

# COMMUNICATIONS

## Binding Affinity and Kinetic Analysis of Targeted Small Molecule-Modified Nanoparticles

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Nanoparticles bearing surface-conjugated targeting ligands are increasingly being explored for a variety of biomedical applications. The multivalent conjugation of targeting ligands on the surface of nanoparticles is presumed to enhance binding to the desired target. However, given the complexities inherent in the interactions of nanoparticle surfaces with proteins, and the structural diversity of nanoparticle scaffolds and targeting ligands, our understanding of how conjugation of targeting ligands affects nanoparticle binding remains incomplete. Here, we use surface plasmon resonance (SPR) to directly and quantitatively study the affinity and binding kinetics of nanoparticles that display small molecules conjugated to their surface. We studied the interaction between a single protein target and a structurally related series of targeting ligands whose intrinsic affinity varies over a 4500-fold range and performed SPR at protein densities that reflect endogenous receptor densities. We report that even weak small molecule targeting ligands can significantly enhance target-specific avidity (by up to 4 orders of magnitude) through multivalent interactions and also observe a much broader range of kinetic effects than has been previously reported. Quantitative measurement of how the affinity and kinetics of nanoparticle binding vary as a function of different surface conjugations is a rapid, generalizable approach to nanoparticle characterization that can inform the design of nanoparticles for biomedical applications.

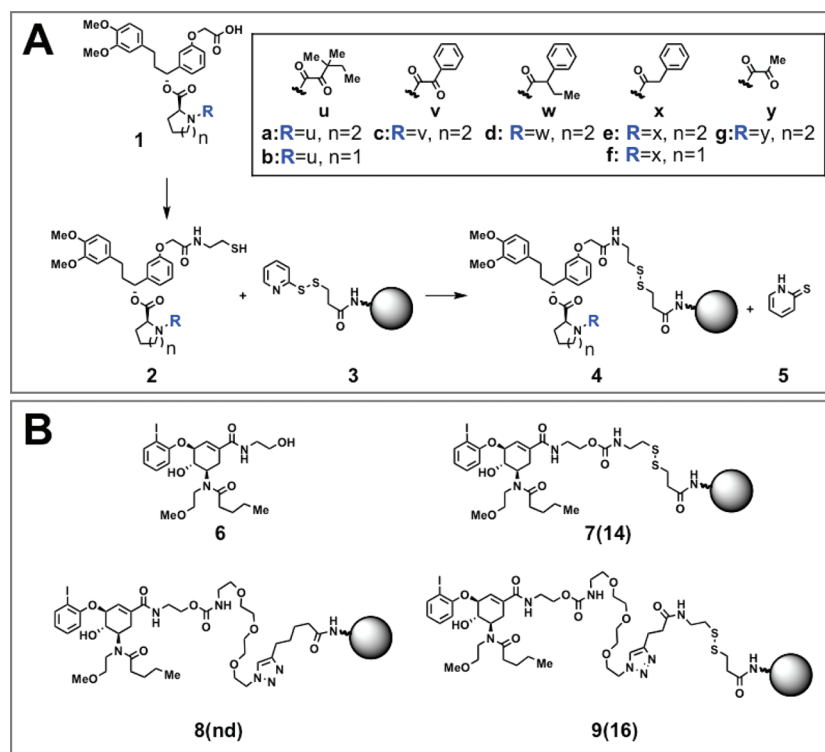
Targeted nanoparticles are an exciting class of materials that are undergoing clinical development as diagnostics, molecular imaging probes, and therapeutic delivery vehicles (1). Targeting is typically achieved through the surface display of multiple high affinity ligands, such as antibodies, peptides, or natural products. Multivalent interactions between the nanoparticles and their targets can increase the affinity of target binding (avidity) (2), much as multivalency in Nature facilitates the attachment of pathogens to host cells, or circulating cells to vascular endothelium (3, 4). More recently, investigators have used synthetic, non-natural product small molecules as targeting ligands on the surface of nanoparticles (5, 6). This approach allows a much more diverse range of chemical matter to be used for targeting and enables libraries of small molecule modified nanoparticles to be rapidly screened for the desired binding properties. Non-natural product small molecules generally have much weaker affinity for their targets (e.g., micromolar  $K_D$ ), so their use as targeting ligands assumes that multivalent avidity effects will enhance the binding affinity. This notion is supported by the observation that multivalency enhances the avidity as well as recognition specificity of weak interactions between carbohydrates and their protein partners (7–9) and increases the biologic activity of a weakly binding therapeutic (10). However, given the complexities inherent in interactions

of nanoparticle surfaces with proteins (11, 12), we sought to develop an approach that could quantitatively describe the binding of a surface-conjugated nanoparticle to its target, and that was sufficiently rapid and generalizable to be widely applicable to the development and characterization of targeted nanoparticles.

Here, we use surface plasmon resonance (SPR) to directly and systematically study the affinity and binding kinetics (13) of nanoparticles that display targeting small molecules conjugated to their surface. To enable direct comparisons, we study a series of structurally related ligands to the same protein, whose intrinsic  $K_D$  varies over a 4500-fold range. Importantly, we perform SPR measurements at target protein densities that are comparable to those reported for known cellular targets. These studies demonstrate that even weak small molecule ligands can increase the avidity of nanoparticle target interactions, but also reveal unanticipated effects on binding kinetics. As targeted nanomaterials move toward clinical application, a quantitative understanding of the structure–activity relationships underlying their avidity and kinetics would inform the future design of targeted nanoparticles.

We chose the well-studied interaction between synthetic derivatives of the natural product FK506 and its target protein, FK506-binding protein 12 (FKBP12). A series of structurally related synthetic FK506 analogues possessing  $K_D$ s that range from 24 nM to 110  $\mu$ M (14) (Scheme 1A) were individually conjugated to a dextranated magnetic nanoparticle (cross-linked iron oxide, or CLIO) originally developed for MR imaging (15)

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Scheme 1. Conjugation of Small Molecules to Nanoparticles<sup>a</sup>

<sup>a</sup> A. Conjugation of a series of synthetic derivatives of FK506 (**1**) by sulfhydryl exchange. B. Small molecule that binds aurora A kinase (**6**) and nanoparticle conjugates obtained by sulfhydryl exchange and Huisgen 1,3-dipolar cycloaddition.

and biosensor applications (16). This nanoparticle has a diameter of approximately 38 nm in aqueous solutions. We conjugated small molecule ligands to sulfhydryl reactive groups on the nanoparticle using sulfhydryl exchange, and ultraviolet quantitation of the amount of released pyridine-2-thione (**5**) allowed calculation of the average number of small molecules conjugated to each nanoparticle (17) (Scheme 1A). (For ligands of class **1**, our conjugation linkers utilized the same carboxylic acid moiety that has been previously used to modify this class of compounds while preserving their affinity for FKBP12 (18–21).) Nanoparticles with low and high small molecule valency (approximately 3–5 vs 13–18 small molecules per nanoparticle, respectively) were selected for further study; the number of small molecules conjugated per nanoparticle is depicted in parentheses (e.g., **4a(17)** corresponds to particle **4a** with 17 conjugated small molecules).

Binding of small molecule conjugated nanoparticles to their target protein is specific and exquisitely sensitive to the density of protein immobilized onto the SPR sensor chip. Since our target protein is immobilized to the sensor chip as an FKBP12-GST (glutathione S-transferase) fusion protein, we tested nanoparticles for nonspecific binding to GST and saw no binding to either unmodified or small molecule conjugated nanoparticles (Figure 1A,B). In contrast, at protein densities commonly used for SPR experiments, targeted nanoparticles remain bound to an FKBP12-GST surface (hereafter called an FKBP12 surface) with no appreciable dissociation even after more than 12 min (Figure 1C). At lower protein densities (e.g., 100 response units, corresponding to approximately 1 protein per 648 nm<sup>2</sup>), we observe a measurable (but still slow) off-rate that allows calculation of dissociation kinetics (Figure 1C). Of note, this low protein density is still the same order of magnitude at which physiologically relevant receptors are expressed on cell surfaces. For instance, the HER2 receptor is expressed in SKBR-3 breast cancer cells at a density of  $\sim 1 \times 10^6$  receptors per cell (22); assuming a single-sided disk 15  $\mu\text{m}$  in diameter, this corresponds

to a HER2 receptor every 707 nm<sup>2</sup>. An analogous calculation for folate receptors on KB cells ( $\sim 2.5 \times 10^6$  receptors per cell (23)) corresponds to a receptor every 283 nm<sup>2</sup>.

We measured the association rate  $k_a$ , dissociation rate  $k_d$ , and dissociation constant  $K_D$  ( $k_d/k_a$ ) for the interaction of FKBP12 with each small molecule (**1a–1g**), as well as with those same small molecules conjugated to CLIO nanoparticles (**4a–4g**) (Scheme 1A). For each experiment, small molecules or nanoparticles were flowed in parallel over a reference sensor bearing GST (see Supporting Information); the minimal signal arising from GST interaction is then subtracted out to yield SPR tracings that reflect specific interactions with FKBP12 (Figure 1D,E). Small molecule and nanoparticle sensorgrams were analyzed with a Langmuir 1:1 binding kinetic analysis, using software provided by the instrument manufacturer (Biacore).  $K_{D(\text{free})}$  for all free ligands are in excellent agreement with previously published values, ranging from 24 nM to 110  $\mu\text{M}$  (Table 1). By comparison, the conjugated nanoparticles show profound changes in both association and dissociation kinetics, with only the weakest free ligand, compound **1g**, failing to bind FKBP12 when conjugated to a nanoparticle. As expected,  $k_d$  for the multivalent nanoparticles are all markedly decreased relative to the corresponding free ligand (up to 3400-fold slower for **4a(17)** vs **1a**). (Dissociation of FKBP12-GST from the surface is negligible during the time course of these experiments; see Supporting Information.) In contrast,  $k_a$  for different nanoparticles show widely divergent effects. For instance, nanoparticle **4a(17)** has a 400-fold slower  $k_a$  compared to the free ligand **1a**, while nanoparticle **4f(20)** has a 39-fold faster  $k_a$  compared to the free ligand **1f**.

$K_{D(\text{multivalent})}$  (calculated from  $k_d$  and  $k_a$ ) for all of the nanoparticles is in the single digit nanomolar range. To relate  $K_{D(\text{multivalent})}$  to  $K_{D(\text{free})}$  in cases where the precise number of ligand–target interactions is not known, Whitesides and colleagues defined an enhancement factor  $\beta$ , which increases as the relative affinity of a multivalent interaction increases:  $\beta =$

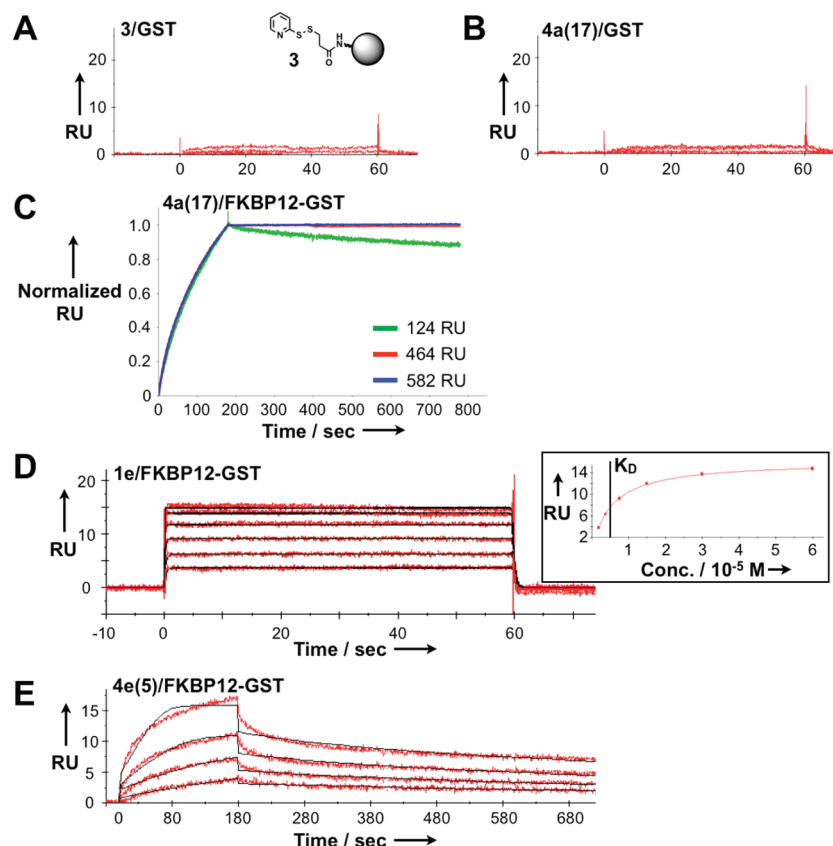


**Figure 1.** Specific protein–nanoparticle binding documented in SPR sensorgrams. A. Nanoparticle intermediate **3** does not bind to a control surface of GST. B. Targeted nanoparticle **4a(17)** does not bind to a GST surface. C. Sensorgrams for targeted nanoparticle **4a(17)** at different immobilization densities of FKBP12-GST. Significant binding occurs; appreciable dissociation only occurs at the lowest protein density tested. D. Representative sensorgram for a free small molecule (**1e**) flowed over an FKBP12-GST surface. Inset: Depiction of calculating  $K_D$  from steady-state affinity studies. E. Representative sensorgram for multivalent nanoparticle **4e(5)**. In parts D and E, red curves depict experimental data at different analyte concentrations; fitted curves modeled to describe a 1:1 binding event are overlaid in black.

**Table 1. Equilibrium Affinity and Rate Constants Derived from a Langmuir 1:1 Binding Kinetic Analysis<sup>a</sup>**

| analyte (valency) | $k_a$ ( $M^{-1} s^{-1}$ ) | $k_d$ ( $s^{-1}$ )    | $K_D$ (M) multivalent | $\beta$ | $K_D$ (M) ligand      | $\chi^2$ (RU <sup>2</sup> ) |
|-------------------|---------------------------|-----------------------|-----------------------|---------|-----------------------|-----------------------------|
| <b>1a</b>         | $2.31 \times 10^7$        | 0.557                 | --                    | --      | $2.41 \times 10^{-8}$ | 0.11                        |
| <b>4a(5)</b>      | $4.88 \times 10^4$        | $2.78 \times 10^{-4}$ | $5.68 \times 10^{-9}$ | 4       | --                    | 0.45                        |
| <b>4a(17)</b>     | $5.76 \times 10^4$        | $1.64 \times 10^{-4}$ | $2.84 \times 10^{-9}$ | 8       | --                    | 0.60                        |
| <b>1b</b>         | $1.04 \times 10^6$        | 0.812                 | --                    | --      | $7.85 \times 10^{-7}$ | 0.17                        |
| <b>4b(4)</b>      | $6.64 \times 10^5$        | $2.86 \times 10^{-3}$ | $4.30 \times 10^{-9}$ | 182     | --                    | 0.17                        |
| <b>4b(9)</b>      | $6.62 \times 10^5$        | $2.08 \times 10^{-3}$ | $3.14 \times 10^{-9}$ | 250     | --                    | 0.40                        |
| <b>1c</b>         | $1.11 \times 10^6$        | 0.117                 | --                    | --      | $1.05 \times 10^{-7}$ | 0.37                        |
| <b>4c(4)</b>      | $1.13 \times 10^5$        | $7.64 \times 10^{-4}$ | $6.77 \times 10^{-9}$ | 16      | --                    | 0.29                        |
| <b>4c(13)</b>     | $1.21 \times 10^5$        | $3.58 \times 10^{-4}$ | $2.96 \times 10^{-9}$ | 36      | --                    | 0.33                        |
| <b>1d</b>         | $1.70 \times 10^4$        | 0.0500                | --                    | --      | $2.94 \times 10^{-6}$ | 0.29                        |
| <b>4d(6)</b>      | $2.19 \times 10^5$        | $1.62 \times 10^{-3}$ | $7.39 \times 10^{-9}$ | 398     | --                    | 0.05                        |
| <b>1e</b>         | $5.23 \times 10^5$        | 2.67                  | --                    | --      | $5.11 \times 10^{-6}$ | 0.35                        |
| <b>4e(5)</b>      | $3.46 \times 10^5$        | $2.15 \times 10^{-3}$ | $6.20 \times 10^{-9}$ | 824     | --                    | 0.19                        |
| <b>4e(18)</b>     | $7.29 \times 10^5$        | $4.43 \times 10^{-3}$ | $6.09 \times 10^{-9}$ | 840     | --                    | 0.26                        |
| <b>1f</b>         | $5.62 \times 10^4$        | 2.21                  | --                    | --      | $3.94 \times 10^{-5}$ | 0.61                        |
| <b>4f(20)</b>     | $2.17 \times 10^6$        | $9.01 \times 10^{-3}$ | $4.15 \times 10^{-9}$ | 9498    | --                    | 0.72                        |
| <b>1g</b>         | $1.90 \times 10^4$        | 2.08                  | --                    | --      | $1.10 \times 10^{-4}$ | 0.58                        |
| <b>6</b>          | $1.79 \times 10^4$        | 0.0775                | --                    | --      | $4.33 \times 10^{-6}$ | 0.37                        |
| <b>8(nd)</b>      | $2.13 \times 10^6$        | $8.32 \times 10^{-3}$ | $3.91 \times 10^{-9}$ | 1107    | --                    | 0.64                        |
| <b>9(16)</b>      | $4.20 \times 10^5$        | $3.82 \times 10^{-3}$ | $9.08 \times 10^{-9}$ | 477     | --                    | 0.58                        |

<sup>a</sup> Corresponding groups of free ligand and conjugated nanoparticles are listed in alphabetical order (a–g). Nanoparticle names have the measured number of small molecules conjugated per nanoparticle (valency) in parentheses.  $\beta = K_{D(\text{free})}/K_{D(\text{multivalent})}$ . Valency was not determined for nanoparticle **8(nd)**.

$K_{D(\text{free})}/K_{D(\text{multivalent})}$  (**3**). For the multivalent nanoparticles tested here,  $\beta$  ranges from approximately 4 to 9500; the largest  $\beta$  is for ligand **1f**, with a  $K_D$  of 39  $\mu\text{M}$  as a free ligand and a  $K_{D(\text{multivalent})}$  of 4.15 nM as nanoparticle **4f(20)**. Thus, nanoparticles bearing ligands with weak intrinsic affinity demonstrate greater  $\beta$  and increased affinity (avidity).

The rate map in Figure 2 graphically summarizes several features of the data: (i) A series of multivalent nanoparticles, all binding to the same protein target with approximately the same  $K_{D(\text{multivalent})}$ , can nonetheless display markedly different kinetics. (ii) Conjugating free ligands (open symbols) to form the corresponding multivalent nanoparticles (closed symbols) has dramati-

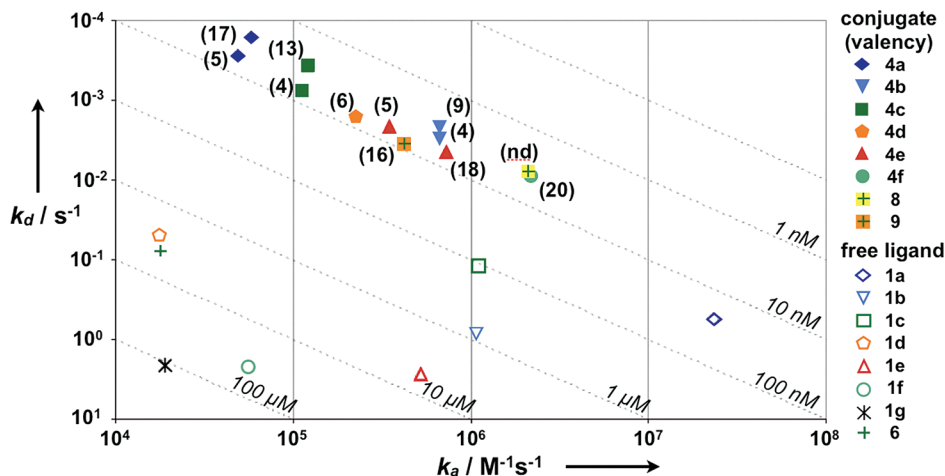


**Figure 2.** Rate maps summarizing binding affinity and kinetics. Different combinations of  $k_a$  and  $k_d$  that result in the same  $K_D$  are indicated by dashed lines. Data for free ligands are depicted by open symbols; for each ligand, the corresponding nanoparticles are depicted by a solid symbol of the same shape, with conjugation valency listed in parentheses next to the solid symbol. Valency of **8(nd)** was not determined.

**Table 2.** Equilibrium Affinity and Rate Constants Derived from a Tertiary Interaction (Bivalent) Kinetic Analysis<sup>a</sup>

| analyte       | $k_{a1}$ ( $M^{-1} s^{-1}$ ) | $k_{d1}$ ( $s^{-1}$ ) | $k_{a2}$ ( $M^{-1} s^{-1}$ ) <sup>b</sup> | $k_{d2}$ ( $s^{-1}$ ) | $K_{D1}$ (M)          | $K_{D2}$ (M)           | $K_{D1}/K_{D2}$       | $\chi^2$ (RU <sup>2</sup> ) |
|---------------|------------------------------|-----------------------|-------------------------------------------|-----------------------|-----------------------|------------------------|-----------------------|-----------------------------|
| <b>4a(5)</b>  | $2.61 \times 10^4$           | $3.41 \times 10^{-4}$ | $4.88 \times 10^4$                        | 0.106                 | $1.31 \times 10^{-8}$ | $2.17 \times 10^{-6}$  | $6.03 \times 10^{-3}$ | 0.40                        |
| <b>4a(17)</b> | $3.15 \times 10^4$           | $2.31 \times 10^{-4}$ | $1.03 \times 10^5$                        | 0.109                 | $7.33 \times 10^{-9}$ | $1.06 \times 10^{-6}$  | $6.91 \times 10^{-3}$ | 0.44                        |
| <b>4b(4)</b>  | $7.88 \times 10^4$           | $2.33 \times 10^{-2}$ | $2.70 \times 10^4$                        | $1.23 \times 10^{-3}$ | $2.95 \times 10^{-7}$ | $4.69 \times 10^{-8}$  | 6.28E+00              | 0.08                        |
| <b>4b(9)</b>  | $2.17 \times 10^5$           | $3.09 \times 10^{-3}$ | $8.35 \times 10^3$                        | $6.43 \times 10^{-6}$ | $1.42 \times 10^{-8}$ | $7.70 \times 10^{-10}$ | $1.84 \times 10^1$    | 0.3                         |
| <b>4c(4)</b>  | $2.95 \times 10^4$           | $7.99 \times 10^{-4}$ | 2.09                                      | $1.17 \times 10^{-3}$ | $2.71 \times 10^{-8}$ | $5.62 \times 10^{-4}$  | $4.82 \times 10^{-5}$ | 0.37                        |
| <b>4c(13)</b> | $5.95 \times 10^4$           | $3.67 \times 10^{-4}$ | 5.26                                      | $1.04 \times 10^{-3}$ | $6.16 \times 10^{-9}$ | $1.98 \times 10^{-4}$  | $3.11 \times 10^{-5}$ | 0.35                        |
| <b>4d(6)</b>  | $6.10 \times 10^4$           | $3.98 \times 10^{-2}$ | $8.05 \times 10^4$                        | $3.92 \times 10^{-4}$ | $6.52 \times 10^{-7}$ | $4.87 \times 10^{-9}$  | $1.33 \times 10^2$    | 0.05                        |
| <b>4e(5)</b>  | $2.24 \times 10^4$           | $3.49 \times 10^{-2}$ | $6.55 \times 10^3$                        | $4.64 \times 10^{-4}$ | $1.56 \times 10^{-6}$ | $7.08 \times 10^{-8}$  | $2.20 \times 10^1$    | 0.08                        |
| <b>4e(18)</b> | $4.66 \times 10^4$           | $3.91 \times 10^{-2}$ | $7.26 \times 10^3$                        | $6.93 \times 10^{-4}$ | $8.39 \times 10^{-7}$ | $9.55 \times 10^{-8}$  | 8.78                  | 0.11                        |
| <b>4f(20)</b> | $1.62 \times 10^5$           | $1.56 \times 10^{-1}$ | $6.41 \times 10^3$                        | $1.75 \times 10^{-3}$ | $9.63 \times 10^{-7}$ | $2.73 \times 10^{-7}$  | 3.52                  | 0.27                        |
| <b>9(16)</b>  | $1.89 \times 10^4$           | $2.57 \times 10^{-3}$ | $1.91 \times 10^3$                        | $1.30 \times 10^{-5}$ | $1.36 \times 10^{-7}$ | $6.84 \times 10^{-9}$  | $1.99 \times 10^1$    | 0.34                        |

<sup>a</sup> Bivalent interactions between A (analyte) and B (immobilized ligand) are described by the following equations: (1)  $A + B \rightleftharpoons AB$  (with rate constants  $k_{a1}$  and  $k_{d1}$ , and  $K_{D1} = k_{d1}/k_{a1}$ ), (2)  $AB + B \rightleftharpoons AB_2$  (with rate constants  $k_{a2}$  and  $k_{d2}$ , and  $K_{D2} = k_{d2}/k_{a2}$ ). <sup>b</sup>  $k_{a2}$  (RU<sup>-1</sup> s<sup>-1</sup>) was converted approximately to  $k_{a2}$  (M<sup>-1</sup> s<sup>-1</sup>) as follows:  $k_{a2}$  (M<sup>-1</sup> s<sup>-1</sup>) =  $k_{a2}$  (RU<sup>-1</sup> s<sup>-1</sup>)  $\times$  MW  $\times$  100.

cally diverse effects on interaction kinetics;  $k_{d(\text{multivalent})}$  decreases to a variable extent, and  $k_{a(\text{multivalent})}$  can increase, decrease, or remain largely unchanged. (iii) Under the tested protein densities, adding more small molecule ligands to each nanoparticle has a modest effect on both kinetics and affinity.

We also extended our analysis to a relatively uncharacterized synthetic small molecule screening “hit”. Compound **6** (Scheme 1B), discovered through a biochemical affinity screen, binds aurora A kinase with  $K_{D(\text{free})} = 4 \mu\text{M}$  (24). We conjugated **6** to CLIO using thiol exchange (conjugates **7(14)** and **9(16)**) or Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition (“click” chemistry, conjugate **8**) (Scheme 1B). (Conjugation of **6** to CLIO utilized the same primary alcohol that was used to immobilize **6** in the affinity screen that led to its discovery (19, 24).) Nanoparticle **7(14)** does not bind to immobilized aurora A kinase (data not shown). In contrast, nanoparticles **9(16)** (with a similar but longer linker than the inactive **7(14)**) and **8** both show significantly increased avidity, with  $K_{D(\text{multivalent})} = 9$  and 4 nM, and  $\beta = 480$  and 1100, respectively (Table 1 and Figure 2). Thus, in this second model system, multivalent attachment of a weakly binding screening “hit” results in large  $\beta$  factors and enhanced avidity toward the compound’s target. The rate map in Figure 2 illustrates how different methods of conjugating the same molecule (e.g., in nanoparticles **9(16)** and **8**) can result in significantly divergent effects on binding kinetics (especially with regard to  $k_a$ ) (Figure 2).

Because it is not possible to precisely determine the number of interactions each nanoparticle makes with immobilized ligands, all of the above sensorgrams used a simple binary interaction model (similar to one used for other nanoparticle

studies (23); full details in Supporting Information). In fact, a binary model fits the data quite well with very small residual differences (between experimental and fitted curves; residual curves are shown in Supporting Information) and small  $\chi^2$  values (minimized sum of the squared residual difference). This is consistent with our choice of relatively sparse protein densities to mimic physiologic receptor densities. To estimate the contribution of higher-order interactions, we reanalyzed the data using a tertiary interaction model (i.e., corresponding to a bivalent interaction between the nanoparticle and protein) as follows (the full binding model is described in Supporting Information):

(1)  $A + B \rightleftharpoons AB$  (with rate constants  $k_{a1}$  and  $k_{d1}$ , and  $K_{D1} = k_{d1}/k_{a1}$ )

(2)  $AB + B \rightleftharpoons AB_2$  (with rate constants  $k_{a2}$  and  $k_{d2}$ , and  $K_{D2} = k_{d2}/k_{a2}$ ).

For the conjugated nanoparticles **4a–4f** and **9**, the ratio of  $K_{D1}/K_{D2}$  correlates with  $K_{D(\text{ligand})}$ , the dissociation constant for the free ligand ( $R^2 = 0.57$ ; Table 2 and Figure 3A). That is, the bivalent mode of binding contributes more to the overall nanoparticle interaction (greater  $K_{D1}/K_{D2}$  ratio) for nanoparticles bearing ligands of weaker intrinsic affinity (larger  $K_{D(\text{ligand})}$ ). This is consistent with our finding that nanoparticles bearing weaker ligands also exhibit the greatest  $\beta$  and enhancement of avidity. Interestingly, the avidity effect for weaker ligands appears to be mediated primarily by the dissociation rate of the bivalent interaction. The dissociation constant for the free ligand ( $K_{D(\text{ligand})}$ ) shows a strong correlation with  $k_{d1}/k_{d2}$  ( $R^2 = 0.76$ ; for weaker free ligands,  $k_{d1} \gg k_{d2}$ ) but no correlation with  $k_{a1}/k_{a2}$  ( $R^2 = 0.01$ ) (Table 2 and Figure 3B,C). While it is uncertain

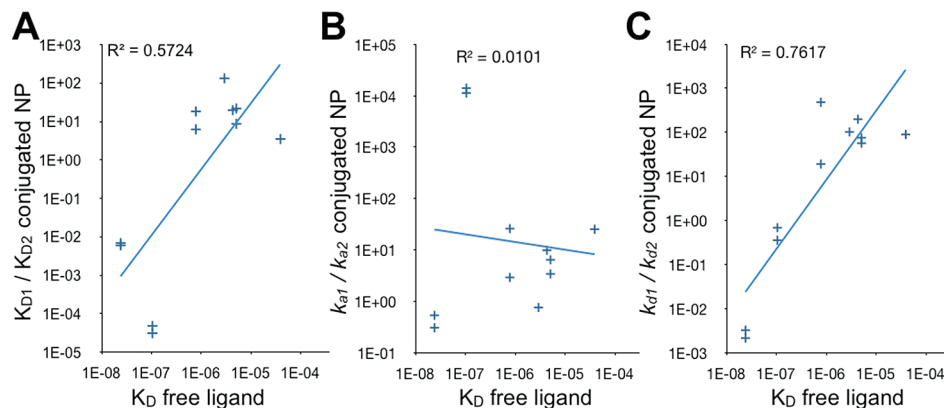


**Figure 3.** Relative contribution of monovalent vs bivalent reaction terms to nanoparticle binding as a function of the intrinsic  $K_D$  of the free ligand. A. As the intrinsic affinity of the free ligand becomes weaker,  $K_{D1}/K_{D2}$  increases ( $R^2 = 0.57$ ). B. No correlation between  $k_{d1}/k_{d2}$  and intrinsic affinity of the free ligand. C. As the intrinsic affinity of the free ligand becomes weaker,  $k_{a1}/k_{a2}$  increases ( $R^2 = 0.76$ ).

whether the nanoparticles interact through bivalent or higher-order interactions, this analysis confirms that multivalent interactions make important contributions to nanoparticle avidity.

Predicting the structure–activity relationships (SAR) for multivalent ligands has not been straightforward, even for relatively small systems such as dimers of the antibiotic vancomycin (25). This problem is exacerbated as functionalized nanoparticles increasingly utilize chemically and structurally diverse scaffolds (e.g., dextranated nanoparticles, dendrimers, albumin, liposomes) and targeting ligands (e.g., antibodies, peptides, aptamers, or low-affinity small molecules). Here, we have performed SPR on relatively sparse but physiologic protein surfaces (22, 23) to very sensitively detect how subtle variations in targeting ligands can affect binding kinetics and affinity. Our target protein densities are approximately 100-fold sparser than those used to document very large avidity effects for folate-targeted dendrimers (23), and our findings provide a complementary view of avidity at a distinct, physiologically relevant regime of protein concentrations. Using even slightly denser (e.g., 4-fold) protein surfaces, we observed essentially no dissociation of nanoparticles (Figure 1C), which suggests that our results may underestimate the maximal avidity obtainable with these particles, as well as the contribution of higher ligand valencies. Other factors, such as deformable and fluid membranes that could facilitate close apposition of cellular receptors, could further increase avidity.

We demonstrate strong avidity effects ( $\beta$  of up to 4 orders of magnitude) for nanoparticles conjugated to relatively weak (micromolar  $K_D$ ) small molecule ligands, including “hits” from a high-throughput screen. We observed a dramatic decrease in  $k_d$  for the nanoparticles relative to the free ligand, consistent with other reports (23); however, we observed a much wider variety of effects on  $k_a$  than has been reported previously, including markedly increased, markedly decreased, or unchanged  $k_a$  relative to the free ligands. These unanticipated effects on binding kinetics were revealed because of the deliberate constraints of our experiment (a common protein target, shared nanoparticle scaffold, similar valency, and identical conjugation chemistry), which were imposed to allow detailed comparisons of multivalent binding. The kinetic differences displayed by our series of related functionalized nanoparticles could translate into functional differences. For instance, nanoparticle affinity and association and dissociation rates can all affect how deeply an intravascular circulating agent penetrates into tissue before its target sites become effectively saturated (26); the relative contribution of these variables depends on several *in vivo* factors, including capillary permeability, particle size, and target density.

Overall, this approach can be generalized rapidly to a variety of ligand–target combinations, as it does not require *de novo* development of a cell-based assay for each target. Systematic application of this approach could reveal principles that guide nanoparticle design. For instance, our studies suggest that a view in which nanoparticles are largely modular assemblies of scaffold, linker, and targeting ligand may be too simplistic; instead, SPR studies could clarify how different structural components collaborate to determine binding kinetics and affinities. Our data also suggest testable hypotheses about how nanoparticles with distinct binding and kinetic properties will behave in complex *in vitro* and *in vivo* systems. This in turn has broad implications for the design and synthesis of functionalized nanoparticles for the imaging, diagnosis, and treatment of disease.

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**Supporting Information Available:** Experimental procedures, characterization data, and NMR spectra for all novel compounds, as well as sensorgrams for small molecules and nanoparticles. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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