Biophysical Considerations in the Rational Design and Cellular Targeting of Flexible Polymeric Nanoparticles

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How nanoparticle (NP) mechanical properties impact multivalent ligand–receptor-mediated binding to cell surfaces, the avidity, propensity for internalization, and effects due to crowding remains unknown or unquantified. Through computational analyses, the effects of NP composition from soft, deformable NPs to rigid spheres, effect of tethers, the crowding of NPs at the membrane surface, and the cell membrane properties such as cytoskeletal interactions are addressed. Analyses of binding mechanisms of three distinct NPs that differ in type and rigidity (core-corona flexible NP, rigid NP, and rigid-tethered NP) but are otherwise similar in size and ligand surface density are reported; moreover, for the case of flexible NP, NP stiffness is tuned by varying the internal crosslinking density. Biophysical modeling of NP binding to membranes together with thermodynamic analysis powered by free energy calculations is employed, and it is shown that efficient cellular targeting and uptake of NP functionalized with targeting ligand molecules can be shaped by factors including NP flexibility and crowding, receptor–ligand binding avidity, state of the membrane cytoskeleton, and curvature inducing proteins. Rational design principles that confer tension, membrane excess area, and cytoskeletal sensing properties to the NP which can be exploited for cell-specific targeting of NP are uncovered.

1. Introduction

An understanding of the interaction of nanoparticles (NPs) with a biological cell membrane is crucial for the establishment of advanced therapeutics such as cancer immunotherapy and gene therapy[1–3] and their application as biomarkers for imaging and detection.[4–6] Functional nanoparticles are finding applications in genome editing, immune modulation, cell therapies, and molecular diagnostics to stratify patients based on biomarkers. However, the functional NPs’ design, optimization, and deployment are hampered by our lack of understanding of their biological barriers and pharmacokinetics.[7] While such barriers have contributions that are systemic and span multiple scales, the specificity for a given application has essential contributions from the cellular scale. Cellular targeting is governed by avidity (adhesion) and uptake (internalization), which depend on the properties of the NPs and the cellular microenvironment.

Adhesion between an NP and a cell surface typically involves the simultaneous binding[8,9] of many hundreds of ligands on the NP’s surface to a similar number of receptors on the cell surface.[9–13] The physiological outcome of such multivalent adhesion, which can mediate diverse phenomena, including cell crowding and cell uptake,[14] depends on the strength of ligand–receptor binding interactions. Other factors that can modulate multivalent adhesion strength include physicochemical properties of both the NPs and the cell surfaces.[15] Previous
investigations have shown that the NP’s shape and size are critical design parameters for the interaction of the particles with the membranes. However, in addition to controlling the external particle shape, it is also essential to consider how the internal structure affects the avidity, in addition to the receptor–ligand binding strength. With this perspective, we consider a new class of crosslinked polymeric NPs called nanogel particles (NGPs), making them deformable and elastic. We mainly focus on a biocompatible NGP comprising a lysozyme-rich core with dextran brushes capable of hosting guest molecules, including small hydrophobic drugs and contrast imaging agents. Furthermore, when these NPs are bound to the cell surface, they undergo deformation. Studies in culture and in vivo have provided significant evidence for the relationship between the mechanical properties of NGPs and the enhanced targeting of NGPs to topologies that are entirely inaccessible to their counterpart rigid particles. The extent of the deformation of NGPs may then be controlled either by tuning the polymer crosslinking density (i.e., elasticity) or impacted by the cell surface mechanical properties. The crosslinked polymer networks can offer control over size, rigidity, drug encapsulation, and storage stability in biomedical applications, but their design and optimization remain largely empirical.

NP–cell adhesion is also expected to be influenced by the state of the cytoskeleton or the extracellular matrix, which (in the current study) is represented as the pinning of a deformable membrane with cytoskeletal proteins that bind the cell membrane to the substrate or extracellular matrix. An earlier study demonstrates that the pinning induces confinement in the membrane undulations and, as a result, leads to novel and nontrivial membrane topographies, which could impact the binding and the uptake of the NP to the cell membrane.

The uptake of functionalized NPs into the cell membrane is accompanied by membrane shape evolution, including the formation of buds, invaginations, or protrusions, that are later pinched off from the membrane vesicles through protein-mediated scission. NPs enclosed in such vesicles can be transported to different locations inside the cell, commonly referred to as intracellular transport. Biophysical and thermodynamic principles governing NP adhesion and uptake in response to both NP and membrane mechanical properties are addressed in this study.

Although it is clear that the stiffness of NPs functionalized with targeting antibodies has significant effects on their interaction with cells, relatively little is known about the role of NP flexibility on drug delivery. This effect governed by mechanics could be crucial to the design of NPs for targeting to diseased tissues. In this context, few studies have investigated the role that NP stiffness plays in NP interactions with diseased endothelial cells and tissue targeting behavior. While the NGP construct has been physically characterized and realized as a good vehicle for drug delivery in vivo in mice models, its optimization for targeting specific tissues is far from clinical translation. These data underscore the need to clarify the unique mechanisms through which deformable NPs may impart benefits and opportunities for engineering the biodistribution and tissue targeting.

Quantitative characterization of mechanisms modulating NGP adhesion to the host cell membrane requires a model that accurately estimates the adhesion affinity from the underlying receptor–ligand binding parameters. In this context, we have recently developed a statistical mechanics based computational framework for multivalent adhesion of functionalized rigid, rigid-tethered, and flexible NPs to a membrane surface that takes into account mechanical properties of both the NP and the cell surfaces as well as the biophysical factors of membrane anchorages. We have also proposed new thermodynamic methods to compute entropy–enthalpy compensation for the related problem of binding affinity of NPs to the cell surface.

We bring these novel computational techniques to the current study and address central questions regarding the roles of NP composition from very soft, deformable NPs to rigid spheres, effect of tethers, the crowding of NPs at the membrane surface, and the cell membrane properties such as membrane tension, and cytoskeletal interactions. We aim to shed light on the potential of NPs’ structure and mechanics to control the binding and ultimately the uptake of the NPs, therefore opening up avenues for rationally engineering and fine-tuning of new systems in targeted drug delivery.

2. Results

We consider three different types of NPs in this study: flexible, rigid, and rigid-tethered NPs (Figure 1a). The flexible NPs represent lysozyme-core/dextran-shell polymer NPs and are modeled as star polymers. The stiffness of the flexible NP is engineered by physically crosslinking the polymer chains to create an NP microstructure. Experimentally, we vary the stiffness of the flexible NP from 0.43 to 15.02 kPa; the corresponding NP models were developed in our earlier work, and the structures, given in Figure 1b, are referred to as FL1 to FL5. The rigid NP corresponds to a ligand-coated spherical particle, and in rigid-tethered NP, the ligands are attached to the rigid NP via polymer tethers with variable molecular weight. RG and RT in Figure 1b correspond to the rigid and rigid-tethered NPs, respectively; see methods for details of the NP models.

The equilibrium conformations of a cell membrane are described by the parameters that govern the physicochemical properties in our model: namely, the bending rigidity \( k \), excess area \( A_{ex} \), and the presence of membrane cytoskeleton-linker-proteins, \( N_{p} \). The membrane bending rigidity is taken to be \( 20k_{B}T \). The dimensionless quantity \( A_{ex} \) defined as \( A_{ex} = \frac{A-A_{p}}{A} \), is the available excess area in the surface and is a representative of tension experienced by the cell membrane. Here, \( A \) is the total curvilinear area, and \( A_{p} \) is the projected area of the equilibrated membrane patch. We obtain the membrane configurations for different \( A_{ex} \) by keeping \( A_{p} \) fixed and varying \( A \). The range of \( A_{ex} \) explored here is 6%–34%, which was noted for most endothelial cells. The equilibrium conformations of the membranes are also determined by the state of cytoskeletal pinning and adhesive interactions between the membrane and extracellular matrix. The presence of these pinning or adhesion sites, their mobility, and binding–unbinding dynamics limit the out-of-plane fluctuations of the membrane and induces protrusions or invaginations in the membrane. To understand the role of such membrane adhesions, we introduce pinning sites.
at randomly selected discrete points on the membrane and vary the density of pinning ($N_{\text{pin}}$).

### 3. Effect of NP Stiffness and Membrane Tension on NP Avidity to the Cell Surface

We begin our discussion by the avidity distribution of the multivalent NPs. The equilibrium conformations and fluctuations of both NPs and cell membranes are quantified by recording i) the probability distribution of multivalent binding interactions between the ligands on the NP and the receptors expressed on the cell surface and ii) the adhesion free energy computed by including both the enthalpy gain, that enhances binding, and the entropy loss, that inhibits binding of NPs.

#### 3.1. Roles of Membrane Excess Area and NP Stiffness on the Multivalency of Binding

To evaluate the binding multivalency of ligand-functionalized flexible/rigid/rigid-tethered NPs, we computed the probability distribution of the number of simultaneous binding interactions between the ligand–receptor pairs (Figure 2a,b; Movies S1–S5, Supporting Information). Here, we demonstrate that flexible NPs exhibit maximal binding multivalency to the membrane in an excess area independent manner. In the case of rigid NPs, the number of bound ligand–receptor pairs are very low, and the multivalency does not show a significant increase with $A_{\text{ex}}$. Compared to rigid and flexible NPs, rigid-tethered NPs exhibit a broad multivalency distribution $P(m)$ that is bimodal. In this case, $A_{\text{ex}}$ plays a crucial role in binding, showing higher multivalency at higher $A_{\text{ex}}$. Our results show that the formation of receptor–ligand bonds in the case of rigid-tethered NPs induces noticeable membrane invaginations (insets of Figure 2a,b) at the site of binding and nearly internalizes the NP at high $A_{\text{ex}}$. The membrane deformations at a higher excess area allow the rigid-tethered NPs to gain a high number of bound ligand–receptor pairs, as high as the bond formation observed for flexible NPs. Representative snapshots of flexible NPs (inset of Figure 2a,b) suggests that a pancake-like configuration is often adopted for almost all stiffness of NPs to maximize the surface coverage and therefore the binding enhancement. The probability of multivalent binding interactions for flexible NPs and their snapshots with different stiffness are provided in Figure S1 (Supporting Information).

#### 3.2. Free Energy Analysis

To predict the overall binding affinity (also referred to as the binding avidity) of the NPs, we quantify the losses in the
configurational entropies and the gains in the enthalpies of a ligand-coated NP bound to a substrate. Our earlier studies have shown that interactions between the NP and the cell surface are governed by entropy–enthalpy compensation associated with receptor–ligand translation, NP flexibility, and membrane undulations.[16,32,33] The free energy landscape for NP binding is analyzed by explicitly computing the total free energy of binding as described in the Discussion section in the Supporting Information, also see refs. [34,35].

Free energy analysis for different type NPs is shown in Figure 2c–e. We observe a significant enthalpy–entropy compensation in the binding of flexible NPs to the membrane, and it depends on the stiffness of the flexible NP and $A_{ex}$ of the membrane. That is, flexible NPs experience a significant loss of configurational entropy due to polymer flexibility (Figure 2e), an ingredient specific to the flexible NPs. This loss of configurational entropy restricts conformational fluctuations, but results in a higher multivalency of binding (Figure 2a,b), and a favorable free energy of binding (Figure 2c). This trade-off is unique to flexible NPs and is absent in both rigid and rigid-tethered NPs.

When a rigid-tethered NP is bound to a membrane, the enthalpy gain between complementary receptor–ligand pairs significantly exceeds the loss of total entropy (Figure 2c,d), resulting in a stronger binding free energy (Figure 2c). Moreover, upon adhesion, the rigid-tethered NPs draw the available excess membrane area to form invaginations that wrap around NPs, resulting in a substantial increase in enthalpy of binding (Figure 2d). In effect, the results suggest stronger binding free energy for rigid-tethered NPs.

Furthermore, the multivalent binding interactions between a rigid NP and a cell membrane (both excess areas) result in a small enthalpic gain (Figure 2d) that is completely offset by the equivalent loss in entropy (Figure 2e), resulting in no significant change in free energy.
We hypothesize that the stiffness of NPs, type of NPs, and membrane $A_{\text{ex}}$ impose barriers to the binding of multiple NPs.
to the cell surface. To test this hypothesis, we evaluate the effects of NP density (as crowding agents) on the NP–membrane conformational state and binding interactions by increasing the concentration of NPs from one to eight NPs, as depicted in Figure 3a,b and Figure S3 (see also Supporting Information; Movies S6–S11, Supporting Information). As noted, it is not necessary that the involved NPs interact with each other, such as through specific binding or association. Rather, NPs influence each other indirectly through membrane deformations caused by their adhesion. Figure 3a,b (FL1 and FL2) and Figure S3a,b (Supporting Information) for the flexible NPs show that when a relatively small number of NPs are present, a pancake-like configuration is still adopted to maximize the surface coverage and, therefore, resulting in enhanced multivalency. With an increase in the number of flexible NPs, the surface gets more crowded, and as multiple NPs share the available receptors, the NP chains tend to interdigitate, resulting in a brush-like configuration.

Figure 3. Crowding of functionalized NPs bound to membrane. a,b) Snapshots of multiple flexible NPs with different stiffness, multiple rigid NPs, and multiple rigid-tethered NPs, with number of antibody = 162/NP, binding to two characteristically cell membrane types, having distinct excess areas ($A_{ex}$), with 500 ICAM receptors per $0.25 \mu m^2$; the corresponding movies can be found in Movies S6–S11 (Supporting Information). c–h) Effect of crowding on the relative free energy, enthalpy, and entropy of binding per NP ($\Delta F/NP$, $\Delta H/NP$, $T\Delta S/NP$) as a function of membrane excess area ($A_{ex}$). Note: FL1–FL5 correspond to flexible NPs with different stiffness and RG and RT correspond to rigid and rigid-tethered NPs, respectively.
Looking at the snapshots of rigid NPs (Figure 3a,b, RG; Figure S3c,d, Supporting Information) and the average number of multivalency in Figure S4c (Supporting Information), the number of bound receptor–ligand pairs per NP does not change by increasing the number of NPs, and the interacting pairs do not induce a morphological change at the binding site.

Though an increasing number of NPs did not alter membrane morphology significantly for both flexible and rigid NPs (Figure 3a,b, FL1–FL5 and RG), the adhesion of rigid-tethered NPs (Figure 3a,b, RT; Figure S3e,f, Supporting Information) is impacted by crowding. As the available excess area of the membrane and number receptors are shared by multiple NPs, each NP makes smaller membrane deformations than the single rigid-tethered NP case.

The free energy analysis of multiple NPs is shown in Figure 3c–h per NP. The increased density of NPs does not significantly influence the entropy–enthalpy compensation of rigid NPs, and they have the lowest binding strength out of all the three types of NPs (Figure 3c,f). For flexible and rigid-tethered NPs, though we have seen a significant enthalpy gain over entropy loss in the case of single NPs, the crowding of NPs results in a weaker binding energy per NP (Figure 3c,f).

Our results and analysis for flexible NPs suggest that an increase in NP density can result in a stronger binding affinity per receptor–ligand bond (Figure S4a,b, Supporting Information). However, the overall avidity is determined by the total free energy which is the product of free energy per bond and the multivalency. As the particle density increases the number of multivalent receptor–ligand bond per flexible NP dramatically decreases (Figure S4c, Supporting Information) and as a result the flexible NPs experience weaker overall binding affinity per NP. Comparing FL1 and FL5 of a flexible NP, upon the presence of crowding agents, FL5 of flexible NPs shows a relatively stronger overall binding avidity than FL1. Therefore, the stiffness of flexible NPs manifests as an important design factor for the enhanced binding avidity of flexible NPs in crowded environments. These results further depict that the overall binding avidity per NP (Figure S4c–h, Supporting Information) and per receptor–ligand bond (Figure S4a,b, Supporting Information) for FL5 is about the same for the rigid-tethered NPs.

4. Effect of Membrane Morphologies in NP Adhesion and Uptake

The adhesion proteins, membrane-cytoskeleton linker proteins, and membrane-ECM attachment proteins play a significant role in defining cell membranes’ morphology. The pinning of the membrane due to such proteins not only restricts membrane undulations but induce protrusions and invaginations.[24] In the model, the adhesion interaction of the membrane with the cytoskeleton/ECM is incorporated by assigning bell–bond interaction between selected number of membrane vertices and the adhesion surface. The pinning sites are allowed to diffuse and perform binding/unbinding dynamics to mimic the characteristics of linker proteins. The equilibrium conformations of a membrane patch under the influence of such interactions as a function of $A_{m}$ and pinning density $N_{p}$ is given in Figure S5 (Supporting Information). Here we focus on the adhesion of different types of NPs on pinning-induced membrane morphologies. Figure 4 shows the snapshot of bound NPs on the membrane for low, intermediate, and high excess areas when $N_{p} = 0.05, 0.1$ (see Supporting Information for details of the model and conformational dynamics; Movies S12–S14, Supporting Information).

Comparing our results of adhesion without pinning to that with pinning, we find that both the membrane conformations (Figure 4a,b) and the multivalency distribution (Figure S6, Supporting Information) show that the binding of NPs on the membrane is more sensitive to $A_{m}$ than to the density of pinning sites. For low $A_{m}$ (Figure 4a), the pinning-induced membrane conformations remain unaltered upon binding for all type NPs except for rigid-tethered NPs. In rigid-tethered NPs, the pinning and NP-adhesion act cooperatively such that the pinning-induced invaginations coincide with the membrane area that wraps the NPs. Besides, the pinning changes the bimodal distribution of rigid-tethered NPs into unimodal (Figure S6, Supporting Information). This change in binding interactions is due to the ironing out of membrane undulations by pinning sites that allow maximum membrane area for wrapping the NPs and much more homogeneous binding conformations across the replicates.

Free energy analysis of the membrane-bound NPs in the presence of pinning sites is shown in Figure 4. Similar to the previous cases, rigid NPs exhibit the lowest binding avidity. At low $A_{m}$ of the membrane (Figure 4a), the binding avidity of flexible NPs is comparable in magnitude to rigid-tethered NPs. As the pinning density increases to densities 0 and 0.5. As the pinning density increases to 0.1 (Figure 4a,b), FL5 suffers from lower enthalpy gain and FL1 benefits from lower entropy loss. The result of this enthalpy–entropy compensation promotes stronger avidity for FL1.

5. Effect of Curvature-Inducing Proteins at the Site of NP Uptake in the Cell Surface

Curvature effective proteins are known to play a significant role in the adhesion and internalization of nanocarriers and viruses; clathrin-mediated endocytosis being the best-known example.[38–40] Previous studies of internalization of NPs in the presence of curvature-inducing proteins explored the effect of shape and aspect ratio in the uptake of particles.[15] Here we focus on the effect of NP flexibility and membrane deformation when the receptor–ligand binding interaction between the NP and the membrane initiates a protein coat assembly at the surface. In our model system, the assembled protein coats induce a curvature 0.06 nm$^{-1}$ on the membrane, a choice motivated by and justified in a previous study reporting clathrin-mediated endocytosis.[40]

The representative conformations of the membrane–NP system and the multivalency for low and intermediate excess areas are shown in Figure 5. The presence of curvature-inducing proteins causes a significant increase in multivalency.
only in the case of rigid-tethered NPs, for both values of $A_{ex}$. In this case, the proteins stabilize a higher curvature around the NPs, allowing for more binding events. The cooperative action of proteins and the adhesion interaction of ligands and
receptors results in the uptake of rigid-tethered NPs at high $A_{ex}$, whereas the available area limits the low $A_{ex}$ case due to the higher tension experienced by the membrane. For flexible NPs, the binding multivalency has already been saturated, and hence the additional protein-induced curvature does not affect the multivalency significantly. However, the binding-induced assembly of proteins causes a large membrane deformation, a curvature high enough to cover and uptake the NP at high $A_{ex}$. The effective curvature of the invaginations in this case is larger compared to rigid tethered NPs. This is due to the fact that, the bound receptors and hence the coat proteins are more localized in the case of flexible NPs when compared to rigid-tethered NPs. In the case of rigid NPs, as the multivalency is lower, the observed effect of protein coat is negligible.

6. Comparison with Experimental Data

Computational methodologies based on Monte Carlo (MC) or/and coarse-grained molecular dynamics (CGMD) protocols for the binding of functionalized NPs to both functionalized compliant and noncompliant surfaces have been developed and extensively validated in previous works.[16,31,32,34,41] We highlighted a summary of published articles in Table S1 (Supporting Information) which have reported experimental validation of the rigid and flexible models. Therefore, we present the validation of the rigid-tethered NP binding below.

As explained in the Methods, we have performed the endothelial cell culture experiment for the binding of antibody-PEGylated gold NPs, having distinct molecular weights, to the Cho-1CAM cells. The average size of coated gold NPs, determined by the dynamic light scattering, for PEG with molecular weights of 2, 5, and 10 kDa is 5274, 64.6, and 76.2 nm, respectively. We plot the number of bound gold NPs against the corresponding total amount added in Figure S7a (Supporting Information).

In our recent studies, we looked at the role of NP size on the binding affinity of functionalized rigid-tethered NPs to the cell surface. This phenomenon was demonstrated in Figure 9b of ref. [32] by measuring the equilibrium multivalency for NPs having five different sizes $R_{rigid-tethered} = 40, 150, 250, 350,$ and $500$ nm, and two different spring constants $k_{rigid-tethered} = 0.1$ and $1$ N m$^{-1}$. The corresponding total number of ligands on the NPs were selected to be $N_l = 162, 1458, 4050, 7938,$ and $16200$ such that NPs have a uniform ligand density of 14%. We utilize that information and plot the number of bound ligands versus the total number of ligands added per NP in Figure S7c (Supporting Information).

For the comparison purpose, we choose antibody-PEGylated gold NPs with a molecular weight of 5 kDa (i.e., $R_{rigid-tethered} \approx 40$ nm) (Figure S7b, Supporting Information), where 35 numbers of ligands were conjugated to each gold NP. The effect of increasing number of ligands on the binding interactions of NPs with the cell surface in the simulation is considered by increasing the size of a single NP. This effect in the experiment is investigated by increasing the number of NPs of the same size. In Figure S7d (Supporting Information), we compare the number of bound ligands against the total number of ligands added. This comparison result is normalized by the average number of ligands and is in excellent agreement.

7. Discussion and Conclusions

Using a biophysically inspired coarse-grained subcellular model and thermodynamic analysis, we demonstrate that numerous factors such as NP type, stiffness, and concentration,
state of the membrane cytoskeleton, and curvature remodeling proteins can selectively induce cellular targeting and uptake of functionalized NPs. We report tunable exploration of binding and uptake of three distinct NPs that differ in type and rigidity (flexible, rigid, and rigid-tethered) but are otherwise similar in size and ligand surface density.

Based on our results, the fact that the flexible NPs can spread and flatten on the target surface and maximize multivalent interactions may have advantages in affinity targeting over the rigid-tethered NPs. However, we also find that the collaborative action of curvature-inducing proteins and the receptor–ligand binding interactions facilitate complete membrane wrapping (enveloping) of flexible and rigid-tethered NPs, which can be exploited for tuning NP uptake by cells. Our study reveals that the adhesion of a flexible NP depends nontrivially on engineered changes in NP stiffness. Therefore, the rational engineering of NP flexibility requires understanding the dependence of the underlying enthalpic and entropic terms that dominate. The dominant effects are context-specific (i.e., depend on NP type, cytoskeletal state, and on the membrane excess area), and the crowding density of NPs. We discuss below the specific learnings from our exploration of the individual effects and provide conclusions that can directly benefit in the rational design of flexible NPs in applications warranting the optimization of binding avidity, binding selectivity (specificity), and cellular uptake.

Nonintuitive Dependence of NP Binding on Stiffness: Our results show that the NP strain energy exhibits a maximum at intermediate stiffness (FL4), see bead–bead interaction energy in Table 1, and remains low for low as well as high stiffness NP causing the trends to be nonsystematic. NPs exceeding this intermediate stiffness threshold have different binding thermodynamics compared to NPs with stiffness below the threshold. Significant enthalpy–entropy associated with multivalent binding, receptor diffusion, and membrane undulations (especially amidst crowding) make the predictions context specific and dependent on membrane properties (tension) and cytoskeletal state.

Tension or Excess Area Sensors: In the absence of tethers or if tethers mediating ligand–receptor interactions are of comparable stiffness to the NP, then there is minimal effect of the membrane excess area on binding avidity (FL1-FL3 and RG). If the tether is softer than NP stiffness (FL4, FL5, and RT) then there is a definite dependence of binding on membrane excess area. In this latter case, membrane/cytoskeletal tension can be exploited as a switch to regulate NP binding. Cortical tension, therefore, impacts NP binding for some (but not all) NP stiffness, which can be exploited for targeting NPs to specific cell types.

Effect of Crowding: The effect of increasing NP concentration is more pronounced in the presence of flexible or rigid-tethered NPs as they exhibit significantly greater binding avidity relative to their rigid counterpart. However, since the available excess area of the membrane and number receptors are shared by multiple flexible or rigid-tethered NPs, each NP experiences weaker binding energy than the single NP case.

All of the NP models we have investigated with the exception of the rigid NP (RG) are limited by the availability of receptors on the membrane surface for binding owing to the large multivalency. Saturating multivalency in crowded conditions would imply a local density of receptors that is greater than the average surface density, which translates to an entropic penalty, a scenario that manifests as a strong dependence of crowding on the binding avidity.

In addition, we have uncovered the effect of crowding is further mediated by the membrane excess area for FL5, RT but not for FL1, RG (among the four models investigated for this compound effect of crowding and $A_{ex}^f$). We conclude that for models of intermediate stiffness (neither too rigid nor too flexible), membrane mediated interactions further induce negative cooperativity on binding implying that the tension sensing properties of such NPs are impacted by crowding. The mechanism that mediates such an effect is also evident from our results. In this case, the local deformation fields on the membrane upon NP binding confer the negative cooperativity in binding in a crowded environment owing to the fields interacting with each other; such interactions are long ranged and hence are in play even under modest crowding scenarios. However, the negative cooperativity does not manifest for NP of low stiffness (FL1 or rigid NP (RG), where the induced deformation field is weak.

Effect of Cytoskeleton-Induced Heterogeneity: When subject to cytoskeletal pinning interactions, the adhesion behavior is strikingly different among the various of NPs. Both flexible and rigid-tethered NPs in the presence or absence of pinning sites exhibit favorable binding avidity to the cell surface. However, the pinning sites on the membrane limit the out-of-plane fluctuations of the membrane. We find that the membrane tension and the pinning density exquisitely influence how the stiffness of flexible NPs can alter the energy of adhesion of flexible NPs. The binding induced membrane deformations of the rigid NP (RG) remains unvarying amidst pinning while the rigid-tethered NP (RT) display full wrapping compared to the other NP models which do not show wrapping. Based on the binding free energy, we conclude that rigid and rigid-tethered NPs can be sensors of cytoskeletal state in all cases while the sensitivity of flexible NPs changes depending on the crosslinking density, excess area and the pinning.

Degree of crosslinking in flexible NP can be used to engineer the sensing of the cytoskeletal state. Our results suggest that uncrosslinked NP (FL1) shows higher selectivity in binding to membrane with greater pinning density while the opposite is true when the degree of crosslinking was increased (FL5).

NP Uptake through Curvature Effector Proteins: The protein coat assemblies consisting of curvature effector proteins introduced in our model as curvature fields promote the formation of membrane invaginations and uptake. The observed multivalency distribution and membrane conformations suggest that flexible and rigid-tethered NPs will have low barriers for NP uptake when curvature effector proteins are recruited to the site of NP binding.

However, a possible scenario could involve the curvature effector proteins relying on the induced curvature upon NP binding to be recruited through a curvature sensing mechanism. Under such a mechanism, only NP with high stiffness (FL5) or rigid-tethered NP (RT) would be able to recruit the curvature effector proteins at the binding site and therefore benefit from the additional extrinsically (i.e., curvature...
not coupled to multivalent adhesion) induced curvature for NP uptake. However, alternative mechanisms regulating access to spatially clustered receptors or noncanonical internalization routes can favor the uptake of other NP types.\cite{22,45,46}

**Conclusion:** Our computational analysis shows that tuning NP stiffness impacts enthalpic and entropic contributions to multivalent adhesion and uptake through both cytoskeletal and membrane dependent mechanisms. The results also show cellular targeting and uptake can be rationally engineered by the tuning of both NP and cell mechanical properties. These results collectively suggest that flexibility of NPs can be leveraged to engineer essential drug delivery properties of NPs, and NP stiffness can be best utilized in tandem with modifications of other physical and chemical parameters to create more advanced NP delivery systems. Design principles informed through our studies open up avenues for rationally engineering and tuning of new systems in targeted drug delivery.

8. Experimental Section

**Computational Models, Endothelial Cell Culture, Binding of ligand-Coated NPs:** Computational Methods: We have developed a multiscale computational framework to estimate and assess the binding affinity and accessibility of functionalized flexible NPs to undulating cell membranes.\cite{10}

The conformational state of the multivalent NPs adhering to cell surfaces is resolved through a coupled Metropolis Monte Carlo (MC)-Brownian dynamics method, and the microstates of the NP-membrane systems are sampled at thermal equilibrium. A detailed description of the computational model and analysis, and a summary of the detailed parameter set are made available in the Supporting Information and Table S2. The simulations are performed in a 500 × 500 × 620 nm\(^3\) box with periodic boundary conditions along the membrane surface. We first equilibrate the NP and membrane independently. We allow the membrane to equilibrate for 5 × 10\(^8\) MC steps and then for the NP near the cell surface and collect the data after the system relaxes for 5 × 10\(^8\) MCS. Each ensemble is 1 × 10\(^8\) steps of sampling and for each set of conditions, we generate four independent simulation trajectories. To evaluate the error in the calculated quantities, we compute the average value for each ensemble and then compute the standard deviation over the four ensembles. As the decay time for height correlation in membrane is less than 1 × 10\(^8\) MC steps, four ensembles with 1 × 10\(^8\) steps of sampling are expected to give enough statistical sampling. The possibility of kinetic trapping of NPs has been shown to be low (for unpinched systems) using the free energy analysis of rigid NPs in.\cite{10}

The computations for a typical trajectory require three CPU-weeks on a single core of a 2.7 GHz processor.

**Rigid Polystyrene and Flexible Nanoparticles (NPGs):** Methods for preparation, characterization, and assaying of binding, uptake, and targeting of rigid and flexible particles were described in earlier works\cite{22,35,36,45} and will not be repeated here. We discuss the protocols for rigid-tethered particles because experimental results for such particles are presented for the first time here.

**Preparation of 50 nm Gold Nanoparticles (AuNPs):** To prepare 50 nm AuNPs, first, 2 mL of 1% HAuCl\(_4\) solution was added in 200 mL of DI water in a conical flask. Next, 6 mL of 15 nm seed particles solution was added to the solution while stirring. Finally, 440 μL 1% sodium citrate and 2 mL of 0.03 M hydroquinone were added, respectively. The solution was left an hour on a stir plate to observe color transition.

**Expression and Purification of Protein Z L17 Mutations:** The protein Z L17 sequence and the pEVOL-pBpp plasmid (Addgene.org) were cloned into the pSTEPL plasmid, all of which were cotransformed into the E. coli bacteria: New England Bio-labs). Bacterial starter cultures were grown in a shaker in 2 mL LB + 100 μg mL\(^{-1}\) ampicillin + 25 μg mL\(^{-1}\) chloramphenicol at 37 °C overnight. Bacterial starter cultures were then added at a 1:1000 dilution to Autoinduction LB Broth Base containing Trace Elements (Formedium), 100 μg mL\(^{-1}\) ampicillin, and 25 μg mL\(^{-1}\) chloramphenicol. L-Benzoyl-phenylalanine (Bachem, King of Prussia, PA) was added into the bacterial culture for an ultimate concentration of 0.1%. Furthermore, arabinose was added into the culture to an ultimate concentration of 0.1% to create the pEVOL plasmid. Protein Z L17 was expressed at 37 °C in a shaker for 24 h and pelleted by centrifugation.

For lysis, cell pellets were resuspended in B-PER (Thermo Fisher Scientific) with 200 μg mL\(^{-1}\) lysozyme, 4 μg mL\(^{-1}\) DNase, and 1 complete Mini EDTA-free Protease Inhibitor Tablet (Roche). Resuspended pellets were incubated at room temperature for 30 min and then put in the freezer to increase the lysis efficiency. The lysates clarified by centrifugation were incubated with cobalt resin (TALON Metal Affinity Resin, Clontech, 1.2 mL per 100 mL expression culture) for 30 min at room temperature for binding. Following the incubation, resin was washed by PBS. For labeling protein Z L17, 500 μL PBS + 30 μL CaCl\(_2\) + 200 μL GGSKD (BDCO)/NH\(_2\) peptide (NEO Scientific) pH 7.4 was added and incubated for 4–6 h at 37 °C. At the end of this time, the product was purified from the labeling reagent using a 3 kDa molecular weight cutoff (MWCO) filter (Amicon Ultra, Millipore, Temecula, CA). Purified DBCO labeled protein Z L17 was checked by SDS-PAGE gel, and concentration was quantified by BCA assay (Pierce, Rockford, IL).

**Copper-Free Click Chemistry:** For copper-free click conjugation, three reactions were performed with different molecular weight Thiol-PFG-Azide (2, 5, and 10 kDa). DBCO labeled protein Z, and different molecular weight Thiol-PFG-Azide were mixed with a 1:3 molar ratio overnight at room temperature. The next day, reactions were used for antibody crosslinking.

**Preparation of Antibody-PEG-Thiol:** Protein Z clicked PEG-Thiol mixes were incubated with R6.5 at the molar ratio of 1 IgG to 8 Protein Z-PEG Thiol in a clear centrifuge tube on ice for 2 h with 365 nm UV light through the use of a UVP CL-1000L UV crosslinker (Upland, CA). To purify the crosslinked products from access proteins and PE-Thiol mix a molecular weight cut-off (MWCO) microfilter (Amicon Ultra, Millipore, Temecula, CA) was used. The purified crosslinked products then were analyzed using SDS-PAGE electrophoresis. The samples were boiled for 5 min with buffer (Bio-rad, Hercules, CA) loaded by SDS-PAGE, which contains 1:20 dilution of beta-mercaptoethanol (Bio-rad). The samples were then operated for 30 min at fixed 180 V.

**Antibody-PEG-Thiol Radiolabeling:** Antibody-PEG-Thiol radiolabeling was performed with Na\(^{251}\) using Pierce Iodination Beads. Using Quick Spin Protein Columns (G-25 Sephadex, Roche Applied Science, Indianapolis, IN), the Free 125I was cleared. As a 1:1 ratio of antibody-PEG-Thiol mixtures was washed with PBS before the use of a UVP CL-1000L UV crosslinker (Upland, CA). To purify the crosslinked products from access proteins and PE-Thiol mix a molecular weight cut-off (MWCO) microfilter (Amicon Ultra, Millipore, Temecula, CA) was used. The purified crosslinked products then were analyzed using SDS-PAGE electrophoresis. The samples were boiled for 5 min with buffer (Bio-rad, Hercules, CA) loaded by SDS-PAGE, which contains 1:20 dilution of beta-mercaptoethanol (Bio-rad). The samples were then operated for 30 min at fixed 180 V.

**Gold Radiolabeling:** Direct labeling of gold nanoparticles was carried out through chemisorption of iodine-125.\cite{23,25} Gold nanoparticles were also radiolabeled with Na\(^{251}\) using Pierce Iodination Beads. Free 125I was purified by three washes in distilled H\(_2\)O and centrifugation at 3000 × g for 30 min. Finally, the radiochemical purity was specified as the area of the free 125I divided by the area sum of the total.

**Antibody-PEGylation of AuNPs:** Antibody-PEG-thiol mieoties were conjugated at different number ratios to gold nanoparticles in PBS. Thiolated polystyrene glycol (PEG-SH) was used to cover unused bare gold surfaces with decreased nonspecific interplays. The particles were incubated for 15 min under agitation on a shaker. The antibody-conjugated particles were washed three times in PBS with 1% BSA/0.1% Tween and centrifugation at 3000 × g for 30 min.

**Binding of Targeted Gold NPs:** Twenty-four well plates (Corning Inc., Corning, NY) were used to plate Cho-ICAM cells (105 cells cm\(^{-2}\)). The radiolabeled gold NPs were then subjected to the Cho-ICAM cells. Once incubated at 4 °C, the cells were triple washed, and then lysed with lysis buffer (1% Triton X-100, 1 μl NaOH). Figure S8 (Supporting Information) shows the bound gold NPs versus the total amount of added gold NPs.
Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

avidity, entropy, Monte Carlo simulation, nanoparticle flexibility, polymeric nanoparticles

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