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Understanding biophysical behaviours of microfluidic-synthesized nanoparticles at nano-biointerface

Shirin Soleimani[a], Mohammad Mahdi Hasani-Sadrabadi[b,c,*], Fatemeh Sadat Majedi[d], Erfan Dashtimoghadam[e], Mahdi Tondar[f], Karl I. Jacob[b,c,g,*]

[a] Department of Biomedical Engineering, University of Calgary, Calgary, Alberta, Canada
[b] Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, USA
[c] G.W. Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, USA
[d] Department of Bioengineering, University of California at Los Angeles, Los Angeles, CA, USA
[e] Department of Developmental Sciences, Marquette University School of Dentistry, Milwaukee, WI, USA
[f] Department of Biochemistry and Molecular & Cellular Biology, Georgetown University, Washington DC, USA
[g] School of Materials Science and Engineering, Georgia Institute of Technology, Atlanta, GA, USA

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ABSTRACT

Encapsulating drugs in nanoparticles (NPs) provide some advantages over free drugs; for example the probability of distribution in off-target tissues decreases and drugs remain safe from environment degrading factors. Upon entering the bioenvironment, NPs establish a number of interactions with their surroundings based on their physicochemical properties. Here we demonstrate how the size-surface charge interplay of chitosan NPs affects the protein corona formation and endocytosis pathway in the HeLa cells at non-toxic concentrations. Generally, large NPs (102 and 161 nm) with low surface charge (+6.7 and +3.6 mV) exhibited weaker tendency for endocytosis compared with smaller ones (63 and 83 nm with 10 and 9.3 mV surface charge, respectively). This is mainly because the interactions of larger NPs with the plasma membrane were too weak to release enough free energy required for cellular internalization. Furthermore, we tested the upright and inverted cell culture configurations to better understand the impact of the sedimentation and diffusion velocities of NPs on the resulting cellular uptake pattern in both serum free and serum containing culture medias. Considering the different particokinetics, the amount of internalized NPs in upright and inverted positions differed in all cases by a factor of approximately three (for 161 nm particles), or less for smaller ones. Ultimately, our results offer a paradigm for analyzing the biobehavior of NPs under the precise control of their physicochemical characteristics.

1. Introduction

The effectiveness of nanomedicine in developing therapeutic systems, with nanoscale components (e.g. NPs) that can reach the pathologic sites, depends on our ability to tailor such systems with a unique set of properties, including composition, structure, size, radius of curvature, shape and charge [1]. Yet, very little is known about how these parameters influence the final pharmacodynamics and pharmacokinetics of NPs [2]. When the biological target for treatment is localized in the subcellular organelles, the plasma membrane plays a significant role because it acts as a bulwark that is only selectively permeable to small, uncharged molecules. To be effective, NPs must cross the plasma membrane to reach the action site of the drug, which requires careful design of NPs with specific properties that enables them to enter cells and reach their intracellular targets [3].

Despite the striking advances in nanoscience, relatively little is known about the interactions that occur during the cellular uptake. One of the most studied uptake mechanisms is endocytosis, an energy-dependent uptake process in which the cell membrane protrusions spread over NPs and forms vesicles known as endosomes that carry the engulfed NPs into the cellular interior [4]. Different endocytic pathways in non-phagocytic cells (e.g. caveolae/clathrin mediated, caveolae/clathrin independent and pinocytosis) have distinct components and mechanisms [5]. Additionally, it has been reported that some NPs may slip directly through the plasma membrane of eukaryotic cells, which is similar to the processes

* Corresponding authors at: Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, USA
E-mail addresses: mahdi.hasani@gatech.edu, mahdi.hasani@gmail.com (M.M. Hasani-Sadrabadi), karl.jacob@mse.gatech.edu (K.I. Jacob).

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exhibited by bacteria crossing the plasma membrane [6]. Clearly, cell type is an important parameter determining the internalization mechanism, but, the physicochemical properties of NPs are also key players of cellular uptake [2,7]. Particle size is an important parameter considering the space available in these endocytic compartments [8], but the interplay of different physicochemical characteristics plays a more decisive role.

Accumulation of NPs in pathologic sites can be adjusted both actively and passively [9]. The passive homing mechanism of NPs in cancer is based on the enhanced permeability and retention effect (EPR) that allows an NP to penetrate into a tumor tissue due to the vascular hyperpermeability (380–780 nm in diameter pores) as well as the chance of taking long residence time within the tumor due to the decreased, sluggish lymphatic drainage from the interstitial space [10]. To take advantage of this phenomenon, the size of NPs needs to be optimized. Large NPs cannot pass through vascular fenestrations, or even if they could, they would not get far beyond the vessels to distribute throughout the whole tumor environment [11]. In contrast, small NPs have the ability to penetrate deep into tumors, but these particles can only remain there transiently [12,13]. To establish a balance between an efficient distribution of NPs and a sufficiently long residence time, an optimum size is required for NPs [9].

In addition to size, surface charge is another significant factor responsible for charge–charge interactions, which results in the formation of NP aggregations. Sedimentation of NPs above cells, a factor that is often ignored, makes the final results less reliable and causes deviation from the in vivo conditions [14].

NPs with high surface energy will strongly interact with biomolecules present in the bioenvironment and become surrounded by them; a masquerade called protein corona. Thus, what approaches the cells is a corona coated, not a bare, particle. [15–17]. Hence, the coverage of NPs with a natural physiological coating layer can affect the cellular internalization patterns and NPs' final effects on cells' viability [18,19].

In order to explore the effect of particle size on the NPs' biobehavior, NPs with different sizes were selected for this study. To efficiently investigate the effect of size, the fundamental objective is to employ a fabrication procedure with high control over the NPs' size and monodispersity since even minute size changes may alter many non-specific interactions that occur at the cell–particle interface. To precisely fabricate the NPs, we employed a microfluidic technique taking advantage of a solvent free cross-junction device that can generate a narrow mixing regime via a hydrodynamically focused flow to form chemically and physically tuned monodisperse chitosan NPs [20–23]. This system allows to have a better understanding of the effects that key particle variables (size, chemical composition and surface charge) have on the cell entering mechanisms.

2. Experimental—materials and methods

2.1. Particle preparation

The chitosan nanoparticles were fabricated according to a previously published protocol [20]. Briefly, a solution of acetic acid (50 mL, 1% w/v; Sigma–Aldrich) containing one-gram chitosan (CS; M_n = 280 kg/mol, degree of deacetylation 83%, Fluka) was stirred for 12 h. The resulting solution was filtered with a 0.45-μm Nylon syringe filter. The solution pH was maintained at 5.5 by the drop-wise addition of sodium hydroxide (Sigma–Aldrich). 300 mg palmitic acid *N*-hydroxysuccinimide ester (Sigma–Aldrich) in absolute ethanol (Sigma–Aldrich) solution was added drop-wise to the chitosan solution at 98 °C under reflux. The ethanol was allowed to dissolve and distribute homogeneously throughout the solution by stirring for 48 h. The solution temperature was then decreased to reach to the room temperature. Then, adding acetone under the pH of 9.0 precipitated chitosan in the solution. The resultant polymer precipitate was filtered twice, washed with an excess of acetone, and vacuum-dried at room temperature. The product was analyzed by 1 H NMR (Bruker 400 MHz) and the palmitoyl groups placement on chitosan chains were determined using the ninhydrin assay [24]. The high molecular weight chitosan supplement (HMCS) was dissolved in aqueous acetic acid. Then, 0.5 mL acetic acid/acetate buffer (4 M, pH = 5.5) was added into 0.5 mL of the prepared solution. The test tubes were filled with 1 mL of ninhydrin reagent (Sigma–Aldrich) and placed in a boiling water bath for 20 min. The solutions were then cooled and their absorbance was read at 570 nm and compared with the unmodified chitosan solution, the control sample, and the acetic acid/acetate buffer, the blank sample [25].

The microfluidic device for the fabrication of nanoparticles consists of two inlets for water at a basic pH (pH 9.0) and one main inlet for the entrance of HMCS solution at an acidic pH (pH 4.5). The resulting outlet nanoparticle stream was collected in disposable cuvettes (Eppendorf) and used for further analysis. A critical parameter in determining nanoparticle size is the mixing time, τ_{mix}, which can be estimated from the equation:

$$\tau_{mix} = \frac{W^2}{9D} \frac{1}{(1 + \frac{1}{R})^2} \quad (1)$$

where W is the main channel width (150 μm), D is the diffusion constant (10^{-9} m^2/s) and R is the ratio of the polymeric stream and basic water flow rates (R = 0.03–0.2).

For comparison with the microfluidic fabrication, bulk synthesized samples were also prepared as follows: the polymeric solutions were prepared by a constant stirred- dissolving of 2.5 mg/ml HMCS in 1% w/v acetic acid, and the nanoprecipitated HMCS molecules resulted at a pH of 7.4 by the drop-wise addition of 1 M NaOH.

The fluorescently-labeled HMCS was fabricated based on the reaction between the fluorescein isothiocyanate (FITC) and the chitosan [26]. The prepared FITC in methanol (2 mg/ml) was added gradually to the solution of 1% w/v acetic acid containing the HMCS. After 5 h of reaction in dark at room temperature, the FITC-labeled chitosan was precipitated in 0.2 M NaOH and separated from the unreacted FITC via a Sephadex G-50 column with 1/15 M phosphate buffer/0.2 M NaCl as an elution solvent. The fractions containing the labeled polymers were collected and dialyzed against deionized water using a 3 kDa molecular weight cut-off dialysis cartridge (Thermo Scientific, Rockford, IL). The process was continued for almost 4 days until no fluorescence was detected in the supernatant. The resulting sample was ultimately freeze-dried.

2.2. Particle size and surface charge analysis

Dynamic light scattering (DLS) and zeta potential measurements were performed using a Zetasizer (Zetasizer 3000HS, Malvern Instruments Ltd., Worcestershire, UK) in the backscattering mode at 173 °C for the particles dispersed both in water and FBS at a concentration of 0.3 mg/ml. Three measurements were performed for each sample.

2.3. Turbidimetry

The measurement of transmittance of the nanoparticle suspensions was performed via the spectrophotometry method (Shimadzu UVmini-1240 UV–vis spectrophotometer) at 25 °C.