Hyaluronic acid (HA) presentation as a tool to modulate and control the receptor-mediated uptake of HA-coated nanoparticles

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ABSTRACT

The natural turnover of free hyaluronic acid (HA) is predominantly based on its CD44-mediated internalisation in leukocytes. In a phagocytic cell model (RAW 264.7 murine macrophages) we here provide conclusive evidence that this receptor-mediated mechanism endocytosis is responsible also of the uptake of materials where HA is used as a coating agent, in this case chitosan/triphosphate nanoparticles on whose surface HA is electrostatically adsorbed. Alginate-coated nanoparticles were used as a control and they appeared to undergo a qualitatively similar endocytic process, which was mediated by a different scavenging receptor yet to be identified. In this general picture, an important, modulating role appears to be played by how receptors can cluster around individual nanoparticles. The CD44 slow representation (24–48 h) enforces a limit in the amount of available HA internalisation receptors; therefore a higher affinity, and hence a higher degree of clustering, would yield a lower number of internalised nanoparticles. HA presentation can be varied by acting on nanoparticle structure/morphology, and our data suggest that a better presentation may be linked to both higher affinity and lower capacity/uptake rate. Paradoxically, this result would suggest that particles with a lower affinity for CD44 may allow a more efficient HA-mediated delivery of payloads.

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1. Introduction

Hyaluronic acid (HA), a major glycosaminoglycan (GAG) constituent of extracellular matrices, is increasingly seen as a potential agent to target conditions (e.g. several solid tumours) characterised by the overexpression of its main receptor, CD44 [1,2]. A variety of HA-based carriers have been used for the purpose of targeting tumoural cells, e.g. HA-butirrate [3], HA-paclitaxel [4] conjugates, self-assembled [5], PLGA-based [6] or disulfide cross-linked nanoparticles [7], and PEGylated HA derivatives [8]. This field has been recently reviewed by Choi et al. [9] and Ossipov et al. [10]. In the majority of cases, the role of CD44 in the internalisation of HA-based carrier structures is inferred from the preferential

internalisation in CD44 (over)expressing cell lines: for example, from the comparison of MDA-MB-231 vs. ZR-75-1 breast cancer cell lines [11], of HepB3 and HepG2 hepatoma cell lines [12], or of SCC7 squamous cell carcinoma cells and fibroblasts or HA-saturated cells [5]. Two important caveats exist: a) the slow representation of CD44 and hence its ease of saturation is a cause of concern, since, as we have recently shown, this drawback cannot be circumvented by the addition of ancillary internalisation ligands [13]; b) CD44 is heavily expressed not only by tumoural cells, but also by inflammatory cells [14], e.g. playing a major role in the clearance of apoptotic cells by macrophages [15]; the expression of CD44 by ubiquitous cells such as leukocytes may be seen as a problem, since it would inherently reduce the targeting efficiency of tumoural cells, but may also open new avenues by allowing to target (activated) leukocytes.

A yet unanswered question is whether inflammatory cells would be able to recognise HA when it is present on the surface of a nano-carrier, since its binding to leucocyte CD44 is affected by several morphological variables, such as HA molecular weight

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[16] and spatial arrangement (e.g. HA cables vs. pericellular coats [17,18]), or the possibility of receptor clustering around it [19]. Indeed the mode of ligand presentation is a major determinant of the uptake of nanocarriers, as it has been demonstrated also using coarse-grained simulations [20]. Previous literature studies have suggested that HA-coated nanoparticles can be internalised in a receptor-mediated fashion [21,22], but a final proof is still lacking. Further, if we hypothesise that internalisation is mediated by a slowly represented receptor, the extent of its clustering around individual nanoparticles determines their uptake rate and affinity: high level of clustering would correspond to high affinity, but this will limit the overall capacity, thus determining an apparently slow uptake (Scheme 1). In turn, the degree of receptor clustering would depend on nanoparticle dimension and on the accessibility of HA residues and their individual affinity for the receptors.

In this study, we have used the polyelectrolyte complexation of chitosan with triphosphate (TPP) to produce nanoparticles, which were then employed as a cationic core to complex HA and deliver HA-coated chitosan/TPP nanoparticles (CS–TPP//HA). The electrostatic adsorption of HA on CS–TPP nanoparticles can be modulated by several variables of the preparative process [23], possibly influencing its mode of presentation and its interactions with cell receptors. Therefore, we have investigated the effect of chitosan molecular weight and of two different preparative methods on the toxicity, internalisation kinetics and mechanism of CS–TPP//HA nanoparticles in a phagocyte model (RAW 264.7 macrophages). In addition, nanoparticles coated by an anionic, non-CD44 binding polysaccharide of similar size (alginate, CS–TPP//Alg) were used as a control.

In terms of molecular weight, we have employed chitosan with $\overline{M}_v = 684$ kDa (~85% deacetylated), 325 kDa (~92% deacetylated) and 25 kDa (~85% deacetylated, produced through nitrite controlled degradation of the highest molecular weight). Chitosan size is likely to influence the internal structure of the nanoparticles: higher molecular weight is likely to be less efficiently cross-linkable and therefore to produce more porous particles (larger dimensional variations in response to changes in ionic strength); on its turn, this can lead to better HA penetration as opposed to surface adsorption, therefore influencing its presentation to cell receptors.

Table 1

Key differences between preparative methods A and B.

	Addition of TPP	pH during CS/TPP complexation	pH during coating	Removal of excess of coating agent via dialysis
Method A	Injection into chitosan solution	5.0	5.0	Yes
Method B	Dropwise addition	4.0	7.2	No

In terms of preparative method, we have followed two procedures, whose key differences are summarised in Table 1.

Method A was optimised in a previous study [23] and implemented with chitosan of $\overline{M}_v = 684$, 325 and 24 kDa; by using the same pH = 5 for all solutions, it allows to minimise the influence of different mixing conditions, while by minimising the charge density on both cationic and anionic components, it provides the conditions for a likely slower but more controlled complexation. On the contrary, method B (in this case implemented with chitosan of $\overline{M}_v = 325$ kDa) provides a higher charge density to chitosan during TPP complexation and to the anionic coating agent during nanoparticle coating; therefore, in this method the phenomena of polyelectrolyte complexation are likely to occur faster. Further, the different mode of addition of TPP (injection of the liquid into chitosan solution in method A vs. its dropwise addition in method B) may result in different mixing kinetics and thus contributes to structural differences in the nanoparticle structure. Last, method B is performed under fully aseptic conditions and the nanoparticles can be directly applied to cell experiments, while in method A they are purified by dialysis and then sterile-filtered; method B is therefore more rapid and robust with less variables being introduced, and it is therefore suited for the preparation of the nanoparticle dispersion immediately before administration (advantageous for sensitive payloads and because controlled storage conditions are not required) but the presence of non-adsorbed HA or alginate may affect the results.

2. Materials and methods

2.1. Materials

Middle-viscosity chitosan of $\overline{M}_v = 684$ kDa (Mark–Houwink coefficients: $(K = 1.57 \times 10^{-5} \text{ L g}^{-1} \text{ and } a = 0.79)$ [24]) and degree of deacetylation 85% was purchased from Sigma (Sigma–Aldrich, Gillingham, UK). Lower molecular weight chitosan ($\overline{M}_v = 25$ kDa) was obtained by oxidative degradation of middle-viscosity chitosan (1% w/v in 0.1 M HCl) using 3 mM sodium nitrite. All chitosan samples were purified as described in previous reports. Chitosan of $\overline{M}_v = 325$ kDa (degree of deacetylation 92%), sodium alginate ($\overline{M}_v = 400$ kDa) and hyaluronic acid (HA; $\overline{M}_v = 360$ kDa) were provided by Medipol (Medipol SA, Ecublens, Switzerland).

Sodium triphosphate pentabasic (TPP), dimethyl sulfoxide (DMSO) and rhodamine B isothiocyanate (RITC) were purchased from Sigma–Aldrich (Gillingham, UK). [3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] (MTS) kit was purchased from Promega (Madison, WI, USA).

RAW 264.7 macrophages (ECACC, UK), Dulbecco's modified eagle medium (DMEM), the quantipro BCA assay kit, Triton X-100, antibiotic–antimitotic solution, sodium pyruvate, D-glucose, 4',6-diamidino-2-phenylindole (DAPI) and endocytosis portals inhibitors (cytochalasin D, filipin, 5-(N-ethyl-N-isopropyl)amiloride (EIPA), nocodazole, chlorpromazine, bafilomycin A1 and sodium azide) were purchased from Sigma–Aldrich. Foetal bovine serum (FBS), LysoTracker Green DND-26, L-glutamine and anti-fade mounting gel were purchased from Invitrogen (Paisley, UK). Monoclonal antibody anti-CD44 [KM81] and paraformaldehyde were respectively brought from Abcam (Cambridge, UK) and VWR (Lutterworth, UK).

2.2. Fluorescent labelling of chitosan

An amount of chitosan corresponding to 0.48 mmol of amine groups (100 mg for CS684) were dissolved in 18 mL of 0.10 M acetic acid solution and the pH was then adjusted to 4 with 0.1 M NaOH. 10 mg of RITC (0.019 mmol of isothiocyanate groups) dissolved in 2 mL of DMSO were added slowly to the chitosan solution using a

Scheme 1. Under conditions of slow receptor representation, the amount of available receptors is a crucial factor to determine the uptake kinetics of e.g. an HA-coated nanoparticle. A limited number of interaction sites, e.g. because of poor presentation of HA, would allow the internalisation of a larger amount of less strongly bound nanoparticles (bottom left); on the contrary, strong interactions due to the clustering of many receptors around each nanoparticle may lead to a small number of nanoparticles being internalised (bottom right).

dropping funnel. The reaction was left under stirring for 12 h protected from light. The reaction mixture was then diluted with 20 mL of deionised water and dialysed against deionised water (pH = 6.5) using an RC dialysis membrane of MWCO 10 kDa (SpectraPor, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) until the conductivity and pH values of the waste water reached the values of pure water. The sample was then freeze-dried and stored at 4 °C. The degree of functionalisation was determined by measuring the fluorescence intensity of the RITC-chitosan solution, using a calibration of fluorescein ($\lambda_{\text{ex}} = 540$ nm; $\lambda_{\text{em}} = 620$ nm). Typically, 1.2–1.7% mol of D-glucosamine units were derivatised.

2.3. Preparation of nanoparticles

2.3.1. Method A

A 0.069% wt. RITC-labelled chitosan solution was prepared by dissolving purified RITC-labelled chitosan in 4.6 mM HCl. The pH was adjusted to 5 by the addition of appropriate volumes of 0.1 M NaOH. The solution was kept under magnetic stirring overnight and sonicated for 40 min. TPP was prepared as a 0.1% wt. solution in deionised water, correcting the pH to 5 using appropriate volumes of HCl 0.1 M. Both solutions were filtered through a 0.22 μm pore size filter. The nanoparticles were prepared by addition of the TPP solution 0.1% wt. to the chitosan solution in a 1:9 TPP to chitosan mass ratio, e.g. for a final volume of 3 mL of nanoparticle solution, 214 μL of TPP solution is added to 2786 μL of chitosan solution, where the final concentrations of chitosan and TPP are 0.064 and 0.0071% wt., respectively. The complexation was carried under magnetic agitation (750 rpm), for 30 min at 25 °C. The final dispersion was sonicated for 40 min and then left undisturbed for additional 16 h. Then the nanoparticle dispersion was dialysed against deionised water (MWCO 1000 kDa) before being surface decorated by HA (360 kDa) or sodium alginate (400 kDa). For the surface coating process, 5 mL of a 0.025% wt. dispersion of CS–TPP nanoparticles in 100 mM acetate buffer at pH = 5 were added under vigorous magnetic stirring (30 min, 1200 rpm) to an equal volume of HA or sodium alginate both at a concentration of 0.15% wt. in the same buffer. The dispersions were then dialysed against deionised water (pH = 6.5, membranes with MWCO 1000 kDa).

2.3.2. Method B

The preparative method is based on a patented aseptic process [25]. RITC-labelled chitosan (325 kDa) was dissolved in deionised water (final concentration is 0.1% w/v) by the addition of 1 M HCl until the pH was stabilised below 4. The dispersion was kept under magnetic stirring until complete dissolution. TPP 0.1% (w/v) in deionised water at pH $\approx$ 6.5 was added dropwise to the chitosan solution, in a 1:9 TPP to chitosan mass ratio while keeping the pH under 4 by the addition of proper volumes of 0.1 M HCl. The suspension was kept under stirring for an hour and then diluted with deionised water at a 1:1 (v/v) ratio. The dispersion was then added dropwise to 0.1% (w/v) sterile filtered aqueous HA or alginate solutions at a volume ratio of 1:1 (v/v) under vigorous agitation. 0.1 M NaOH was used to adjust the pH of the final nanoparticle suspension to 7.2. Finally, the suspension was kept under magnetic stirring for one additional hour at room temperature.

2.4. Physico-chemical characterisation

Hydrodynamic diameter (Z-average size), size polydispersity (PDI) and zeta potential measurements were always performed on three independent samples at a temperature of 25 °C using a Zetasizer Nano ZS instrument (Model ZEN3600, Malvern Instruments Ltd., UK) equipped with a solid state HeNe laser ($\lambda = 633$ nm) and measuring at a scattering angle of 173°.

Atomic force microscopy (AFM) images were acquired at 25 °C in air using a Molecular Force Probe 3D AFM (MFP-3D, Asylum Research, Santa Barbara, CA). Si-Icon cantilever (model NP–S10, Bruker) with a nominal spring constant, tip radius, tip height and resonance frequency, of 0.06 N/m, 20 nm, 2.5–8 μm , 18 kHz respectively, was used. Contact mode images of several regions were acquired (512 $\times$ 512 pixels per inch), at a scan frequency of 1 Hz and a gain of 10.

2.5. Cellular interactions of nanoparticles

2.5.1. General cell culture

RAW 264.7 macrophages were maintained as a semi-adherent cell culture in standard conditions for cell culture (37 °C, 5% CO₂, in humidified atmosphere) in Dulbecco's modified eagle minimal essential medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2×10^{-3} M glutamine, 100 U mL⁻¹ penicillin and 100 U mL⁻¹ streptomycin. Cells were detached by scrapping and adjusted to the required concentration of viable cells, by counting in a haemocytometer.

2.5.2. Cell viability assays

RITC-labelled chitosan was employed in the preparation of nanoparticles through both methods A and B as described above. Nanoparticles with required concentrations (750, 500, 325, 200, 100, 10, 1 and 0 $\mu\text{g/mL}$) were prepared in full phenol red-free medium (10% FBS, 1% L-glutamate, 1% antibiotic/antimicrobial) (see Supplementary information).

RAW 264.7 macrophages of passages 10–15 were seeded in a 96-well plate at a density of 10,000 cells/well and cultured in DMEM medium containing 10% FBS, 1% antibiotic/antimicrobial solution, and 1% L-glutamate and incubated under standard sterile conditions for cell culture (5% CO₂, 37 °C) until 70% confluency. The medium was then removed and the cells were washed with PBS. The cells were then incubated with nanoparticles in full phenol-red free DMEM in standard conditions for cell culture for 0, 1, 2, 4, 8, 16 and 24 h. At the completion of the incubation, the cells were washed with PBS and incubated further for 2 h in serum-free medium containing MTS 5% (v/v). Cell viability was measured colourimetrically using the conversion of MTS to a soluble coloured formazan. The absorbance readings were acquired with a Synergy2 Biotek plate reader using Gen5 software. The quantity of formazan produced as measured by the absorbance at 490 nm is proportional to both the number and the metabolic activity of living cells in culture. Cell viability measurements were normalised by the amount of total protein content in each well. Total protein content was quantified using the Quantiprot BCA assay kit. Briefly, the cells were washed with PBS, and incubated for 15 min, in 100 μL cell lysis buffer (0.5% Triton X-100 in PBS), to which 100 μL of Quantiprot solution (prepared following the instructions of the manufacturer) was added. After 2 h incubation at 37 °C, the absorbance was recorded. Cell viability was expressed as the ratio of the absorbance reading for nanoparticle treated cells to that for control non-treated cells.

2.5.3. Quantification of cellular uptake (fluorimetry)

RITC-labelled chitosan samples were incorporated in nanoparticle preparation methods A and B to highlight the effect of the preparative procedures, chitosan molecular weight and surface chemistry on the cellular fate of nanoparticles. Following the same procedure as previously described for cell viability studies, the uptake experiments were performed on concentrations ranging from 10 to 250 $\mu\text{g/mL}$ of nanoparticles in full phenol red-free DMEM medium. After specified incubation times (1, 2, 4, 8, 16 and 24 h), the supernatant solution was removed and the cells were washed three times with PBS and lysed with 100 μL of 0.5% Triton X-100 in 0.2 M NaOH. The amount of membrane bound and internalised nanoparticles was estimated on the basis of the fluorescence of the cell lysates ($\lambda_{\text{ex}} = 540$ nm; $\lambda_{\text{em}} = 620$ nm); nanoparticles dispersed in cell lysates were used to provide a calibration for the fluorescence data. The amount of internalised nanoparticles was normalised by the total protein content, which was quantified using the Quantiprot BCA assay kit as described previously. For Michaelis–Menten fitting, the amount of nanoparticles internalised after 2 and 8 h was divided by the exposure time to respectively provide an initial uptake rate and an overall uptake rate.

2.5.4. Intracellular localization

RAW 264.7 macrophages in full DMEM medium were seeded in slide-flasks at a density of 300,000 cells per flask and incubated for 24 h in standard conditions for cell culture. The medium was then removed and the cells were washed with PBS. The cells were then incubated with nanoparticles in full phenol red-free DMEM at a final concentration of 250 $\mu\text{g/mL}$ for 4 h. At the completion of incubation, the medium was discarded and cells were washed with PBS. Cells were then incubated with 0.2% wt. trypan blue solution for 1 min at room temperature and washed further three times with PBS. LysoTracker Green (100 nm) in plain phenol red-free DMEM was then incubated with the cells for 10 min in standard conditions for cell culture. The medium was carefully discarded and the cells were washed with PBS and fixed with 4% wt. paraformaldehyde in PBS for 30 min at room temperature. Fixed cells were washed with PBS and incubated with DAPI for nuclei staining. Cells were then washed with PBS and mounted using an anti-fade mounting gel. The slides were observed under a microscope (Delta Vision RT deconvolution microscope, Applied Precision, Issaquah, USA) using a 60 $\times$ /1.42 Plan Apo objective and 360/475 nm, 490/528 nm and 555/617 nm filter sets (Chroma 8600v2/89000, Chroma Technology, USA). The images were acquired using a Coolsnap HQ (Photometrics, Tucson, USA) camera at a resolution of 512 $\times$ 512 pixels per inch with a Z optical spacing of 0.2 mm. Softworx software was used to deconvolute the raw images.

2.5.5. Mechanism of nanoparticle uptake

The mechanism of nanoparticle internalization was studied by measuring the relative cellular uptake of fluorescent nanoparticles in RAW 264.7 macrophages after the incubation of cells with a small library of selective inhibitors of different endocytosis mechanisms: cells were treated with each inhibitor before being incubated with the nanoparticles, and afterwards the fluorescence emission of cell lysates was measured. The relative uptake of nanoparticles was expressed as the ratio of the fluorescence reading for endocytic inhibitor treated cells to that for control non-treated cells. Macrophages were seeded in 96 well-plate and incubated as described in the previous section. Cells were pre-incubated for 30 min with plain DMEM medium containing (inhibitors) cytochalasin D (1 μM), filipin (5 $\mu\text{g/mL}$), nocodazole (0.1 $\mu\text{g/mL}$), bafilomycin A1 (200 nM), chlorpromazine (65 $\mu\text{g/mL}$), sucrose (0.45 M) or sodium azide (10 mM), or for 1 h with plain DMEM medium containing (inhibitors) EIPA (100 μM) or anti-CD44 antibody [KM81] (20 $\mu\text{g/mL}$). The medium was then discarded and the cells were gently washed with PBS, and further incubated for 2 h with full phenol red-free DMEM containing nanoparticles at a final concentration of 250 $\mu\text{g/mL}$. The cells were then washed with ice-cold PBS and lysed with 100 μL of 0.5% Triton X-100 in 0.2 M NaOH for 15 min at room temperature. Fluorescence readings and BCA assay were carried out as described previously.

3. Results and discussion

3.1. Nanoparticle characterisation

3.1.1. Effect of chitosan molecular weight (method A)

The dimension of CS–TPP (uncoated) nanoparticles increased with increasing chitosan molecular weight (Table 2, top); this effect is ascribed to a less effective cross-linking action of TPP (gel point reached at a lower TPP content) for larger chitosan macromolecules, thus yielding larger but more porous particles for the same chitosan content. This phenomenon has already been shown for chitosan/TPP microparticles, where the TPP content decreases with increasing chitosan molecular weight [26].

All CS–TPP//HA (HA-coated) nanoparticles showed a similar dimension and charge, independently on chitosan molecular weight (Table 2, middle); in relation to their parent particles, this corresponded to a significant size increase for CS(25), to a negligible change for CS(325) and to a slight decrease for CS(684). We ascribe this effect to the effect of chitosan molecular weight on the nanoparticle structure: HA would hardly penetrate into compact particles, and indeed CS(25)–TPP//HA showed a crown of loosely bound HA, which is ultimately responsible of the increase in size (Fig. 1, left); on the other hand more porous particles such as CS(684)–TPP//HA may better accommodate the anionic polysaccharide in their internal structure, yielding no apparent external crown and a more cross-linked and hence shrunken core (Fig. 1, right); a more comprehensive analysis of particle morphology is the subject of a forthcoming publication. However, although present in a different morphology, HA will dictate both surface composition and charge in all cases.

Alginate has twice the charge density of HA and correspondingly should adsorb more rapidly and more efficiently on the cationic CS–TPP nanoparticles. Indeed, CS–TPP//Alg (alginate-coated) nanoparticles showed a higher surface charge, but also a much larger size than the corresponding HA-coated ones (Table 2, bottom). This effect is most likely due to agglomeration during coating, as a consequence of the increased attraction between partially coated particles.

3.1.2. Effect of preparative method (method A vs. method B)

We have compared the two preparative methods using CS(325). In a brief summary, the key differences are as follows:

Table 2

Physical characteristics^a of nanoparticles as a function of chitosan molecular weight (25, 325 and 684 kDa), coating material (HA/alginate) and preparative method (method A/method B).

Chitosan MW (kDa)/ preparative method	CS25/ method A	CS325/ method A	CS325/ method B ^b	CS684/ method A
CS–TPP				
Z-average size (nm)	144 ± 28	263 ± 38	487 ± 52	330 ± 47
PDI ^c	0.27 ± 0.01	0.25 ± 0.02	0.44 ± 0.02	0.30 ± 0.01
ζ potential (mV)	+37 ± 4	+41 ± 3	+47 ± 2	+41 ± 6
CS–TPP//HA				
Z-average size (nm)	317 ± 5	303 ± 59	268 ± 43	292 ± 7
PDI ^c	0.27 ± 0.01	0.28 ± 0.02	0.20 ± 0.02	0.25 ± 0.03
ζ potential (mV)	–52 ± 6	–58 ± 2	–39 ± 1	–53 ± 4
CS–TPP//Alg				
Z-average size (nm)	696 ± 84	730 ± 95	499 ± 21	671 ± 70
PDI ^c	0.33 ± 0.09	0.30 ± 0.01	0.34 ± 0.11	0.31 ± 0.01
ζ potential (mV)	–76 ± 3	–78 ± 8	–56 ± 6	–72 ± 2

^a Measurements performed on 250 µg/mL nanoparticle dispersions in deionised water.

^b Using method B, the CS–TPP nanoparticles were prepared at pH = 4 in deionised water and the resulting, non-dialysed dispersions were measured at that pH. The CS–TPP//HA and Alg nanoparticles were prepared at pH = 7.2 in deionised water and the resulting, non-dialysed dispersions were measured at that pH.

^c PDI: polydispersity index. It is calculated in a scale 0 (monodisperse distribution) to 1 (very broad distribution).

method A – pH = 5 for both chitosan and TPP (charge = –3), the two solutions are rapidly mixed because one is injected into the other. Method B – chitosan at pH = 4 (more positively charged) and TPP at pH = 6.5–7 (charge ≈ –4); one solution (TPP) is added dropwise into the other, which may result in a kinetically controlled process; no dialysis. The conditions of method A are supposed to provide smaller and possibly more compact nanoparticles because of slower complexation (reduced charge density in both partners) but better mixing; those of method B were designed to provide a more rapid complexation, possibly leading to larger and more porous objects, and an easier aseptic process.

Indeed, the CS–TPP nanoparticles produced through method B were considerably larger and with a broader size distribution than those obtained through method A (Table 2). On the other hand, they considerably shrunk upon HA coating and produced smaller and less negatively charged objects than their method A counterparts; this effect is possibly a consequence of the higher porosity of method B particles, which would allow polyanions to better diffuse into their bulk, further cross-linking and thus shrinking them. However, we cannot exclude an osmotically-driven shrinkage due to the presence of excess (free) polyanions in the medium, since particles prepared through method B are not purified via dialysis.

Finally, it is noteworthy that also for method B nanoparticles the use of the more negatively charged alginate vs. that of HA likely caused nanoparticle agglomeration.

3.1.3. Effect of medium

In cell culture medium (DMEM supplemented with 10% FBS), all coated nanoparticles considerably shrunk. This effect is well-known [22] and stems from a reduced nanoparticle swelling due to the increased external osmotic pressure.

For method A the size reduction (compare top and bottom graphs in Fig. 2) was most significant for alginate-coated nanoparticles, whose agglomerated structure would allow much shrinkage to occur between individual particles.

However, all method A nanoparticles showed a comparable final size in cell culture medium, with the majority of the distribution comprised between 100 and 400 nm.

On the other hand, method B nanoparticles shrunk to significantly lower values (Fig. 3; in no case the contribution of <100 nm nanoparticles is negligible), which would confirm them to have a looser, more porous internal structure. For any comparison of their effects on cells, it is therefore important to note that method B nanoparticles may differ because of morphological, structural but also dimensional factors.

3.2. Cellular interactions

3.2.1. Effects on macrophage metabolic activity (cytotoxicity)

We have studied the effect of nanoparticles on the activity of RAW 264.7 macrophages, a cell line with high sensitivity to potentially toxic chemicals [27,28], as a function of nanoparticle concentration, time of exposure, chitosan molecular weight, nature of the anionic polysaccharide and preparative method. This analysis was performed by measuring the macrophage mitochondrial reductase activity (MTS assay) normalised by the cell protein content (BCA assay), thus providing an assessment of the average metabolic activity per cell as a function of the nanoparticle exposure (Fig. 4).

First, no significant difference was recorded for any method A nanoparticles, independently from chitosan molecular weight and from the nature of the coating (either HA or alginate): the macrophages showed no significant reduction in metabolic activity (viability) upon exposure to nanoparticles for concentrations up to 750 µg/mL and for times up to 24 h (Fig. 4, A–F). The absence of a

Fig. 1. Deflection images of CS(25)–TPP/HA (left) and CS(684)–TPP/HA (right) nanoparticles obtained via contact mode AFM. The two kinds of nanoparticles show a similar flattened morphology on a mica substrate, but those containing lower molecular weight chitosan exhibit an additional, <2 nm thick crown that we interpret as loosely bound HA. Please note that deflection images do not describe the actual contour of an object but rather the slope of the sample surface; however, they are considerably more sensitive to resolving fine details than height images and were therefore chosen for the purpose of this visualization; the corresponding height images can be found in [Supporting information](#), Fig. 1SI.

chitosan molecular weight dependency is not surprising, since this result has also been recorded for uncoated nanoparticles [29].

Since uncoated chitosan–TPP nanoparticles were reported to significantly reduce the viability of the less sensitive J774 macrophages at 300 μg/mL (24 h) [22], the negligible toxicity of the coated particles suggests that for all chitosan molecular weights, both anionic polysaccharides effectively shielded the underlying chitosan core.

On the other hand, nanoparticles produced with method B showed a rather high toxicity, which depended both on the dose and on the exposure time (Fig. 4, G–H); at 24 h HA- and Alg-coated nanoparticles yielding IC₅₀ values of 270 and 480 μg/mL,

respectively. Method B particles showed larger shrinkage in media and a considerably lower (less negative) ζ potential; these points would indicate larger porosity and lower exposure of the anionic polysaccharides and/or possible exposure of chitosan, which could be the cause of the increased toxicity. It is noteworthy that HA-coated nanoparticles caused a stronger reduction in cell viability than the alginate-coated ones, which could possibly be due to a more efficient internalisation.

3.2.2. Kinetics of nanoparticle uptake

The uptake of nanoparticles was evaluated by measuring the fluorescence of cell lysates (normalised against the protein content)

Fig. 2. Size distribution of CS–TPP/HA and CS–TPP/Alg nanoparticles prepared using method A and dispersed in deionised water or in cell culture medium (DMEM with 10% v/v FBS). Nanoparticle concentration: 250 μg/mL. Please note that the increase in scattering intensity below 70 nm is due to FBS proteins.

Fig. 3. Size distribution of CS-TPP/HA and CS-TPP/Alg nanoparticles (CS325) dispersed in deionised water or culture medium (DMEM with 10% v/v FBS). Nanoparticle concentration: 250 $\mu\text{g}/\text{mL}$. Please note that the increase in scattering intensity below 70 nm is due to FBS proteins.

as a function of nanoparticle concentration, time of exposure, chitosan molecular weight, nature of the anionic polysaccharide and preparative method (Fig. 5). It is noteworthy that this analysis does not allow discrimination between intracellular and surface-bound materials. In all cases, the uptake increased with incubation time and reached a plateau after 8 h, as previously recorded for HA-coated CS-TPP nanoparticles using J774.2 macrophages [22]; please note that in this study we have used the more active RAW 264.7 macrophages, which show a considerably higher nanoparticle uptake (hundreds vs. tens of $\mu\text{g}/\text{mg}$ of protein).

The presence of a plateau suggests an uptake based on a saturable cell receptor, which for HA-based systems has been hypothesised to be the slowly represented CD44 [13]. After binding, CD44 is internalised and then degraded with its ligands; it generally takes 24–48 h for the cell to represent it again, thus during this time window the capacity of CD44-mediated uptake will considerably diminish [13,22]. For alginate-coated nanoparticles, we can only speculate the involvement of a similarly saturable scavenging receptor.

We have first focused on the analysis of the uptake at saturation.

The plateau values increased with increasing nanoparticle concentration, although slowly (a 25-fold increase in concentration corresponded to a 1.5–4.5-fold increase in uptake). This dependency suggests the occurrence of a variable mode of binding: a higher particle concentration may lead to a lower number of clustered receptors per particle and thus a larger uptake per cell (the number of receptors per cell being assumed constant).

HA-coated nanoparticles always showed 20–70% higher saturation values than alginate-coated ones; this suggests a somewhat higher efficiency for the HA receptor than the putative alginate receptor.

The highest saturation values were recorded for high molecular weight (684 kDa) chitosan nanoparticles (compare Fig. 5, E–F with A–B). This effect is possibly related to the deeper penetration of the

polyanions in these particles, which would reduce their accessibility and therefore determine a lower number of interaction sites per particle, that is, a larger number of particles per cell is allowed; however, we cannot completely exclude some direct exposure of chitosan, which can be internalised through the mannose receptor in macrophages [30], as well as in other cell types [31].

The plateau uptake of method B nanoparticles (Fig. 5, G and H) was particularly low, which could be due to the presence of free (=in solution, unbound) HA or alginate competing with nanoparticles for the same cell receptors. However, even if the internalisation experiments were conducted under the conditions of nanoparticle concentrations/exposure time compatible with untouched viability, we cannot completely exclude effects due to reduction of macrophage metabolic activity.

In order to gather additional quantitative information on the binding kinetics, we have studied how nanoparticle uptake at a given time depended on nanoparticle concentration; the shape of the corresponding curves was strongly influenced by the chitosan molecular weight (Fig. 6, left) and by the preparative method (Fig. 6, right).

The uptake after 2 and 8 h was respectively used to calculate an initial and an overall uptake rate, whose concentration dependency was fitted using a Michaelis–Menten model [19,25]. This treatment provides an asymptotic internalisation rate at high particle concentration (V_{max}) and a value inversely related to the average nanoparticle affinity for macrophages (K_m) (see [Supplementary information](#), Figs. 2SI and 3SI). Despite its probably excessive simplicity, this model has been widely applied to study a range of receptor-mediated processes, e.g. the internalisation of proteins [32,33] or bile salt/drug complexes [34].

Either obtained for the initial phase (0–2 h) or for the overall kinetics (0–8 h) the results are substantially analogous (Table 3) and indicate three trends:

Fig. 4. Average cell viability (MTS; mitochondrial activity normalised against protein content) of RAW 264.7 macrophages incubated with CS–TPP//HA and CS–TPP//Alg nanoparticles as a function of time of exposure, chitosan molecular weight, nature of the anionic polysaccharide and nanoparticle preparative method. Data ($n = 3$) are expressed relative to control experiments.

First, HA-coated particles showed a 10–50% larger $V_{\max}$ and a 20–80% higher affinity (lower K_m) than alginate-coated ones. The difference in $V_{\max}$ is probably due to the availability of a different number of receptors for the two polysaccharides. The difference in affinity can be due to a different clustering behaviour of HA and alginate receptors: the latter ones may be less prone to cluster

around the same nanoparticle, decreasing the scavenging efficiency (affinity) at low particle concentration.

Second, the dependency of uptake kinetics on chitosan molecular weight can be explained in a similar fashion, with the accessibility of HA or alginate being reduced in high molecular weight chitosan particles and therefore allowing for more internalisation

Fig. 5. Uptake of CS-TPP/HA (A, C, E and G) and CS-TPP/Alg (B, D, F and H) nanoparticles by RAW 264.7 macrophages as a function of time, nanoparticle, chitosan molecular weight and preparative method (compare methods A and B for CS325). $n = 3$. The uptake was measured as the fluorescence of cell lysates (using a calibration of nanoparticles in cell lysates) and was normalized against the amount of total protein content: The data therefore provide an average amount of internalised material "per cell", assuming the cellular protein content to be roughly constant. Please note that method A CS684 nanoparticles exhibit the highest uptake and method B CS325 ones the lowest, and correspondingly the vertical scales are not the same as for the other nanoparticle types.

points (higher V_{max}) with lower affinity (higher K_m). An explanation for the much reduced affinity of CS(325)-TPP coated nanoparticles can only be tentative and invoke a tighter binding of the anionic polysaccharides: CS325 has the highest content of acetyl groups that, by promoting hydrophobic association, may also increase the local density of the cationic groups.

Third, method B nanoparticles were internalised with a slower kinetics (3–5 times smaller V_{max}) but much higher affinity (10–40 times lower K_m) than those with identical composition obtained with method A. As previously mentioned, their uptake kinetics can be heavily influenced by the competition with excess of (non-bound) polyanions in solution, which may allow internalisation

Fig. 6. Uptake of chitosan nanoparticles in RAW 264.7 macrophages after 8 h incubation as a function of nanoparticle concentration, chitosan molecular weight and method of preparation (right).

only of particles with affinity higher than that of free HA/alginate; however, the considerably smaller diameter of most particles (Fig. 3, bottom graphs) may also facilitate receptor clustering leading to higher affinity.

3.2.3. Mechanism of internalisation

As recently reviewed [35], the use of selective endocytic inhibitors can be applied to assess the mechanism of internalization of nanomaterials. Here, we have quantitatively assessed the effect of inhibitors by first exposing RAW 264.7 macrophages to them and then measuring the fluorescence of cell lysates after a 2 h incubation with the nanoparticles (Fig. 7).

It is noteworthy that chitosan molecular weight and method of preparation did not appear to affect the mechanism of internalisation, which on the contrary seemed to depend on the chemistry of the adsorbed polyanion.

Sodium azide, cytochalasin D, nocodazole and bafilomycin A1 were used as positive controls: by interfering respectively with all energy dependent processes [36], F-actin polymerization microtubule formation/active vesicular transport [37,38] and endosomal acidification/receptor recycling [13,39]. They are general inhibitors of all or most forms of endocytosis and intracellular trafficking. Sodium azide and cytochalasin D very significantly reduced the internalisation of all kinds of particles (20–40% values of the control), showing the uptake to be an active, endocytic process. The lower influence of nocodazole (10–15% and 15–23% reduction in uptake for HA-coated and Alg-coated nanoparticles, respectively) and bafilomycin A1 (35–50% and 20–30% reduction in uptake for HA-coated and Alg-coated nanoparticles respectively) is probably

ascribable to the relatively short time of the test, which allows only for a limited extent of intracellular trafficking; however, their clear inhibitory effect indicates that all particles are likely trafficked in endosomes (nocodazole) and that their endocytosis is probably a receptor-mediated process jammed by inhibition of acidification (bafilomycin). Indeed, fluorescence microscopy showed a significant co-localisation of the nanoparticles with late endosomes/lysosomes (Fig. 8). It is noteworthy that the fluorescence associated to chitosan typically covered larger areas around those associated to LysoTracker fluorescence (see the red halos around the yellow dots in the merged pictures of Fig. 8), which is a sign of lysosomal escape.

Chlorpromazine and hypertonic sucrose (0.45 M) interfere with the formation of clathrin-coated pits [40,41] and are therefore widely considered as inhibitors of the clathrin-mediated endocytosis. In no case, they appeared to reduce uptake, suggesting, therefore, no involvement of clathrin in the internalisation process. It is worth pointing out that this may be a cell-specific process. For example, RAW 264.7 macrophages have shown clathrin-dependent internalisation of nanomaterials such as superparamagnetic iron oxide nanoparticles [42], while other endo/phagocytic processes are not affected by clathrin inhibition [43]. On the other hand, in a previous study by our group, J774.2 macrophages showed a 20–30% reduction of HA-coated CS(477)–TPP nanoparticles internalisation with clathrin inhibition via hypertonic sucrose or potassium-free buffer [22], a difference that we are inclined to ascribe to the different endocytic behaviour of the two cell lines.

Pre-incubation with 5-(N-ethyl-N-isopropyl)amiloride (EIPA), which suppresses endocytosis via macropinocytosis [13,44], did not affect the internalisation of HA-coated nanoparticles, while resulted in a 10–20% decrease in alginate-coated ones, with no statistically significant difference attributable to chitosan molecular weight or method of preparation. Due to this negligible or relatively little effect, macropinocytosis was concluded not to be a major internalisation route for any of the nanoparticle formulations.

Filipin is a cholesterol-sequesterant that inhibits lipid raft-dependent internalisation processes such as caveolae-mediated endocytosis [41,45]. Filipin had no effect on the internalisation of HA-coated nanoparticles and caused a rather slight decrease in alginate-coated nanoparticles (alginate-coated nanoparticles uptake was reduced by 16 and 10% for 25 and 684 kDa chitosan, respectively), which possibly suggests some involvement of lipid raft-mediated endocytosis in the uptake of alginate-coated nanoparticles.

Finally, the use of a mouse anti-CD44 antibody [KM81] produced a 65% reduction in the uptake of HA-coated nanoparticles

Table 3

Kinetic parameters (Michaelis–Menten model) for the uptake of CS–TPP//HA and CS–TPP//Alg nanoparticles.

Preparative method	Nanoparticles	Michaelis–Menten fitting at 2 h		Michaelis–Menten fitting at 8 h	
		V_{max} (μg/mg h)	K_m (μg/mL)	V_{max} (μg/mg h)	K_m (μg/mL)
A	CS(684)–TPP//HA	114 ± 21	57 ± 30	65 ± 4	25 ± 5
	CS(684)–TPP//Alg	110 ± 15	72 ± 25	51.5 ± 5	33 ± 11
	CS(325)–TPP//HA	84 ± 20	204 ± 84	35 ± 4	76 ± 20
	CS(325)–TPP//Alg	70 ± 14	136 ± 53	25 ± 3.4	84 ± 27
	CS(25)–TPP//HA	94 ± 17	49 ± 13	27 ± 0.5	4.5 ± 0.6
	CS(25)–TPP//Alg	42 ± 3	5 ± 2	21 ± 1	5.6 ± 2
B	CS(325)–TPP//HA	13 ± 1	5 ± 2.7	8.6 ± 0.2	4.7 ± 1
	CS(325)–TPP//Alg	9.7 ± 1	13 ± 4.2	6.4 ± 0.2	8.8 ± 2

Fig. 7. Effect of endocytosis inhibitors on the 2 h uptake of HA- and alginate-coated CS-TPP nanoparticles in RAW 264.7 macrophages as a function of chitosan molecular weight (A and B) and method of preparation (C and D). The uptake was measured as the fluorescence of cell lysates (using a calibration of nanoparticles in cell lysates); it was then normalised against the amount of total cellular protein content and expressed in relation to control experiments performed without inhibitors. $n = 3$.

while only marginally affected (–5%) the internalisation of alginate-coated ones. This result finally proved that the internalisation of the HA-coated particles is a CD44-mediated process.

From the above analysis it appears that HA-coated nanoparticles were internalised in RAW 264.7 macrophages through a

CD44-mediated process leading to acidified late endosomes. The precise nature of the internalisation mechanism is not completely clarified and shows differences from that in J774 macrophages, which was at least partially clathrin-dependent [22].

Fig. 8. Intracellular localisation of CS(325)-TPP/HA and CS(325)-TPP/Alg nanoparticles (method A) in RAW 264.7 macrophages after a 4 h incubation. Blue: nuclei (DAPI); Green: late endosomes and lysosomes (LysoTracker Green); Red: chitosan (rhodamine). Please note that trypan blue was used to quench the fluorescence of surface-bound nanoparticles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

In this study, we believe to have demonstrated that, firstly, the internalisation of HA-coated nanoparticles is a CD44-mediated process; their similar kinetic profile suggests that the uptake of alginate-coated nanoparticles is also a process mediated by a saturable (scavenging) receptor. Secondly, an identical ligand, e.g. HA with the same molecular weight, can be presented in a different arrangement on the nanoparticle surface, due to morphological differences arising, for example, from the method of preparation, or from the use of chitosan with different molecular weights. These differences do not appear to affect the nature of the uptake, which for HA-coated nanoparticles appear to be still based on binding to CD44. On the other hand, the different ligand presentation seems to have major effects on the mode of interactions affecting affinity and speed of uptake and also cytotoxicity. We believe this to be a consequence of the limited number and of the slow representation of the internalisation receptors; under these conditions, a lower number of receptors would cluster around nanoparticles with poorly presented HA resulting in lower affinity but higher overall number of bound particles.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2013.03.065>.

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