldin P, Hascall VC, Karamanos NK, Skandalis SS, Markwald RR, et al. HA/CD44 interactions as potential targets for cancer therapy. The FEBS Journal 2011, 278(9):1429–1443

[6] Sy MS, Guo YJ, Stamenkovic I. Inhibition of tumor growth in vivo with a soluble CD44-immunoglobulin fusion protein. The Journal of Experimental Medicine 1992, 176(2):623–627.

[7] Platt VM, Szoka FC. Anticancer Therapeutics: Targeting Macromolecules and Nanocarriers to Hyaluronan or CD44, a Hyaluronan Receptor. Molecular Pharmaceutics 2008, 5 (4):474–486.

[8] Ouasti S, Kingham PJ, Terenghi G, Tirelli N. The CD44/integrings interplay and the significance of receptor binding and re-presentation in the uptake of RGD-functionalized hyaluronic acid. Biomat 2012;33:1120-1134.

[9] Mickler FM, Möckl L, Ruthardt N, Ogris M, Wagner E, Braüchl C. Tuning Nanoparticle Uptake: Live-Cell Imaging Reveals Two Distinct Endocytosis Mechanisms Mediated by Natural and Artificial EGFR Targeting Ligand. Nano Lett. 2012;12:3417-3423.
[10] Zhang Y, Hensel M. Evaluation of nanoparticles as endocytic tracers in cellular microbiology. Nanoscale 2013;5:9296-9309.
[11] Portis AM, Carballo G, Baker JL, Chan C, Walton SP. Confocal microscopy for the analysis of siRNA delivery by polymeric nanoparticles. Microsc Res Tech. 2010;73(9):878–885.
[12] Almalik A, Day PJ, Tirelli N. HA-coated chitosan nanoparticles for CD44-mediated nucleic acid delivery. Macromol Biosci 2013;13:1671-1680.
[13] Almalik A, Karimi S, Ouasti S, Donno R, Wandrey C, Day PJ, Tirelli N. Hyaluronic acid (HA) presentation as a tool to modulate and control the receptor-mediated uptake of HA-coated nanoparticles. Biomat 2013;34(21):5369-5380.