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Augmented cellular trafficking and endosomal escape of porous silicon nanoparticles via zwitterionic bilayer polymer surface engineering

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ABSTRACT

The development of a stable vehicle with low toxicity, high cellular internalization, efficient endosomal escape, and optimal drug release profile is a key bottleneck in nanomedicine. To overcome all these problems, we have developed a successful layer-by-layer method to covalently conjugate polyethyleneimine (PEI) and poly(methyl vinyl ether-co-maleic acid) (PMVE-MA) copolymer on the surface of undecylenic acid functionalized thermally hydrocarbonized porous silicon nanoparticles (UnTHCPSi NPs), forming a bilayer zwitterionic nanocomposite containing free positive charge groups of hyperbranched PEI disguised by the PMVE-MA polymer. The surface smoothness, charge and hydrophilicity of the developed NPs considerably improved the colloidal and plasma stabilities via enhanced suspensibility and charge repulsion. Furthermore, despite the surface negative charge of the bilayer polymer-conjugated NPs, the cellular trafficking and endosomal escape were significantly increased in both MDA-MB-231 and MCF-7 breast cancer cells. Remarkably, we also showed that the conjugation of surface free amine groups of the highly toxic UnTHCPSi-PEI (Un-P) NPs to the carboxylic groups of PMVE-MA renders acceptable safety features to the system and preserves the endosomal escape properties via proton sponge mechanism of the free available amine groups located inside the hyper-branched PEI layer. Moreover, the double layer protection not only controlled the aggregation of the NPs and reduced the toxicity, but also sustained the drug release of an anticancer drug, methotrexate, with further improved cytotoxicity profile of the drug-loaded particles. These results provide a proof-of-concept evidence that such zwitterionic polymer-based PSi nanocomposites can be extensively used as a promising candidate for cytosolic drug delivery.

1. Introduction

The success in cancer nano-therapy is extremely dependent on the development of carriers that are able to efficiently deliver therapeutic agents to the cytosolic compartment of target cells with minimal toxicity [1]. Among many different types of nanoparticles (NPs) applied for this aim, honeycomb-like porous silicon (PSi) NPs show remarkable advantages, including high surface area, stable nanostructure, tunable pore diameter, two functional surfaces (external particle surface and internal pore surface), modifiable shape and size, effective protection of the therapeutic cargos from undesirable degradation, as well as superior safety at concentrations adequate for pharmacological applications [2–5].

Currently, despite the above mentioned advantages, there are still concerns regarding the potential of PSi NPs at the cellular level due to the low cellular interaction and entry into the cells [6,7]. In addition, the internalized PSi NPs usually become entrapped inside endosomes and ultimately end up in the lysosome, a biological compartment in charge for enzymatic degradation and inactivation of different compounds [8]. Therefore, these nanostructures will possess a high potential for cancer therapy, if rendering the ability to breach cellular membrane and reach the cytoplasm or nucleus of the cell, a difficult task to achieve due to the complexity of the biological barriers [9,10]. Since many types of developed NPs with favorable physicochemical properties *in vitro* cannot be successfully

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applied *in vivo* owing to the limited intracellular functionality, numerous attempts have been made to find new approaches to attenuate this drawback by enhancing their cellular uptake and endosomal escape [11,12]. For example, cell penetrating peptides have been widely used for cellular uptake enhancement despite several disadvantages, such as low metabolic stability, possible immunogenicity, and dependency of their membrane translocation ability on the amino acids arrangement and site of conjugation with the NPs [13–15]. To overcome these problems, new alternative strategies are essential to achieve favorable therapeutic effect for the nanomedicines. Surface polymeric functionalization is one of the desirable methods that can be applied not only to affect the NP's properties by manipulation of the intrinsic size, shape, charge, smoothness, hydrophilicity/hydrophobicity, homogeneity, and stability, but also to act as a driving force for improving cellular internalization, endosomal escape, and drug release profile with the final aim of achieving a subtle therapeutic effect [16–18].

Although many reports in the literature have demonstrated the ability of some specific types of positively charged polymers, such as polyethyleneimine (PEI), for successful improvement of cellular internalization and endosomal escape [19,20], these materials have shown drawbacks in terms of making overt pores in the lipid bilayers, which can eventually lead to cellular toxicity by disturbing the concentration balance of ions and proteins that are essential to maintain the normal function of the cells [21,22], Thus, the application of specific polymer-conjugated nanostructures that are capable to avoid cellular toxicity while increasing cellular trafficking and release their payloads in the cytoplasm is essential for designing superior nanomedicines. Despite rapidly increasing progress in the polymer and copolymer based drug delivery systems with different compositions, shapes, and properties [23–25], there is still lack of deep understanding about the fate of surface polymeric functionalized NPs at the cellular level. Thus, clarification of the benefits associated to the surface functionalization of NPs with different polymers may bring new advantages to this burgeoning area of research.

Despite current attempts to reduce cellular toxicity and improve cellular interaction using functionalization of the PSi NPs with bioadhesive negatively charged polymers [26], combining these properties with endosomal escape behavior is crucial for effective therapeutic effect. Accordingly, this study reports the preparation, characterization and *in vitro* fate of a new class of bilayer polymer-conjugated PSi NPs developed using the surface conjugation of PEI and poly(methyl vinyl ether-co-maleic acid) (PMVE-MA). The covalent attachment of the copolymers to the PSi's surface was made via the 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride/N-hydroxysuccinimide (EDC/NHS) mediated covalent binding. The developed bilayer nanocomposite, namely UnTHCPSi-PEI-PMVE-MA NPs (Un-P-P), was studied in terms of cytotoxicity effect on cancer cells, stability in the aqueous buffer and in human plasma, cellular internalization, drug release, and antiproliferation effect on cancer cells.

2. Material and methods

2.1. Fabrication of the UnTHCPSi NPs

The preparation and characterization of PSi NPs have been previously described in detail elsewhere (Scheme 1) [2,7,27]. Details can be found in Supporting Information.

2.2. Preparation of UnTHCPSi-PEI (Un-P) NPs

The carboxyl groups of UnTHCPSi NPs were covalently conjugated to the amine groups of branched PEI (average Mw ~ 25,000), as shown in Scheme 1. To successfully accomplish the covalent conjugation, 1.5 mg of UnTHCPSi NPs was dispersed in 4 mL of 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) saline buffer at pH 5.2. Following dispersion, 8 μL and 6 mg of EDC and NHS, respectively, were added and mixed for 2 h to activate the carboxyl groups of the PSi NPs. Finally, the surface activated PSi NPs were exposed to an excess of hyper-branched PEI with a ratio of 1:10 (NPs:polymer) and vigorously stirred (800 rpm) at room temperature (RT) overnight. Since the amount of PEI used to covalently cross-link to the NPs was very high, we assume that all carboxyl groups of the PSi NPs were reacted with the polymer, leaving no free carboxyl group for further conjugation. To remove the excess of unconjugated polymer, the surface modified UnTHCPSi NPs were extensively rinsed with MiliQ-water, and re-suspended in Hank's balanced salt solution (HBSS)–(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer (pH 7.4).

2.3. Formation of UnTHCPSi-PEI-PMVE-MA (Un-P-P) nanocomposites

The preparation of Un-P-P nanocomposites was achieved by the addition of PMVE-MA copolymer onto the PEI-functionalized UnTHCPSi NPs, as shown in Scheme 1. Briefly, PMVE-MA copolymer was first obtained from poly(methyl vinyl ether-co-maleic anhydrate) (PMVE-MAh, average Mw ~ 216,000) by dissolving the later one in HBSS–HEPES buffer (pH 5.2) at 70 °C for 3 h. Next, PMVE-MA polymer was activated for 2 h by addition of EDC/NHS to the solution, and subsequently, added with a ratio of 1:1 to Un-P NPs dispersed in the same buffer. The obtained polymer-conjugated PSi NPs were washed twice with MilliQ-water by repeated centrifugation at 15,000 rpm for 5 min and redispersed by pipetting in order to ensure that no ungrafted polymer or free reagents were present in the final obtained product. The NPs were then redispersed in HBSS–HEPES buffer (pH 7.4).

2.4. Characterization of the NPs

The hydrodynamic diameter (Z-average), PDI and surface zeta-potential of the NPs were measured using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK). Typically, polymer-conjugated and unconjugated PSi NPs were centrifuged and redispersed in MilliQ-water with a final concentration of 30 μg/mL prior to the measurements. All measurements were repeated at least three times.

The chemical composition and interaction of the UnTHPSi NPs and the polymers were studied by ATR–FTIR. The ATR–FTIR spectra of all samples were obtained using a Bruker VERTEX 70 series FTIR spectrometer (Bruker Optics, Germany) with a horizontal ATR sampling accessory (MIRacle, Pike Technology, Inc.). The ATR–FTIR spectra were recorded in the wavenumber region of 4000–650 cm^{-1} with a resolution of 4 cm^{-1} at RT using OPUS 5.5 software. Prior to each measurement, all the NPs were left to dry at RT for 48 h.

The morphology of the NPs was studied using TEM (Jeol JEM-1400, Jeol Ltd., Japan). Samples were prepared in ethanol and dropped on a carbon coated copper TEM grid before air-drying at RT overnight. The colloidal stability of the NPs was also screened by leaving the samples at RT for 4 h after preparation.

2.5. Stability in human plasma and aqueous solution

To evaluate the impact of the PSi NPs' stability, 300 μg of the bare and polymer-conjugated PSi NPs were dispersed in 200 μL of PBS (pH 7.4). The NPs were then mixed with 1500 μL of human plasma and kept at 37 °C for 2 h under stirring at 800 rpm. Sampling was performed at different pre-determined time intervals (15, 30, 60, 90 and 120 min) to measure the particle size and PDI using Zetasizer Nano ZS instrument. Anonymous donor human plasma was obtained from the Finnish Red Cross Blood Service. For colloidal stability investigation in aqueous solution, all types of the NPs were dispersed in PBS (pH 7.4) and the particle size and PDI were measured in the above mentioned time points.

2.6. Fluorescent labeling of the NPs

Fluorescein isothiocyanate (FITC) was conjugated to the NPs for imaging purposes. FITC was added to a mixture of HEPES (0.1 M; pH 7.5) and ethanol (1:4, v/v). Then, FITC-conjugated NPs were obtained by addition of the NPs to the FITC solution in a 10:1 ratio.

2.7. Cell lines and culture conditions

For the *in vitro* studies, MDA-MB-231 and MCF-7 breast cancer cells were cultured according to the protocols described in detail in the Supporting Information.

2.8. Cell viability studies

To assess the biocompatibility of the NPs, their toxicity towards MCF-7 and MDA-MB-231 cells was evaluated by measuring the ATP activity as described elsewhere [27,28], and are explained in more detailed in Supporting Information. Therapeutic efficiency of the developed nanocarriers was investigated by the cell proliferation evaluation of the cells after a 6 h exposure to free methotrexate (MTX) and drug-loaded NPs using the same protocol applied for cell viability determination.

2.9. Cellular uptake imaging

TEM imaging was used to evaluate the cellular uptake potential of the NPs after exposure to both MCF-7 and MDA-MB-231 breast cancer cells. In this experiment, 13 mm round shape coverslips were placed at the bottom of 24-well plates (Corning Inc. Life Sciences, USA). Next, 10^5 cells were seeded in Dulbecco's modified Eagle's and Roswell Park Memorial Institute 1640 media for MCF-7 and MDA-MB-231 cells,

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