RESEARCH ARTICLE

Effects of Surface Chemistry Modification using Zwitterionic Coatings on the Surface of Silica Nanoparticles on Prevention of Protein Corona: A Test Study

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Objective(s): The purpose of this study was investigation of the protein corona formation on the surface of zwitterionic nanoparticles when they exposed to bio-fluid like human plasma.

Methods: Silica nanoparticles with zwitterionic surface coating, cysteine and sulfobetaine were employed as zwitterionic ligands, were synthesized and characterized in terms of physicochemical properties. To probe protein corona formation, synthesized nanoparticles were incubated at 37 °C for 1h in human plasma solutions (10 and 55% (v/v)).

Results: Our results show no significant changes in size and zeta potential of nanoparticles after treatment with human plasma and elimination of loosely attached proteins. The size of zwitterionic nanoparticles after incubation with human plasma remained around 100 nm and their zeta potential was near zero. The results from gel-electrophoresis and MALDI-TOF mass spectrometry of nanoparticles after incubation with plasma proved that zwitterionic nanoparticles are non-interacting with proteins.

Conclusions: Our observations confirm the hypothesis that zwitterionic surface modified nanoparticles could provide the potential to regulate the interaction of the nanomaterials with biological systems and successfully overcome the protein corona issue in the field of nanomedicine.

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ABSTRACT

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INTRODUCTION

A big challenge in the field of nanomedicine and using of engineered nanoparticles (NPs) is protein adsorption on the surface of NPs upon exposure to protein rich media like blood, leading the formation of a protein layer at the surface of NPs so called “protein corona” and changing the biological fate of NPs [1-4].

Especially during the last few years, many publications have described efficient and convenient approaches to modify the surface of NPs with different targeting ligands like antibodies, peptides, and aptamers providing smart nanocarriers to recognize specific receptors on target cell surfaces on target cell surfaces as nanocarriers applicable in drug delivery systems [5-7].

Non-specific adsorption of proteins on the surface of NPs when they inject to biological milieu in vivo, is the major reason for discrepancy between the in vitro and in vivo results; in case of protein corona formation on the surface of NPs, any ligands engineered onto NPs surface could mask and cause mistargeting or unintended scavenging by the liver, kidney, or spleen [8-12].

Many approaches have been explored and reported to hamper the effect of an accumulated protein corona; one approach is the design of
surface coating with antifouling properties. One of the most famous coating to avoid non-specific adsorption, is noncharged poly(ethylene glycols) (PEG). Although, adsorption of some certain plasma proteins (e.g., fibrinogen, IgG, and apolipoprotein E) have been observed even with PEG-coated surfaces particularly during biological conditions [13-16].

Very recently, surface chemistry modification with zwitterionic coating has been introduced by Rotello et al. to provide corona free NPs exhibiting high biocompatibility, low toxicity, and long circulation time. In our previous report, to reduce protein corona issue in active targeting of engineered NPs in the presence of biological medium, we have investigated zwitterionic coated NPs which carry a specific targeting ligand [17-22].

Considering these reasons, the use of zwitterionic coated NPs is necessary to overcome the protein corona issue for nanomedical applications. Herein, to extend our previous studies we studied protein adsorption behavior of two zwitterionic modified silica NPs with dissimilar structure, cysteine and sulfobetaine were employed as zwitterionic ligand, in the presence of different percentage of human plasma.

We compared physicochemical properties of zwitterionic silica-NPs and also bare silica-NPs (unmodified NPs) like hydrodynamic size and zeta potential before and after incubation with human plasma. In addition, the protein adsorption on the surface of NPs were investigated by gel electrophoresis and matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MALDI-TOF MS).

MATERIALS AND METHODS

Materials

All the chemicals were purchased from Sigma-Aldrich unless otherwise stated. The chemicals were used as received.

Synthesis of bare silica NPs (Bare-SiNPs)

Bare silica NPs were prepared according to Stöber method (23). Briefly, to produce silica NPs with a diameter of 100 nm, tetraethylorthosilicate (TEOS) (62.5 µL) was added to mixtures consist of methanol (1 mL), DI water (0.36 mL) and concentrated ammonia (0.1 mL). The blended solution stirring for 2 h.

Synthesis of S-(Alkylcarbamoyl)-L-cysteine (Ligand 1)

L-Cysteine hydrochloride monohydrate was dried at 70 °C for 12 h. The dried material (2 mmol) was dissolved in dimethylformamide (4 mL) and treated with 3 (triethoxysilyl)propyl isocyanate (TEPI) (2.1 mmol). The reaction of the stirred solution was kept at room temperature for 3 days and the solid product was washed with DMF (Scheme 1), see our previous study for more details [22].

Synthesis of 3-(Dimethyl(3-(trimethoxysilyl) propyl)-ammonio) propane-1-sulfonate (ligand 2)

The synthesis of ligand 2 was according to Schlenoff et al. [24]. To 1.7 mg (14 mmol) of propane sultone in 15 mL dried tetrahydrofuran (THF) under N2 was added 14 mmol of (N,N dimethyl-3-aminopropyl) trimethoxysilane dropwise. The reaction was stirred vigorously for 2 h at 50 °C. The white precipitate product was used without additional purification and stored under N2. The reaction yield was 86 % (Scheme 2).

![Scheme 1. Synthesis protocol for the preparation of S-(Alkyl carbamoyl)-L-Cysteine (ligand 1)](image1)

![Scheme 2. Synthesis procedure for the preparation of 3-(Dimethyl(3-(trimethoxysilyl) propyl)-ammonio) propane-1-sulfonate (ligand 2)](image2)
Synthesis of Cysteine-Conjugated Silica NPs (Cys-NPs) and Sulfobetaine-Conjugated Silica NPs (SB-NPs)

To modify the surface of Bare-SiNPs and produce Cys-NPs and SB-NPs, ligand 1 (0.009 mmol) and ligand 2 (0.01 mmol) were added to two separate Bare-SiNPs batches respectively, and the reactions were stirred for 12 h (all steps were illustrated in Fig. 1). Then after, all types of SiNP were collected by centrifugation, washed with DI water (3×1 mL), and redispersed in DI water. The NPs placed in a 3.5K molecular weight cutoff dialysis membrane and dialyzed against 50% methanol in water for 48 h to remove excess ammonia. Finally, the sample stored in a dark place at room temperature for further analysis.

Characterization of synthesized Si-NPs and colloidal stability test in PBS

All types of prepared SiNPs were characterized in terms of size using transmission electron microscopy (TEM) and dynamic light scattering (DLS) and zeta potential (Malvern Zetasizer Nano-ZS90). To evaluate colloidal stability of the synthesized SiNP, NPs size were measured in PBS buffer by DLS.

Interaction of synthesized NPs with Human Plasma

To prepare samples HP10 and HP55, an aqueous solution of all NPs (100 μL, 1 mg/mL in DI water) were mixed with 900 μL of human plasma (HP) solution (10% HP in PBS and 55% HP in PBS, respectively); the prepared solutions were incubated at 37 °C for 1 h, and after elimination of both excess and loosely attached proteins by centrifugation and two times washing steps with PBS, their size and zeta potential were measured.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Resulting samples from previous steps were analyzed by SDS-PAGE to determine the proteins adsorbed as the corona composition of the different types of NPs. The samples after treatment with human plasma were washed twice with PBS and resuspended in protein buffer and boiled for 5 min at 100 °C. Then, the same volume of each sample was loaded into 12% polyacrylamide gel before running at 120 V and 80 mA for approximately 100 min. The gels were stained using a standard silver nitrate protocol.

MALDI-TOF mass spectrometry analysis

In order to analyses of protein profiles adsorbed on the surface of SiNPs, resulting NPs after treatment with human plasma and washing steps were mixed with an equal volume of matrix solution

![Fig. 1. The synthesis pathway for surface modification of Bare-SiNPs with ligand 1 and 2 to fabricate Cys-SiNPs and SB-SiNPs was represented.](image-url)
of sinapinic acid/α-Cyano-4-hydroxycinnamic acid in 50% ACN containing 0.1% TFA, then spotted on a MALDI-TOF plate and air dried. Protein profiles were analyzed with a MALDI-TOF mass spectrometer (Applied Biosystems 4800 MALDI TOF/TOF, Nd:YAG 200-HZ laser). The standard protocol was used to investigate and optimize all steps. Separate spectra were obtained for two restricted mass-to-charge (m/z) ranges, corresponding to proteins with molecular mass of 1-10 kDa (“<4kD”) and 10-180 kDa (“>4kD”), in linear mode (positive ion) under specifically optimized instrument settings. Each spectrum was the result of 1000 laser shots, per m/z segment per sample, obtained in three sets of 100 shots (at 20-Hz frequency) to each of three different locations on the surface of the matrix spot.

RESULTS AND DISCUSSION

Synthesis and characterization of Bare-SiNPs, Cys-SiNPs, and SB-SiNPs

Engineered Silica NPs having stealth properties were fabricated and their behavior were compared with Bare-SiNPs. Cysteine, a native compatible amino acid and sulfobetaine group were used as a small zwitterionic coating. Three types of 100nm silica NPs (SiNPs) including unmodified SiNPs (Bare-SiNPs), cysteine coated (Cys-NPs), and sulfobetaine coated (SB-SiNPs) were synthesized. Because of the silanol groups on the surface of the Bare-SiNPs, these nanoparticles could be properly functionalized with a desired silane coupling agent; proposed NPs were prepared by surface modification of Bare-SiNPs directly with resulted silane coupling agent through chemical conjugation of ligand 1 and 2 (see Fig. 1, Scheme 1 and 2). The synthesized SiNPs were also characterized by dynamic light scattering (DLS), zeta potential (ZP) and transmission electron microscopy (TEM) to observe the hydrodynamic size, the surface zeta potential and the size distribution (Fig. 2 and Table 1). The results proved the formation of the nanoparticles with uniform size distribution. The entire synthesized NPs polydispersity index (PDI) values, which was measured by DLS, were below 0.1. Zeta potential measurements indicated that the surfaces of the uncoated SiNPs (Bare-SiNPs) and zwitterionic coated SiNPs were negatively charged with significantly different values; surface modification of Bare-SiNPs considerably decreased negative charge on the surface of silica NPs which covered full of silanol groups confirming successful functionalization.

Colloidal stability of the synthesized SiNPs in PBS buffer were assessed by DLS (Table 1). The results showed different stability between Si-NPs
with various surface chemistries; in this regard, Cys-SiNP and SB-SiNPs presented the highest stability (no aggregates) whereas, in contrast, Bare-SiNPs formed considerable aggregations in PBS. According to our observations, we could strongly suggest that modification of NPs surface chemistry with zwitterionic compounds could significantly increase the colloidal stability of NPs, which is highly important for biological application of engineered NPs.

**Interactions of the synthesized SiNPs with human plasma**

All three types of synthesized SiNPs (i.e., Bare-SiNPs, Cys-SiNP and SB-SiNP) were incubated at 37 °C for 1h in human plasma (HP) solutions (10, and 55% (v/v)) mimicking the protein concentration in both in vitro and in vivo conditions. The size and zeta potential of samples for three types of SiNPs were measured (Table 1). To illustrate whether zwitterionic NPs (Cys-SiNPs and SB-SiNPs) are non-interacting NPs with protein rich media, gel electrophoresis was performed; for gel electrophoresis, we used samples of all NPs after incubation with human plasma and washing steps. As expected, huge differences in the protein adsorption patterns for NPs with different surface chemistry were detected (see Fig. 3). The observed protein bands confirmed formation of protein corona on the surface of Bare-SiNPs; in contrast, NPs with zwitterionic ligands (i.e., Cys-SiNP and SB-SiNPs) demonstrated no/negligible protein bands confirming their interesting capability to inhibit protein corona formation. It is noteworthy to mention that the observed light protein band in the case of Cys-SiNPs probably be related to

<table>
<thead>
<tr>
<th>Types of SiNPs</th>
<th>Size [Dh] (nm)</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare-SiNPs</td>
<td>170 ± 220</td>
<td>-65.1 ± 0.8</td>
</tr>
<tr>
<td>PBS, after 12 hours</td>
<td>100 ± 1.1</td>
<td>-65.1 ± 0.8</td>
</tr>
<tr>
<td>HP 10%</td>
<td>130 ± 6.3</td>
<td>-90.4 ± 1.8</td>
</tr>
<tr>
<td>HP 55%</td>
<td>165 ± 6.7</td>
<td>-99.1 ± 1.9</td>
</tr>
<tr>
<td>DI</td>
<td>101 ± 1.7</td>
<td>-18.2 ± 0.6</td>
</tr>
<tr>
<td>Cys-SiNPs</td>
<td>110 ± 5.2</td>
<td>-20.7 ± 1.6</td>
</tr>
<tr>
<td>PBS, after 12 hours</td>
<td>112 ± 1.1</td>
<td>-20.7 ± 1.6</td>
</tr>
<tr>
<td>HP 10%</td>
<td>114 ± 1.5</td>
<td>-21.2 ± 1.8</td>
</tr>
<tr>
<td>HP 55%</td>
<td>100 ± 1.3</td>
<td>-7.2 ± 0.7</td>
</tr>
<tr>
<td>DI</td>
<td>103 ± 1.1</td>
<td>-7.5 ± 1.1</td>
</tr>
<tr>
<td>SB-SiNPs</td>
<td>103 ± 1.8</td>
<td>-8.8 ± 1.2</td>
</tr>
</tbody>
</table>

![Table 1. Characterization of three types of SiNPs in different media](image)

**Fig. 3.** The SDS-PAGE gel related to washed samples (two times with PBS), Bare-SiNPs (10-55%), Cys-SiNPs (10-55%) and SB-SiNPs (10-55%)
trapping of proteins in centrifugation which cause an increasing their size around 10-13 nm at two levels of human plasma (10, and 55 %) while these changes are around 3 nm indicating different behavior of zwitterionic NPs in shielding protein corona.

**Evaluation of synthesized NPs’ proteins adsorption using MALDI-TOF mass spectrometry**

The presence and molecular mass of polypeptides and proteins adsorbed on the surface of NPs can be directly determined by MALDI-TOF MS at the sensitivities and resolution that would make it a great technique for serum/plasma protein profiling (25, 26). To assess whether zwitterionic NPs are non-interacting with proteins, resulting NPs after incubation of all types of synthesized SiNPs with human plasma and removal of the free unbound and loosely attached proteins were analyzed by MALDI-TOF mass spectrometry. To evaluate both small and large range of molecular mass, we tasted two matrix α-Cyano-4-hydroxycinnamic acid and sinapinic acid respectively. Interestingly, the spectrum of protein corona related to Bare-SiNPs are totally different to ones related to zwitterionic SiNPs. As it can be seen in Fig. 4, several proteins adsorbed on the surface of Bare-SiNPs which is inconsistent with observed protein bands in SDS-PAGE. In contrast, for zwitterionic SiNPs, no peaks were observed (a noisy spectrum around baseline with the intensity of 3.5 were detected).

**CONCLUSIONS**

In conclusion, the role of surface chemistry modification of silica nanoparticles with zwitterionic coatings on the capability of NPs to prevent protein corona formation upon exposure to biological environment have been studied. Our observations revealed that different zwitterionic NPs have different potential to repel protein adsorption. We expect that such studies are likely to open new avenues for probing the fundamental nature of the nano-bio interface through direct interfacing of synthetic engineered NPs and biological environment without issues dependent to protein corona formation.

**ACKNOWLEDGMENTS**

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES