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Surface Presentation of Functional Peptides in Solution Determines Cell Internalization Efficiency of TAT Conjugated Nanoparticles

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Abstract

Functionalizing nanoparticles with cell-penetrating peptides is a popular choice for cellular delivery. We investigated the effects of TAT peptide concentration and arrangement in solution on functionalized nanoparticles' efficacy for membrane permeation. We found that cell internalization correlates with the positive charge distribution achieved prior to nanoparticle encountering interactions with membrane. We identified a combination of solution based properties required to maximize the internalization efficacy of TAT-functionalized nanoparticles.

Introduction

A biologically inspired set of peptides collectively known as cell-penetrating peptides (CPPs) has become a popular tool for cellular delivery. This popularity stems from the ease of peptide synthesis and conjugation onto a range of substrates, such as nanoparticles (NPs) and liposomes, and from their ability to mediate cytosolic and nuclear delivery(1) of a range of cargo moieties including proteins(2, 3) and DNA.(4) Despite such extensive application, the mechanism of action of CPPs is still a subject of debate. One of the most thoroughly studied CPPs is the HIV-derived trans-activator of transcription peptide, TAT.(5, 6) Contrary to initial beliefs, TAT-mediated cell entry is not a purely passive process. Recent studies have conclusively demonstrated that at least some of the uptake is governed by a type of endocytosis.(3) What remains unknown is whether there is a simultaneous energy-independent mode of transport, i.e., direct diffusion through the cell membrane.

Synthetic lipid bilayers have allowed the study of TAT behavior more directly by excluding active uptake processes, however, with contradictory results. Some studies show that TAT can cross artificial bilayers,(7) while others show that it cannot.(8) Further complexity arises when studying the interaction of TAT-functionalized nanomaterials with cell membranes. CPPs mediate the cellular uptake of nanoparticles(9–11) and vesicles(12) to all cellular compartments in vitro,(1) but detailed examinations of how the nanomaterial surface affects peptide efficacy have been limited to date. Functional nanomaterials are attracting strong interest as potential drug delivery vehicles(4, 13, 14) and diagnostic tools.(11, 15) Gold

nanoparticles (AuNPs) are attractive for biomedicine owing to their physical and chemical qualities, enabling their versatile functionalization, easy detection, and elevated biocompatibility.(4, 13, 14, 16–23) Currently, there is a plethora of data on how NPs are internalized by cells based on their size, shape, chemical composition, charge, and surface functionality.(24–31) Well-known cell lines incubated with standard AuNPs can display a variety of outcomes, mostly due to the complex response biological systems enact in response to external and internal stimuli.(18, 26–29) Other factors such as the density of functional groups on the Au surface, the charge of the lipid membrane, the release of bilayer tension, temperature, and ionic strength can influence cell penetration.(32)

To further investigate the effects of the functional peptide density on the interaction of cells with nanoparticles, it is useful to have a nanoscale material system with flexible, quantitatively controllable surface chemistry. Self-assembled peptide monolayers (SAMs) on AuNPs are a simple approach to obtain such nanomaterials. Determining the effects of peptide concentrations on the general properties and functionality of peptide-grafted NPs through experiment remains a challenge though. While experiments provide cell-level evidence on the effects of NPs on membranes, molecular insight into the structure of functionalized NPs prior to their interaction with the membrane is still lacking. Computer simulations can assist in gaining such understanding,(33, 34) although simulations involving nanoparticles, specifically AuNPs, and biological matter are still in their infancy due to the general complexity of the organic–inorganic systems and their relatively large size for current computational capacity.(35–38) Considering the lack of interaction parameters for multifaceted AuNPs, various approximations for modeling AuNP interfaced with organic aqueous systems have been used in the literature.(32, 39–41) Some simplified models for direct permeation of charged nanoparticles through membranes have been developed;(24, 25, 32, 36) however, these models lack the complete atomic details and assume various charge distributions on the NP surfaces. It remains unclear though how such different NP surface charge distributions can be attained in solution, prior to the NP encounter with the cell membrane, as a function of the grafted peptide surface density, conformation, and mobility determined by the functionalized NP interactions with the local medium.

We propose that systematic all-atom simulations of NP in solution for varying peptide grafting densities can shed light on the molecular mechanisms responsible for the resultant charge distribution on the NP surface and the structural properties of the functional peptide layer, which can then be correlated with the NP internalization capacity determined through experiments. Knowledge of such correlations will have a significant impact for rational design of nanoparticles, and the simulations depict a powerful numerical approach enabling experimentalists to not only understand but also design the nanoscale interaction of therapeutic and diagnostic agents. If used as a screening method, this approach will help accumulate the fundamental data on the NP properties and provide a widely sought after link between the atomistic structure of functional NP in solution and their membrane permeating capacity, which can inform NP engineering strategies for targeted applications. With this in mind, we have performed an experimental and computational study on the effects of TAT peptide concentration on the structure and dynamics of the peptide layer on the 3 nm AuNP surface in relation to the NP's membrane permeation capacity.

All-atom molecular dynamics simulations were performed to evaluate the effect of TAT concentration on the structure and presentation of the functional peptide layer on a 3 nm NP in solution. A range of concentrations (up to 9%) of TAT were modeled, with the peptides incorporated within a matrix of the pentapeptide ligand, CALNN, which assists in the solubility and stability of conjugated AuNPs.⁽⁴²⁾ Further details on the computational methodology and setup are included in the Supporting Information. Our simulations showed in most systems the conjugated TAT peptides aligned along the CALNN surface in a random coil conformation due to the attractive electrostatic forces between the positive charges of Arg and Lys and the exposed C-termini of CALNN. The hydrophobic Pro residues near the exposed N-terminus of CALNN–TAT were observed to embed their side-chains into the hydrophobic core (Ala-Leu) of CALNN, which imposed additional mobility restrictions on the CALNN–TAT peptides along the CALNN surface. This “locking” behavior contributed to the CALNN–TAT backbone becoming more rigid while allowing the positively charged residues to be exposed to the solvent due to the surface alignment of the TAT section. The importance of high solvent exposure of the basic residues and structural rigidity of CPPs was highlighted in a recent experimental study,⁽⁴³⁾ which showed that an increase in kinetic uptake by the cell can be attributed to the rigid presentation of Arg side-chains and larger guanidinium group separation in cyclized peptides. Therefore, the observed surface presentation of CALNN–TAT in our systems is also indicative that the NP could have membrane-penetrating propensity.

The general size of the conjugated peptide layer on the NP surface was estimated by calculating the peptides’ radius of gyration and effective diameter (D_e) of the modified NPs (Figure 1). The extent of surface elongation by CALNN–TAT peptides was found to be dependent on the density of CALNN in the proximity of CALNN–TAT and temperature. The simulations showed a reduction in the peptide layer thickness at all grafting densities of CALNN–TAT, suggesting that the NP became more compact over time, lowering the effective diameter. Fluctuations in the layer thickness indicated that the TAT peptide was readily interacting with both the aqueous environment and the CALNN surface due to its hydrophilic character and high flexibility. Generally, the higher temperatures (296 and 308 K) led to a thinner peptide layer than the lower temperatures (285 and 288 K) for all concentrations. The thinnest and most stable peptide layer was observed for the 1.8% and 3.6% systems, due to the ability of CALNN–TAT peptides to freely wrap around the CALNN coated surface at the low TAT concentration. Notably, at 5.4% grafting density some CALNN–TAT peptides remained dynamic for the entire simulation contributing to the larger effective diameter of the NP at this concentration. The simulations showed that higher temperatures led to an increase in the peptide dynamics for intermediate TAT concentrations which contributed to a faster formation of a dense peptide layer and a lower effective NP diameter (Figure 1b). This indicates that specific temperature and concentration of the functional peptide can be employed to enable more efficient NP interactions with the membrane.

It is also important to understand the interatomic interactions that determine the structural and dynamic features of the functional peptide layer in solution in order to be able to control such interactions to achieve the desirable features for membrane intake. Specific interactions between the functional peptides and between the peptides and solvent were evaluated by

calculating the radial distribution functions (RDF) over the equilibrium stage of simulation. Figure 2 shows the results for the 1.8% TAT system and two temperatures (296 and 308 K), which represent the trends exhibited by all studied NP systems. High crowding of CALNN peptides in the proximity of TAT correlated with the hydrogen bonding between the peptides manifested by peaks at ~ 0.2 (H–O distance) and ~ 0.3 nm (O–O distance) (Figure 2a). The peak at ~ 0.5 nm confirmed electrostatic interactions between the positively charged Arg and Lys of TAT and the C-terminus of CALNN (Figure 2b). These contacts were contributing to the elongation of TAT along the CALNN-coated NP surface at low densities. The RDF between CALNN–TAT and water showed three distinct peaks: the first two at ~ 0.2 and ~ 0.3 nm indicated hydrogen bonding between water and TAT, leading to formation of the first hydration shell, and the second at ~ 0.45 nm, suggesting a second hydration shell (Figure 2c). The data suggested that increased TAT interactions with CALNN (peptide crowding) resulted in diminished interactions of TAT with water. It should be noted that explicit ionic species in the simulated solution in addition to the counterions incorporated to neutralize the system can affect the magnitude of electrostatic interactions. To evaluate the effect of ionic strength of the medium we performed simulations in salt buffer for select systems (Table S1). We found the trends in systems simulated in the purely aqueous (plus counterions) and salt buffer media to be qualitatively indicative of the same structural and dynamic features of the peptides.

Several studies have raised the relevance of basic residue solvent exposure in CPPs to membrane permeability (see ref 43 and references therein). However, low solvent exposure of the bare NP surface is needed to render the particles stable in solution and have a low propensity to aggregate. We calculated the solvent accessible surface area (SASA) of individual components for the systems at equilibrium (Figure 3) using a probe radius of 1.4 Å (water) and Lennard-Jones hard-shell radii for each atom to define the surface. Figure 3a shows an increase in the solvent exposure of bare NP as TAT concentration was raised from 1.8% to 9.0%. This result confirmed that in regions with higher density of TAT, the CALNN–TAT interactions lead to a locally increased density of CALNN, causing additional exposure of the bare NP surface (Figure 3e). Increased solvent exposure of the bare NP surface may lead to NPs instability and induce undesirable flocculation. In addition, Figure 3b demonstrates significant reduction in solvent accessible area of CALNN at low concentration of TAT (1.8% to 5.4%). However, CALNN was slightly more solvent exposed at higher concentrations of TAT, which suggested that the tilting of CALNN induced by clustering around TAT at higher concentration contributed to an increase of CALNN solvent exposure. At lower TAT concentrations CALNN peptides remained more extended and perpendicular to the NP surface, which led to a lesser solvent exposure of the lateral CALNN surfaces. Studies have shown that the presence of thiols and cohesive lateral interactions between the CALNN peptides through hydrophobic interactions and/or hydrogen bonding enhanced the NP stability.⁽⁴²⁾ In line with these results, our observations suggested that NPs with lower functional concentrations (less than 7.1% coverage of TAT) should be more stable in solution. Despite the loss of electrostatic repulsion between the particles with high concentration of TAT, due to the neutralizing of the overall NP charge, the increase of bare NP surface exposure and clustering of the CALNN layer exhibited at high concentration of TAT provide plausible explanations as to why the 9% CALNN–TAT

NPs were less stable than the CALNN-only coated NPs in our aggregation assays (Figure S6). To determine TAT's contribution to the NP's solubility additional simulations of CALNN-only coated NP (Au-CALNN) were conducted and the SASA of the bare NP surface and CALNN peptides was calculated. The results showed that SASA of CALNN for the Au-CALNN-only is higher than that in all TAT-functionalized systems. For example, for 1.8% TAT, 11% of the CALNN SASA is taken up by TAT; for 3.6% TAT it is 19%; for 5.6% TAT it is 21%; for 7.1% TAT it is 11%; and for 9.0% TAT it is 22%. Interestingly, the lower concentrations of TAT result in a decrease (up to 9%) in bare NP exposure (1.8–5.4% TAT), while at the higher (7.1–9%) TAT coverage there is a significant (~32%) increase in bare NP surface exposure. This suggests that the lower TAT concentrations may have a beneficial effect on the solubility of the NP complex.

High exposure of charged residues in CPPs is believed to be necessary for cell permeation. (39, 40) To get an insight into this property, the solvent accessible surface of Arg and Lys in TAT was calculated. Figure 3c shows the average solvent accessible area of the charged residues per TAT peptide at each concentration. At 5.4% CALNN-TAT concentration, the positively charged residues were most exposed. The elongation of TAT along the CALNN-coated NP surface resulted in lowering of the total SASA for the other TAT concentrations; however, the basic side-chains remained solvent exposed (Figure 3f). This suggested that the grafted CALNN-TAT peptides were flexible enough to be able to adopt multiple arrangements potentially favorable for their interaction with a membrane. The experimental evidence from cell internalization studies supported the indication that ca. 5.4% CALNN-TAT functionalization granted higher cell permeability, described later (Figure 4). Moreover, a recent study(43) identified that transduction efficiency of Arg-rich peptides increases with higher structural rigidity and that the rates of cell permeation are directly dependent on the Arg concentration and structure of the peptide. This is confirmed by the correlation of our simulation results with the membrane internalization experiments.

Figure 3d shows that with increasing CALNN-TAT concentration there was a higher increase in the solvent exposure by the hydrophilic residues, compared to that of the hydrophobic residues. This further highlighted that with an increase in CALNN-TAT concentration the peptide-coated NP became more hydrophilic overall. The reversal of the ratio of exposed hydrophilic/hydrophobic residues occurred under 3.6% CALNN-TAT coverage. The increase in the NP hydrophilic character had a “patchy” pattern, as seen in Figure 3e, due to the clustering of CALNN and increased exposure of the bare NP surface. The solvent exposure of the individual components in the presence of salt buffer was also evaluated (Table S1). The results showed a ~5–10% reduction in SASA by all constituents, compared to those obtained in aqueous solution, as expected due to the enhanced screening leading to decreased electrostatic interactions.

We also investigated the effects of functional peptide distribution on the NP properties. Seven different arrangements of CALNN-TAT on the NP surface were modeled at 5.4% CALNN-TAT concentration, suggested to possess properties favorable for membrane interactions. We note that the chosen collocations of the peptides are representative of possible local peptide arrangements independent of the overall peptide concentration. These simulations therefore provide an insight into the effects of patterning of functional peptides

and potential regioselectivity at different peptide concentrations.(44) Results from the simulations are presented in Figure S3. Similarly to the simulations of the equidistant peptides, the CALNN–TAT peptides adhered to the CALNN surface with their N-termini interacting strongly with the exposed C-termini of CALNN for different TAT peptide collocations. The attractive forces between the oppositely charged residues contributed to the enhanced stability of the surface elongated TAT peptides. The simulations indicated that the TAT peptides were more mobile in regions of low CALNN density due to the diminished attractive interaction between the bare (hydrophobic) NP surface and the hydrophilic TAT. The general properties, including the solvent exposure of CALNN, basic TAT residues, and hydrophobic–hydrophilic residues were in qualitative agreement to those obtained for the peptides equidistant on the NP surface. However, there was an increase in the NP bare surface exposure where TAT peptides were positioned close together, which can contribute to instability and aggregation of the NPs. This simulated behavior was a result of the like-charge repulsion between the Arg and Lys in TAT suggesting that in the real experimental systems the TAT peptides were more likely to adopt a relatively even distribution on the NP surface during functionalization to avoid such repulsive contacts. This finding can shed light on why the NPs with higher than 5.4% TAT were less efficient in permeating the cell membrane (see Figure 4 for 3 nm NPs and Figure S7, for 5 nm NPs). Indeed, at the high grafting density, NPs present low curvature, essentially “flat”, areas of surface relative to the steric dimensions of the neighboring functional peptides. High local grafting density on such flat surfaces will unavoidably cause repulsive same-charge interactions between the ungrafted ends, and the functional peptide will attempt to avoid such interactions by retreating toward the CALNN surface. However, the higher effective TAT density can hinder its ability to extend along the CALNN surface due to the steric restrictions and will cause TAT to collapse downward while hiding (rather than exposing) the basic residues, as we indeed observed for our densely localized peptide models. In contrast, at lower densities the high curvature of small NPs enables the N-termini of the TAT peptides to be sufficiently apart to avoid repulsive interactions between the like-charges, while the fan-like distribution of the peptides provides sufficient space for the interaction of TAT and CALNN due to the opposite charge attraction. These interactions bring the TAT onto the CALNN surface in an elongated conformation with subsequent immobilization of the TAT peptides along the CALNN surface, while the positively charged residues still remain solvent exposed. Because of these effects, systems with the equally spaced TAT at 5.4% concentration attain the rigidity of the TAT backbone, which, together with the increased basic residue solvent exposure, deliver a combination of properties required for membrane activity observed experimentally.(43) In line with the above rationale, the NP systems with higher functional peptide concentrations were less effective for cell permeation as seen in our experiments with 3 nm NPs (Figure 4). This also offers a possible explanation to the plateauing effectiveness for cell internalization by the 5 nm AuNPs with CALNN–TAT coverage higher than 7.1% (Figure S7).

To evaluate Au–TAT NP internalization experimentally, we prepared AuNPs with self-assembled peptide monolayers of varying CALNN–TAT concentration. To confirm successful functionalization, we subjected the resulting NPs to chemical characterization using Fourier transform infrared (FTIR) spectroscopy (Figure S4). FTIR analysis displayed

clear spectral signature differences between bare and peptide-functionalized AuNPs. For CALNN–TAT, increased spectral differences were clearly visible in the 1050–1250 cm^{-1} region, attributed to additional C–O vibrations (1214 and 1146 cm^{-1}) within the Thr amino acids of CALNN–TAT. The 1600–1750 cm^{-1} spectral region also showed increased spectral differences at 1640 cm^{-1} for CALNN compared to CALNN–TAT, indicating greater N–H and C=O vibrations, due to the greater proportion of these functional bonds within the CALNN peptide. Additionally the 1300–1600 cm^{-1} spectral region showed very similar spectral features indicating negligible influence of AuNPs on spectra for both CALNN and CALNN–TAT. The FTIR analysis supported the hypothesis that CALNN and CALNN–TAT were successfully functionalized to the AuNP.

Analysis of the UV–vis spectra of bare and functionalized AuNPs supported the FTIR findings, displaying a visible, but limited red shift for functionalized AuNPs (Figure S5). The limited absorption cross section of 3 nm AuNPs did not allow to quantify the red shift due to the absence of a stationary point at ~ 520 nm; nonetheless, CALNN-functionalized AuNPs displayed an apparent red shift in the region around 520 nm, while the CALNN–TAT-functionalized AuNPs displayed a larger shift, independent of CALNN–TAT concentration up to 9.0%.

Further investigation of UV–vis absorption as a function of salt concentration allowed to elucidate the stability of functionalized particles in simulated biological conditions. Bare AuNPs aggregated extensively at salt concentrations higher than 50 mM. In contrast, CALNN-functionalized NPs were stable up to 1.5 M NaCl, in keeping with previous findings.⁽⁴²⁾ When the coating solution consisted of 90% CALNN and 10% CALNN–TAT, the NPs were more stable than bare gold but less stable than with full CALNN coverage. These results were supported by the theoretical simulations, which showed that higher density of TAT promoted the aggregation of CALNN, leading to an increased exposure of the bare NP surface, which is unfavorable for NP stability, along with the reduction in electrostatic repulsions due the neutralizing charge of the outer layer of the NPs. Aggregation occurred at about 0.5 M NaCl. These results confirmed that both species were incorporated into the peptide monolayer. When the loading solution contained more than 20% CALNN–TAT, they were unstable at any salt concentration, so all further experiments were performed with less than 10% CALNN–TAT.

We functionalized 3 nm AuNPs with different concentrations of CALNN–TAT and incubated them with cells to test the predictions of the simulations regarding cell internalization. In keeping with the numerical simulations, the CALNN–TAT concentrations examined were 0, 1.8%, 3.6%, 5.4%, 7.1%, and 9%. In all instances (apart from 0%) the molarity of CALNN–TAT was at least 10-fold that of the AuNPs, to guarantee excess CALNN–TAT. The molar ratio of AuNP to total peptide was maintained constant to ensure similar reaction kinetics. We note that the concentration reported refers to the nominal concentration in solution, while the number of CALNN–TAT molecules grafted to each AuNP followed a Poisson distribution. The heterogeneity of the particles in the sample contributed to confound a direct comparison of the simulation with the experiments. Nonetheless, a large portion of particles in each sample would carry a CALNN–TAT

concentration close to the nominal one, and thus, the overall behavior of the conjugated AuNPs can be linked and/or compared to the properties obtained by theoretical simulations.

The functionalized particles were resuspended in cell culture media and fed to HeLa cells in 70% confluent culture. Following 6 h of incubation, the cells were analyzed to evaluate the internalization of AuNPs as a function of CALNN–TAT concentration (Figure 4).

Stereological analysis of the transmission electron micrographs (TEM) showed the absence of 0% CALNN–TAT AuNPs from the cell cytosol, as expected in the absence of CPPs. The concentration of particles localizing in the cytosol progressively increased with increasing concentration of CALNN–TAT, up to 5.4%, at which point the cytosolic concentration of NPs began to decrease (Figure 4a–e). At 5.4% CALNN–TAT, approximately 18 NP μm^{-2} localized in the cell cytosol, more than twice the concentration observed at 9% CALNN–TAT, supporting the simulation predicting higher effectiveness of 5.4% CALNN–TAT at cell penetration (Figure 4e).

Inductively coupled plasma–atomic emission spectroscopy (ICP–AES) on cell lysates digested in Aqua Regia provided the total amount of gold internalized by cells, supporting the findings observed by electron microscopy (Figure 4f). As a bulk characterization technique, ICP–AES complemented well the limited statistical power of TEM analysis. ICP–AES, though, cannot discriminate Au–TAT NPs for their localization and included both cell-penetrating and phagocytized particles. Nonetheless, the Au concentration for 0% CALNN–TAT, where only phagocytosis occurred, was below the ICP–AES detection threshold for Au of 50 ppb, while the maximum concentration (at 5.4% CALNN–TAT) was at 246 ppb. Thus, in the most favorable conditions the concentration of cell-penetrating AuNPs was at least 4-fold the concentration of phagocytized particle (assuming phagocytosis remained constant with changing CALNN–TAT concentration). Under this assumption, ICP–AES outlined the same trend as TEM analysis with maximum cell penetration for 5.4% CALNN–TAT, which was more than double what was observed at 9% CALNN–TAT. It should be noted that the amount of cell penetrating particles is simply the time integral of the rate of cell penetration by particles. Thus, the observed difference in the amount of penetrating particles as a function of TAT concentration indicates that the concentration of TAT peptide on the surface of AuNPs alters cell penetration kinetics. The direct experimental inference of cell penetration kinetics in this study though is partly confounded by two intervening and opposing factors: (i) the observed negotiation of intracellular barriers by TAT functionalized AuNPs, which progressively traffics them to the endolysosomal system and eventually leads to their exocytosis,⁽¹⁾ and (ii) potential time fluctuation in uptake rate as a function of particle size and functionalization. The choice of a 6 h time point attempts to observe a condition as close as possible to a steady state, by keeping (i) within reasonable limits while averaging (ii) over the longest possible time, allowing for equilibration. In summary, the combined microscopic analysis by TEM and bulk analysis by ICP–AES indicated that for 3 nm AuNPs the concentration of CALNN–TAT functionalization significantly affected their ability to penetrate cells. CALNN–TAT was necessary for 3 nm Au NPs to penetrate the cell cytosol, and the optimal concentration lay at ~5.4% as predicted by the numerical simulations.

Discussion

Using computational all-atom molecular dynamics and experiments, we examined the effects of peptide concentration and surface arrangement on the properties of functionalized NPs in solution and their relevance to the efficacy for NP membrane permeation. Experimental quantification of cell penetration relative to CALNN–TAT concentration in the 0–9% range showed maximal penetration occurred for 5.4% CALNN–TAT, supporting the prediction by the numerical simulations.

Our results showed that NPs exhibit unique properties in solution dependent on the functional peptide concentration and structure. The favorable electrostatic interactions between the basic residues of TAT and the negative charge of the terminal carboxylic acid of CALNN assisted the elongation of TAT along the CALNN surface at ~5.4% concentration of TAT. This interaction provided an interfacial support for the TAT peptides, which became more rigid, while their charged side-chains remained exposed to the solvent. Intuitively, we might consider high functional peptide concentrations to be required for cell permeation due to the increased density of charge. However, the simulations demonstrated that the high TAT concentration can alter the solution properties of the functionalized NP, which lead to a reduced stability and lowered ability of NPs to permeate the cell membrane by (1) rendering the NP unstable due to the overall charge neutralization and increased exposure of the bare NP surface, in accordance with our aggregation assay experiments; (2) causing a reduced mobility of the functional peptides, which may prevent them from adopting a favorable orientation for interaction with a membrane; and (3) causing the basic residues to be insufficiently exposed and thus being unavailable for interaction with a membrane.

Our simulations suggested a mechanism responsible for reducing TAT activity at higher concentrations, showing that when TAT peptides were densely collocated the like-charge repulsion caused them to adopt conformations in solution unfavorable for membrane interactions. This finding also suggests that TAT would most likely favor a relatively even distribution during the NP functionalization. At lower functional peptide concentrations the high curvature of small NPs enables the ungrafted TAT termini to be sufficiently apart to avoid repulsive interactions and extend on the negatively charged CALNN surface while exposing the basic residues to the environment. In contrast, denser collocation of functional peptides will cause the peptide conformational collapse downward due to electrostatic repulsion, thus compromising the basic residue exposure needed for membrane crossing. The results highlight the need to maintain a specific balance between functional peptides and surface coverage to produce stable and active NPs. The unique combination of solution-based properties obtained for the 3 nm AuNPs conjugated with 5.4% TAT was termed effective for cell penetration, as confirmed by subsequent experiments.

A direct evaluation of the predictions of the models in terms of the hydrodynamic radius of the particles could have provided further insight into the system but was precluded by the extremely small size of the AuNPs.(45, 46) Dynamic light scattering measurements require both a sufficiently high proportion of scattered light to the total transmitted light and a high ratio of scattered to absorbed light. When the size of colloidal gold decreases, both the extinction coefficient and the contribution of scattering to extinction drop rapidly.(47) These

combined effects made it practically impossible to reliably estimate the size of AuNPs by light scattering techniques below 10 nm. Other established sizing strategies for nanoparticles, such as transmission electron microscopy or differential centrifugal sedimentation, do not measure hydrodynamic radius and are not suitable to validate the model. Since the predictions of the model depend strongly on nanoparticle size, it would be of limited interest to assess the effects on larger, measurable NPs.

Biological systems, such as biological fluids, tissues, organs, and animals, are significantly more complex than the computational and experimental models depicted here. Multiple cell types organize and coordinate to execute functions, dynamically responding to the environment. In such systems, nanoparticles will come in contact with many complex and spatiotemporally variable fluids, capable of altering nanoparticles' composition, behavior, and ultimately their interaction with cells and efficacy. Nonetheless highlighting the correlations between simulation and experimental behavior in this simplified system not only provides guidelines for nanoparticles design but also proves that improvements in the quality of modeling translate into deeper understanding of physical properties in biological systems.

Our combined computational and experimental study provided evidence that altering concentration and distribution of the TAT peptide on the NP surface bestows distinct properties to the particle-peptide nanostructure in solution that influence its membrane permeating propensity. This data strongly supports the need to control the structure and dynamics of functional peptides in solution, prior to cell internalization, in order to achieve the desired activity of peptide-functionalized NPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effective diameter of peptide-decorated nanoparticles; (a) radius of gyration at each temperature for 5.4% CALNN–TAT coverage; (b) schematic showing the effective diameter (D_e) of the nanoparticle at 5.4% and 9.0% TAT grafting densities; the volume occupied by the CALNN peptides is shown as a semitransparent cloud, while the heavy atom stick models of the CALNN–TAT peptides are shown in varying colors. The conformations sampled over the last 5 ns of simulations are shown as superimposed. Data for all other systems are shown in Figure S1.

Figure 2.

Peptide–peptide and peptide–water interactions. Radial distribution functions calculated at 1.8% coverage of TAT at 296 K (blue) and 308 K (gray) representing (a) TAT (NHO) atoms–CALNN (O) atoms; (b) CALNN C-terminus–Arg and Lys atoms (NHO); (c) TAT (NHO)–water (O). Peaks at ~ 0.2 nm indicate H–O bonding, ~ 0.3 nm indicate O–O bonding, and ~ 0.5 nm indicate electrostatic interactions, respectively.

Figure 3.

Functionalized nanoparticle surface exposure and solubility. Average solvent accessible surface area (nm²) with standard deviations (error bars) of individual components at each TAT concentration from the 296 K simulation: (a) bare nanoparticle surface, (b) CALNN peptides, (c) TAT basic residues (Arg and Lys), (d) hydrophobic and hydrophilic residues (due to the scale of the Y-axis, error bars not visible for the small errors of ca. 1%); conceptual illustrations highlighting (e) the bare nanoparticle (yellow) surface exposure and residue arrangement at 5.4% and 9.0% concentration of TAT and (f) basic residue side-chain

exposure to the solvent, with CALNN residues represented as points (blue transparent surface) for clarity. The nonpolar, polar, and basic residues are colored gray, light gray, and blue, respectively.

Figure 4.

Internalization of AuNPs by HeLa cells as a function of CALNN-TAT concentration. (a-d) Transmission electron micrographs of 3 nm diameter AuNPs within the cytosol of HeLa cells for increasing concentration of CALNN-TAT: (a) 1.8%; (b) 3.6%; (c) 5.4%; and (d) 9.0% CALNN-TAT. (e) Electron micrograph-based quantification of AuNPs per unit area within HeLa cells as a function of CALNN-TAT concentration. (f) ICP-AES quantification of Au content within HeLa cells as a function of CALNN-TAT concentration.