Mesoscale computational protocols for the design of highly cooperative bivalent macromolecules

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The last decade has witnessed a swiftly increasing interest in the design and production of novel multivalent molecules as powerful alternatives for conventional antibodies in the fight against cancer and infectious diseases. However, while it is widely accepted that large-scale flexibility (10 – 100 nm) and free/constrained dynamics (100 ns – µs) control the activity of such novel molecules, computational strategies at the mesoscale still lag behind experiments in optimizing the design of crucial features, such as the binding cooperativity (a.k.a. avidity).

In this study, we introduced different coarse-grained models of a polymer-linked, two-nanobody composite molecule, with the aim of laying down the physical bases of a thorough computational drug design protocol at the mesoscale. We show that the calculation of suitable potentials of mean force allows one to apprehend the nature, range and strength of the thermodynamic forces that govern the motion of free and wall-tethered molecules. Furthermore, we develop a simple computational strategy to quantify the encounter/dissociation dynamics between the free end of a wall-tethered molecule and the surface, at the roots of binding cooperativity. This procedure allows one to pinpoint the role of internal flexibility and weak non-specific interactions on the kinetic constants of the NB-wall encounter and dissociation. Finally, we quantify the role and weight of rare events, which are expected to play a major role in real-life situations, such as in the immune synapse, where the binding kinetics is likely dominated by fluctuations.

INTRODUCTION

Single-domain antibodies, also known as nanobodies (NB) [1], are found naturally in camelds and represent an intriguing alternative to design and build novel multivalent and multi-specific immunotherapy agents for targeting tumors and viral infections [2]. In addition to leading to an increase in affinity, coupling two (or more) different binders helps in marginalising the effect of mutation and polymorphism of the target. Linked anti-CD16 nanobodies, C21 and C28 [3], for example, have been shown to be effective against treating breast cancer involving a low HER2 expression, which is resistant to the therapeutic antibody trastuzumab [4].

As the conventional antibodies are large structures, they may not be effective when it comes to situations like a partially exposed tumor [5] [6]. In addition, an uneven distribution of the anti-tumor antibody in the entire tumor region may lead to tumor regrowth [7]. In such situations, therapeutic agents with smaller structures are desirable [8]. A good solution is to use engineered structures composed of antigen-specific nanobodies linked with flexible linkers [9], e.g. realized via a polymer such as [Gly4Ser]n. Such structures will be small and will thus have better ability to penetrate into the tumor microenvironment and reinforce the formation of the immune synapse. Such structures are easy to produce and are more efficient when compared to the bulky conventional antibodies, and can be designed to be multivalent and multispecific [11] [12].

An important property of an antibody is the strength of bivalent binding that it demonstrates, a property known in immunology as avidity. Although it has no unique quantitative definition (let alone whether it is more appropriate to regard it as an equilibrium or a kinetic parameter), avidity can be thought of as the cooperative gain in affinity afforded by double binding. Notably, such measure is intimately connected to the internal flexibility of the multi-domain molecules and to the geometric configuration of binding epitopes [10]. For example, viruses employ various strategies to evade the action of antibodies of the immune system. Some virions, like HIV-1, have a very rapid rate of mutation. Experiments reveal that the enhanced antibody evasion capability of HIV-1 is based not just in its capability to mutate but is a combined effect of mutation along with the spike structure and low spike density [13] [15]. While, mutations reduce the affinity of the natural antibodies towards the target spikes, the spike structure and low density preclude intra- (i.e. multiple epitopes on the same target) and inter-spike cross-linking thus preventing bivalent binding and avidity [16] [17].

Experiments have demonstrated that linking two Fab domains of an antibody through an extended polymer like a DNA oligomer, leads to an increase in divalent binding, for an optimal linker length, and a resulting increase in the efficacy of the antibodies. Wu et al. [18] per-
formed experiments on respiratory syncytial virus, which has a very high Env spike density, and showed that the affinity of low-affinity bivalent Fabs was 2–3 orders of magnitude higher as compared to their monovalent counterparts and the efficiency was not affected by mutations that increased the off-rates nearly 100-fold. The results show that multivalent structures made of polymer linked nanobodies would lead to higher degree of avidity and thus higher efficiency. Galimidi et al. [19] performed experiments on linked Env (HIV-1 envelope glycoproteins) binders and showed that linking can lead to an increase in potency by 2–3 orders of magnitude. Jähnichen et al. [20] developed two different single domain nanobodies that could bind to different sites on the extracellular domain of the CXCR4 coreceptor. They found that joining the two nanobodies with protein linkers resulted in a 27-fold increase in CXCR4 affinity. With further analysis they concluded that the effect is pure avidity resulting from the heterobivalent linking of the two nanobodies. Zhang et al. [21] developed multimers of nanobodies leading to an increase in affinity and several orders of magnitude decrease in the rates of dissociation. Yang et al. [22] performed dissociation rate calculations for the binding between a bivalent antibody and hapten ligands as a function of the ligand density. They found cooperative binding as the hapten density increased and bivalent binding set in. They could determine two different dissociation constants for the double-step antibody-hapten binding process with one dissociation constant being 3-orders of magnitude larger than the other.

When one of the nanobodies in a two-NB construct binds to the receptor at its binding site (epitope), the other linked ligand spends more time in the vicinity, leading to a larger probability of the latter unit to bind to another similar or different epitope, on the same or on a facing surface, depending on whether the system is mono-specific or multi-specific. By hindering free diffusion of the ligands, linking can lead to an increase in rebinding events and strengthen the interaction between interfaces [23]. Further, Bongrand et al. [24] showed that the fraction of divalent attachments between an antibody-coated microsphere and a mono- or divalent ligand-coated surface, that resisted a force of 30 pN for a minimum of 5 seconds, was ∼4 times higher than the number of monovalent attachments.

While the choice of the nanobody depends not only on its affinity towards the target epitope, but also on the nature of bond it forms with the epitope [25], the choice of linker would depend on its flexibility and the geometry of the epitope distribution/configurations. Experimental methods have been developed to create linkers of given stiffness and extension, out of a combination of proteins and peptides [26–29]. Among the most common are the (Gly,Ser)ₙ linkers. Protein linkers have been accommodated into the hinge regions of natural antibodies, thus enabling intra-spike linking to viral receptors [28]. Other biocompatible polymers like PEG are also good candidates to be used to link the nanobodies. The properties of the linker are very important in determining the degree of avidity. Depending on the epitope density on a tumor cell or the distribution of the antibody binding sites on the viral envelope, a linker that is either too flexible or too stiff can lead to under-performance.

With the improvements in computational resources and speed, molecular dynamics (MD) simulations in recent days have been playing an important role in fields like drug discovery [30] and also in unraveling fundamental mechanisms involving very large biological complexes, such as chromatin [31]. MD simulations can be important in determining the optimal properties of the linkers that would lead to an efficient multivalent binding for a particular target. In addition, simulations of a group of linked nanobodies can give important insights into their epitope-binding kinetics as a function of the linker structural properties and other important parameters, like the paratope-epitope binding energy. While the engineered nanobody-linker-nanobody systems are much smaller as compared to the conventional antibodies, simulating a significantly large group of them in atomistic detail would be computationally expensive and cannot be done routinely. Thus, some degree of coarse-graining is important to perform kinetics analysis using MD simulation as a tool.

Keeping the above discussion in mind, here we perform MD simulation of a coarse-grained system consisting of two nanobodies connected by a linker (referred to as a diabody) and study its structural and dynamical properties. We use different levels of coarse graining schemes to represent the diabody. In the simplest representation, we perform simulation of two extended (rigid) spheres connected by a flexible bead-string linker (see Fig. 1). In addition to this, with a future aim to study the dynamics of diabodies in the presence of their target receptors (such as HER2), where a nanobody represented by a hard sphere will be incapable of representing the important features of the diabody-target interaction properly, we perform a finer coarse-graining of the nanobody (see Fig. 1). In this scheme, we represent the nanobody using the shape-based coarse-graining (SBCG) scheme developed by Schulten et al. [32]. Using Umbrella Sampling (US) simulations we calculate the free energy profiles on which various conformations of diabodies tethered to a surface lie. From the MD trajectories we calculate the flight and residence times of the free end of the tethered diabody in a region close to the tethering wall. Comparing the results from the two different models we demonstrate how the coarse-graining scheme could affect the results and, notably, we highlight the role of the “shape” in the wall-domain dynamics.

The paper is organize as follows. In section II we provide the details of our computational methods, models and simulations. In section III we describe and comment our results, mainly concerning the different potentials of mean force and the detailed analyses of the encounter and dissociation kinetics of the free NB of a wall-tethered molecule. In section IV we wrap up our discussion and
FIG. 1. (Color online) The two coarse-grained models of diabody studied in this work. Two nanobodies are linked through a polymer. The nanobody beads are represented in blue and the polymer in green. The maroon beads are the connector beads, which connect the nanobody to the polymer, and paratopes, respectively. The model on the left (named SPH) uses single spherical beads to represent the nanobodies. The model on the right (named SBCG) uses a shape-based coarse-graining algorithm for the nanobodies.

I. METHODS

A. Coarse-graining schemes and simulations

All the data reported in this work were generated from Langevin dynamics simulations performed with LAMMPS [42] in the Lennard-Jones (LJ) unit system, with the unit of length being $\sigma = 3.5$ Å (the size of one monomer of the linker) and the unit of energy being $\epsilon = 100$ K. All the simulations were conducted via Langevin dynamics in the overdamped regime.

In this work, we considered two different coarse-grained representations of a two-NB molecule joined by a flexible linker. In the first approach, the NBs were modeled as rigid spheres of radius 10 (in units of linker monomers), decorated with two smaller fixed spheres at two diametrically opposite ends, representing the NB-linker connecting unit and the parotope, respectively (see Fig. 1). The parotope bead has diameter 1.6 and the connector bead diameter 1. A bead-spring polymer bridges the two connector beads. The beads constituting the polymer linker have unit diameter. This model will be referred to in the following as the SPH model.

The second model was meant to reproduce the shape and large-scale flexibility of the NBs. For that we used the shape-based coarse-graining scheme developed in Schulten’s group [33]. This procedure requires a trajectory from an atomistic equilibrium MD simulation to be sampled and fed as an input. The crystal structure with pdb id: 1qd0 [34] was used as the starting structure for the MD simulation to generate the input structure. This is a camelid heavy chain variable (VHH) domain, in complex with a RR6 dye dimer.

The dye was removed from the complex and the remaining protein was solvated in TIP3P water with a 20 Å buffer, leading to a system size of 40298 atoms, with 13284 water molecules. The solvated system was then neutralized by adding 5 Cl$^-$ ions to generate the starting configuration for the MD simulation (see Fig. 2). The system was minimized for 10000 steps using the conjugate gradient method. During minimization, all the atoms in the protein were constrained to their starting positions. This allowed water molecules to re-organize and eliminate unfavorable contacts with the protein. After minimization, the atoms were assigned velocities generated from a Maxwell distribution at 300 K. The particle mesh Ewald (PME) method with a real space cut-off of 12 Å was used to estimate the energy component from the long-range electrostatic interaction. The system was simulated for 100 ns in the NPT ensemble. A Langevin thermostat was used [35] with a temperature coupling constant of 5 ps$^{-1}$, while the pressure was regulated using the Langevin barostat with a pressure coupling constant of 50 ps$^{-1}$. The simulation was performed using NAMD [36] and the CHARMM 27 [37] force field was used to describe the protein. The MD trajectories were visualized using VMD [38].

An average structure was generated from the snapshots belonging to the last 10 ns of the MD trajectory (see Fig. 2). The average structure of the nanobody was used to generate a shape-based coarse grained (SBCG) model using the procedure formulated by Schulten et al. [33]. This scheme uses topology-conserving algorithm developed for neural networks to generate a coarse-grained representation that reproduces the shape of the protein. In a trade-off between the system size and a good representation of the protein shape, we used 40 beads to represent the 126 residue protein. To generate the system to be simulated two of the CG proteins were connected by a polymer linker with monomer diameter 1, similar to the one used in the SPH model (see Fig. 1). It is to be noted that the diameter of the spherical bead that represents the nanobody in the SBCG model is nearly equal to the geometric average of the major and minor axes of the roughly spheroidal SBCG nanobody. In this sense the two models are equivalent and comparable.

B. Interaction parameters

In general, the total interaction potential of our coarse-grained models had bond, angle and van der Waals
FIG. 2. (Color online) **The coarse-graining procedure.** The crystal structure (PDB id. 1qd0) is used as the starting configuration for the explicit solvent atomistic simulation. Snapshots from the last 10 ns of the trajectory are used to generate an average structure. The average structure is then coarse-grained using the shape-based coarse-graining method. A pair of the coarse-grained structures are connected by a linker (10-, 20- and 30-mer), enclosed in a box with X-Y periodicity, aligned along the Z-direction and tethered to the box base, thus generating the starting configuration for CG simulations.

(vdW) terms, given by

\[
V_{ij}^{\text{bond}} = \frac{1}{2} k_b (r_{ij} - r_0)^2 \tag{1}
\]

\[
V_{ijk}^{\text{angle}} = \frac{1}{2} k_\theta (\theta_{ijk} - \theta_0)^2 \tag{2}
\]

\[
V_{ij}^{\text{vdW}} = 4\epsilon_{ij} \left[ \left( \frac{s_{ij}}{r_{ij}} \right)^{12} - \left( \frac{s_{ij}}{r_{ij}} \right)^6 \right] \tag{3}
\]

where \( k_b \) and \( k_\theta \) are the bond force constant and the angle bending energy, respectively, \( r_0 \) and \( \theta_0 \) are the equilibrium bond length and angle, respectively, \( \epsilon_{ij} \) is the LJ interaction energy and \( s \) is the inter-bead distance at which the LJ potential becomes repulsive (referred to as *repulsive* length in the following), which depends on the combined radii of the interacting beads.

1. **Interaction parameters for the SBCG nanobodies and the linker**

All the CG beads were kept neutral. The connectivity and spring constants for the bonds between the beads were used as generated by the SBCG scheme. It is to be noted that the bonds are not set by a distance-based cut-off scheme, but are in accordance with the bonds present in the atomistic system. This helps in maintaining the flexibility of the nanobody and providing the required flexibility to the loop regions, which may play a defining role in the kinetics of the diabody. Repulsive vdW interactions among the beads were also introduced to ensure that the shape is maintained during the course of the simulation. The vdW radii of the protein beads were generated by comparing the masses of the protein beads to that of the PEG monomer. The repulsion between the constituent beads of the nanobody was represented by a Weeks-Chandler-Andersen potential (WCA) \[39\], i.e. a shifted LJ potential cut off at the minimum, i.e. \( r_{\text{cut}} = 2^{1/6}\sigma \). The angle parameters for the nanobody beads were used as generated by the SBCG scheme.

The linker is represented as a freely-jointed chain with two-body harmonic bond-stretching and three-body harmonic bond-bending potentials. The masses and diameters of the polymer beads were set to 1. The vdW interaction between the bead pairs was set to purely repulsive as represented by a WCA potential. The force constant of the (stiff) bond-stretching potential was set to 54 N/m, which corresponds to an average fluctuation of the bonds at room temperature of about 2 % of the equilibrium length. In order to estimate the appropriate value of the bending rigidity \( k_\theta \), it is expedient to refer to the calculation of the persistence length \( \ell_p \) for the freely jointed chain with angle-bending interactions in the hypothesis of zero correlation between bending and torsion degrees of freedom. Referring to published data for PEG \[11\],
we fixed $k_\theta = 1.8 k_B T$ as the bending coefficient for the linker (see [40] for a detailed discussion).

2. Interaction of the beads with the box walls

The walls of the simulation box in the X and Y directions had periodic boundary condition, while the Z walls were fixed. The Z walls interact with the beads via LJ (12-6) interaction given by

$$V_{vdW} = 4\epsilon \left[ \left( \frac{s_{wall}}{r_{wall}} \right)^{12} - \left( \frac{s_{wall}}{r_{wall}} \right)^{6} \right]$$

Here $r_{wall}$ is the distance of the center of any bead from the wall. The wall interaction parameters, $s_{wall}$, $r_{cutwall}$ and $\epsilon$ define the nature of interaction between different beads and the wall. For the purely repulsive wall, $r_{cutwall} = 2^{1/6}s_{wall}$, while for the attractive wall it equals 2.5 $s_{wall}$ (see also Table [VI]). While the interaction of the nanobodies and paratopes with the Z walls was set to be either repulsive or attractive in different simulations, the linker and connector beads always had repulsive interaction with the walls.

C. Preparing the systems for simulation

The first set of simulations reported involve the calculation of potentials of mean force (PMF) for the diabodies as a function of various reaction coordinates. To perform the PMF calculations, the diabodies were enclosed in cuboidal boxes which were periodic in the X and Y directions, while the Z walls were fixed and repulsive. The diabodies were tethered to the lower Z wall by imposing an attractive LJ interaction between the paratope of one of the nanobodies and a fixed epitope bead attached to the lower Z wall. The simulations were performed for linker lengths of 10, 20, 30, 40 and 50 monomers for the SPH system and 10, 20 and 30 monomers for the SBCG system.

The second kind of simulations were performed on a collection of diabodies to calculate dynamical parameters. $N = 25$ diabodies were tethered to the lower Z-wall of a cuboidal box. The tethering points were arranged in a $5 \times 5$ lattice (see Fig. 2 of supplementary information (SI) [40]). Again, the $x$ and $y$ directions had periodic boundary conditions, while the lower $z$ wall was fixed and either perfectly reflecting or attractive. The linker lengths for different systems were similar to that considered for the PMF calculation, with an additional linker length of 60-mer for the SPH system.

In the rest of the article, the free nanobody is referred to as nbd-1 while the nanobody tethered to the wall is referred to as nbd-2. The connector bead corresponding to nbd1 and nbd2 are named CB1 and CB2 respectively, while the paratopes are referred to as P1 and P2 respectively (see Fig.3). In addition to the various bonded and non-bonded interactions described in the previous section, the angles L10-CB1-P1, P2-CB2-L1, L2-L1-CB2 and L9-L10-CB1 were restrained to 180° using a harmonic angle bending potential of the form of eq. (2) with $\theta_0 = 180^\circ$ employing stiffer bending coefficients as compared to the angles corresponding to the linker. On top of this, for the SBCG systems, the nanobodies were restrained from rotating about their respective long axes by restraining a dihedral formed by L10-CB1 (L1-CB2) and two beads of nbd-1 (nbd-2) to their starting values throughout the simulation. The extra restraints were introduced to mimic the fact that, in real-life systems, the bonds between the linker and the nanobody restrict the angular motion of the nanobody about the CB1/2-P1/2 axis.

D. PMF calculation

The PMF calculations have been performed using the umbrella sampling (US) technique along two different reaction coordinates (RCs). The two RCs are named $\rho_{z-proj}$ and $\rho_{z-y}$. The former is the projection of the vector joining the tethering point to the center of mass of nbd-1 on the z-axis (see Fig.4 (A)). The latter is the projection of the same vector on the $x-y$ plane with the condition that $\rho_{z-proj} = 5 \sigma$, which represents a condition where nbd-1 is close to the wall to which nbd-2 is tethered (see Fig.4 (B)). The RC values varied from 3 to 30, 40, 50, 60 and 70 $\sigma$ for the 10-, 20- and 30-, 40- and 50-mer linker systems, respectively, with windows at gaps of 0.5 $\sigma$, leading to 55, 75, 95, 115 and 135 simulation windows for the SPH systems. For the SBCG systems, the RC values varied from 5 to 35, 45 and 55 $\sigma$ for the 10-,
in independent simulations, we computed the average flight where the paratope remained above the threshold. Taking an average over the 25 nanobodies (nbd-1) and three independent simulations, we computed the average flight and residence times and also calculated the corresponding distributions.

II. RESULTS AND DISCUSSION

We performed a 10 ns long simulation of the SBCG nanobody and used the trajectory to measure the radius of gyration of the coarse-grain model. We found the average over the trajectory to be 3.99 ± 0.06σ. The same calculation over the last 10 ns of the atomistic trajectory of the 1qd0 structure yielded an average value of 4.12 ± 0.02σ, which confirmed the soundness of our SBCG-based approach.

A. Free energy profiles

The PMF profiles as a function of ρz−proj are shown in Fig. 5 (A) and (C) for the SPH and SBCG diabodies, respectively. In spite of the PMF profiles having similar shape, there are a few notable differences. For the 10-mer system, for example, the PMF profile is flat in 15σ ≤ ρz−proj ≤ 25σ for the SBCG system, while the same region for the SPH system occurs at shorter distances, extending between 8σ and 20σ. In addition, for small values of RC, the profiles for the SBCG system have a larger slope as compared to the SPH system, which means that the SBCG nanobody faces a larger (entropic) repulsive force from the fixed wall than the SPH nanobody. The derivative of the PMF profiles is shown in the SI [40]. These differences suggest that the different coarse-graining schemes highlight differences in the dynamics near the tethering wall. In particular, an excessively simple spherical model seems to fall short of capturing important features of the interactions with a boundary.

The PMFs are portrayed as a function of ρz−y in Figs. 6 (B) and (D). The profiles for the two models appear very different. While the PMFs for the SPH systems increase steadily in a linear fashion after ρz−y ~ 15σ, the profiles for the SBCG systems are flatter and start rising at a much later stage. Fig. 6 (A) shows the average of all values of the (normalized) ρx−y values for which PMF(ρx−y) ≤ kB T for ρz−proj = 5σ as a function of the linker length. The error bars gauge the flatness of the PMF profiles, which is seen to vary markedly in the two models. More precisely, the PMF profiles are flat (in the xy plane) for short linkers, while the flatness decreases with increasing linker length. This indicates that a network of nanobodies linked with short linkers may be more effective at inter-epitope binding even for configurations with a large standard deviation in the inter-epitope distances.

To better understand the differences displayed by the two models, we computed the PMF along ρz−y for different values of ρz−proj for the SBCG diabodies with 30-mer linkers. Fig. 6 (B) shows the profiles obtained for

![Figure 4](Image)

**FIG. 4.** (Color online) Reaction coordinates. Schematic representation of (A) ρz−proj and (B) ρx−y. The red bead is the tethering point.
PMF profiles for tethered diabodies. PMF profiles as a function of the $z$-projection of the vector joining the tethering point to the center of mass of the free nanobody for the (A) SPH and (C) SBCG systems. PMF profiles as a function of the projection on the $xy$ plane of the vector joining the tethering point to the center of mass of the free nanobody, with the free NB restrained to stay at a $z$-projection of $5\sigma$ for the (B) SPH and (D) SBCG systems. The $x$-axis is represented in units of $\sigma$.

FIG. 5. (Color online) PMF profiles for tethered diabodies. PMF profiles as a function of the $z$-projection of the vector joining the tethering point to the center of mass of the free nanobody for the (A) SPH and (C) SBCG systems. PMF profiles as a function of the projection on the $xy$ plane of the vector joining the tethering point to the center of mass of the free nanobody, with the free NB restrained to stay at a $z$-projection of $5\sigma$ for the (B) SPH and (D) SBCG systems. The $x$-axis is represented in units of $\sigma$.

$\rho_{z-proj} = 5\sigma$ (nbd-1 in contact with the fixed wall) and $\rho_{z-proj} = 8\sigma$ (nbd-1 separated from the wall). For the larger value of $\rho_{z-proj}$, the PMF profile appears similar to that of the SPH diabody, which indicates that the presence of the wall shapes the PMF profiles of the non-spherical model. We infer that for epitope systems with targets present very close to the cell-membrane, which acts as a soft wall, one might have a larger chance of inter-epitope binding as the wall seems to have a flattening effect on the SBCG PMF profiles. Overall, the differences between the PMF profiles of the two systems demonstrates that molecular shape may play a big role in controlling the interaction with the tethering wall.

One notices a steeper increase of the PMF as a function of $\rho_{x-y}$ at low distances for the 10-mer as compared to other linker lengths. This effect is much more pronounced for the spherical molecules (Figs. 5 (B) and (D)). This indicates that when the linker length is comparable to the dimensions of the nanobodies, it is difficult to approach the epitopes close-by to the one to which the diabody is tethered. It is to be stressed that the slope is larger in case of the SPH system as compared to the SBCG system, which indicates that the shape of the nanobody is expected to play a role when it comes to inter-epitope binding for a high target density, especially for low linker lengths.

In the spirit of computationally aided molecular design, the PMF profiles as a function of $\rho_{x-y}$ can be used to estimate the length of the linker required to efficiently result in multi-epitope binding for a given target geometry. With a knowledge of the epitope size and average inter-epitope distances, one can estimate, with a knowledge of the position of minima of the PMF profiles and also their degree of flatness, what length (or range of lengths) of the linker polymer would result in avidity. Simulations with bending modulus of the linker matching different polymers used as linkers in practical situations, like various peptides or nucleic acids, can help predict the appropriate stiffness of the linker for a given geometrical arrangement of epitopes. Tumor receptors like HER2 [43] have one epitope for natural antibodies while some engineered triple helix proteins called affibodies [44][45] are known to engage a different region on the opposite side [40]. With PMF calculations and knowledge of the position of minima as a function of a suitable reaction coordinates, one can predict, using MD simulation, the linker length that would maximize bispecific binding.

B. $z$-distribution of the free paratope: comparison with models

An important observable that is tightly related to the statistics of flight and residence times is the distribution of the $z$-coordinate of the paratope of the free nanobody (bead P1). We computed these distributions for all the systems from the simulations performed to calculate the flight and residence times (see methods). It is interesting to compare the data for the SPH and SBCG systems with a simple model. The expression for the normalized equilibrium $z$-distribution of the free end of a Gaussian polymer, tethered at a height $z_0$ from a reflecting wall, reads [47]
The effective model is thus a higher representation of large \( z \) values and a reduced representation of small \( z \) values when compared to the tethered polymer. This is an expected consequence of the higher entropic repulsive force exerted by the wall on the free bead, a mechanism akin to the entropic pulling force demonstrated in the disassembling and translocating action of Hsp70s chaperones [49]. More precisely, the conformational space near the wall is restricted more severely for the diabodies, due to presence of the nanobodies at the lower end of the wall for the SPH system (see SI [40]). This would represent a polymer tethered at \( z_0 = 7.5 \sigma \) distance units above the tethering wall and having a contour length equal to the linker and nbd1 combined. Our linker has a persistence length of \( \ell_p \simeq 1.5 \sigma \) (see SI), which leads to a Kuhn length \( b = 2\ell_p = 3 \sigma \).

\[ P(z) = \frac{3}{2\sqrt{2\pi N_k b^2}} e^{-3z^2/2(d^2)} \]  

where we have introduced explicitly the average square end-to-end distance of the free polymer, \( \langle d^2 \rangle = N_k b^2 \).

It is interesting to inquire whether the data for the composite two-NB models can be described by an effective polymer model. The most obvious choice would be a Gaussian polymer with the same flexibility as the diabody linker, contour length equal to the contour length of the diabody measured from CB2 to the free paratope (P1) and tethered at the average height of CB2 above the lower \( z \) wall. A reasonable guess for the length of the effective model is thus \( N_k = N + 2r/b \), where \( N \) is the number of monomers in the linker, \( r \) is half the distance between P1 and CB1 and \( b \) is the Kuhn length of the linker polymer (see again Fig. 3). We set \( z_0 = 7.5 \sigma \), which is the average height for CB2 measured from the wall for the SPH system (see SI [10]). This would represent a polymer tethered at \( z_0 = 7.5 \sigma \) distance units above the tethering wall and having a contour length equal to the linker and nbd1 combined. Our linker has a persistence length of \( \ell_p \simeq 1.5 \sigma \) (see SI), which leads to a Kuhn length \( b = 2\ell_p = 3 \sigma \).

Fig. 7 shows the comparison of the effective model with the data for the SPH and the SBCG systems. It is apparent that the distribution for the diabodies describes a higher representation of large \( z \) values and a reduced representation of small \( z \) values when compared to the tethered polymer. This is an expected consequence of the higher entropic repulsive force exerted by the wall on the free bead, a mechanism akin to the entropic pulling force demonstrated in the disassembling and translocating action of Hsp70s chaperones [49]. More precisely, the conformational space near the wall is restricted more severely for the diabodies, due to presence of the nanobodies at the lower end of the wall for the SPH system (see SI [40]). This would represent a polymer tethered at \( z_0 = 7.5 \sigma \) distance units above the tethering wall and having a contour length equal to the linker and nbd1 combined. Our linker has a persistence length of \( \ell_p \simeq 1.5 \sigma \) (see SI), which leads to a Kuhn length \( b = 2\ell_p = 3 \sigma \).

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the two ends of the polymer, than for a bare polymer with the same contour length and flexibility. The extent of the difference between the simulation and the model suggests extended, composite molecules such as our diabodies, belong to a different universality class altogether. One can, however, use eq. (5) or (6) to determine the effective Kuhn length \( N_\text{eff} \) of an effective polymer with the same persistence length \( \ell_p \) as the linker in the diabodies. For this, we fit the distributions of the 40-, 50- and 60-mer SPH systems with eq. (6) (see SI) with \( b = 3\sigma \), which leads to \( N_\text{eff} \sim 108, 115 \) and 119 for 40-, 50- and 60-mer diabodies respectively.

It is interesting to note that the distributions for the SBCG and the SPH systems differ to a highest extent when the linker length matches the dimensions of the linked NBs, i.e. for the shortest linker (10-mer), while they approach each other rapidly as the linker length increases and almost overlap for the 30-mer linker. This means that, as the statistics of the vertical coordinate above the wall is concerned, for linkers longer than approximately the size of the NBs, an equivalent SPH model can be used, entailing considerable simplification of the simulations.

C. Flight/residence times: quantifying the kinetics of the second binding

The statistics of the flight/residence times (F/RT) of the free NB are crucial observables, as they embody the kinetic determinants of the second binding, hence can help quantify avidity. The F/RTs are defined as stretches of consecutive frames that the paratope of the two ends of the polymer, than for a bare polymer with the same contour length and flexibility. The extent of the difference between the simulation and the model suggests extended, composite molecules such as our diabodies, belong to a different universality class altogether. One can, however, use eq. (5) or (6) to determine the effective Kuhn length \( N_\text{eff} \) of an effective polymer with the same persistence length \( \ell_p \) as the linker in the diabodies. For this, we fit the distributions of the 40-, 50- and 60-mer SPH systems with eq. (6) (see SI) with \( b = 3\sigma \), which leads to \( N_\text{eff} \sim 108, 115 \) and 119 for 40-, 50- and 60-mer diabodies respectively.

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The probability per unit time that the paratope enter the domain \( D \) after a time \( t \) is given by the paratope-wall kinetics of some weak non-specific attraction between the protein and the wall/membrane. Data for such events were accumulated over 30 \( \mu \)s-long trajectories of 25 non-interacting tethered diabodies. A set of 3 such simulations were performed for each linker length. In addition, the simulations were performed for both repulsive and attractive tethering walls (with attractive energies equal to 1.5 \( k_B T \) and 2.5 \( k_B T \)), with the aim of assessing the effect on the paratope-wall kinetics of some weak non-specific attraction between the protein and the wall/membrane.

Fig. 8 (upper panels) illustrates the calculation of the on and off rates as described by formulas (9) and (10). The probability per unit time that the paratope enter the interaction domain appears of the order of tens of \( \mu \)s\(^{-1} \), while the probability per unit time that it exit the same domain turns out to be about ten times higher. Interestingly, the SBCG model shows a higher on-rate than the spherical model (with a pure repulsive wall). At the same time, the exit probability is higher for the SBCG model.

Fig. 8 also demonstrates that a weak non-specific attraction between the NBs and the wall of the order \( \epsilon \sim 1 - 2k_B T \) increases the on-rate (i.e. decreases the overall survival probability in the flight domain) and decreases the off-rate (i.e. makes journeys of the paratope in the interaction domains longer). More specifically, these data refer to a modified SPH-wall system with non-specific isotropic attraction (LJ) between the wall and nbd-1/2 and P1/2. It is interesting to observe that the gain afforded by a weak attractive wall in terms of on-rate, as gauged by \( k_{\text{on}}(\epsilon,N)/k_{\text{on}}(0,N) \), is found to increase with the linker length \( N \). In fact, while, \( k_{\text{on}}(0,N) \) as [50]}

\[
\mathcal{P}_f(t) = -\frac{dS_f(t)}{dt}
\]

\[
\mathcal{P}_r(t) = -\frac{dS_r(t)}{dt}
\] (8)

The inverse mean-exit times can be considered as measures of the escape rate from the corresponding domains. Therefore, combining eqs. (7) and (8), it is possible to estimate the on and off rates directly from the series of flight and residence times, as

\[
k_{\text{on}} = \left[ \int_0^\infty t \mathcal{P}_f(t) \, dt \right]^{-1} = \left[ \int_0^\infty S_f(t) \, dt \right]^{-1}
\] (9)

\[
k_{\text{off}} = \left[ \int_0^\infty t \mathcal{P}_r(t) \, dt \right]^{-1} = \left[ \int_0^\infty S_r(t) \, dt \right]^{-1}
\] (10)

To calculate the flight \( (t_f) \) and residence times \( (t_r) \) numerically, the \( z \)-coordinate of the paratope was monitored through the simulation. A threshold \( z_{th} = 3.5\sigma \) was set to define whether the paratope is in the flight or residence regions. If the paratope stayed above or below the threshold for at least 0.5 ns, the corresponding trajectory stretch was designated to correspond to a flight or residence event, respectively. This additional constraint was tailored specifically to avoid short recrossing events that could bias the statistics unphysically at short times. Data for such events were accumulated over 30 \( \mu \)s-long trajectories of 25 non-interacting tethered diabodies. A set of 3 such simulations were performed for each linker length. In addition, the simulations were performed for both repulsive and attractive tethering walls (with attractive energies equal to 1.5 \( k_B T \) and 2.5 \( k_B T \)), with the aim of assessing the effect on the paratope-wall kinetics of some weak non-specific attraction between the protein and the wall/membrane.
might reason as follows. While those of the residence times much less so. In order
the tails of the flight times depend on the linker length,
abilities in either region. As it shows from the figures,
function in Fig. 9 makes this point very clear in the case of the
the linker and are distributed exponentially. The inset
longer survival events depend markedly on the length of
short survival times in either domain are dominated by
random walk in three dimensions [50]. This means that
expected behaviour for the survival probability of a free
in Fig. 9), irrespective of the linker length. This is the
between the paratope/NB system and the wall. Conversely,
increasing linker length in the presence of attraction be-
possibly reflects the fact that the entropic cost associ-
decreases with \( N \), a weak attraction makes \( k_{\text{on}}(\epsilon, N) \)
nearly insensitive to variations in the linker length. This
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ited time series. In this case, the statistics of return
events will be specified by the configurational probabil-
the paratope be in the relevant regions, either \( z \geq z_{th} \) or \( z < z_{th} \). In turn,
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NB-polymer systems and, in the absence of an appropri-
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\[
P_a(k) = P_\geq (1 - P_\leq)^{k-1} P_\leq \approx P_\leq e^{-P_\leq k} \quad (11)
\]

\[
P_c(k) = P_\leq (1 - P_\geq)^{k-1} P_\geq \approx P_\geq e^{-P_\geq k} \quad (12)
\]

If for the sake of the argument we take the Gaussian
tethered model [6] as a reference case, it is readily seen that

\[
P_\geq = \int_{z_0}^{+\infty} P(z) \, dz = e^{-3z_0^2/2(d^2)} \quad (13)
\]

\[
P_\leq = \int_{-\infty}^{z_0} P(z) \, dz = 1 - e^{-3z_0^2/2(d^2)} \quad (14)
\]

If we introduce the time decay constants \( \tau_f \), \( \tau_r \) of the
exponential tails of the survival probabilities in the flight
and interaction domains, respectively, Eqs. (13) and (14)
entail

\[
\tau_f = \frac{\Delta t_c}{P_\leq} = \Delta t_c \frac{2(d^2)}{3z_0^2} \quad (15)
\]

\[
\tau_r = \frac{\Delta t_c}{P_\geq} = \Delta t_c e^{3z_0^2/2(d^2)} \approx \Delta t_c \left( \frac{1 + 3z_0^2}{2(d^2)} \right) \quad (16)
\]

where \( \Delta t_c \) is a time of the order of the typical correla-
tion time of consecutive frames and in the last passages
we have made use of the fact that \( z_0^2/(d^2) \ll 1 \).

Fig. 8 indeed shows that \( \tau_f \) increases linearly with \( N \)
((d^2) \propto N) for the spherical model, according to the pre-
diction (15). It is interesting to observe that the slope
does not seem to depend on the value of the weak attrac-
tive energy \( \epsilon \). This is expected, as rare, long flight
are dominated by the statistical weight of the configu-
ations of the combined NB/linker molecule away from
the wall. The simple calculation leading to eq. (16) also
correctly explains the observed reduced variability of res-
idence times as the linker length is increased (see again
Fig. 8 bottom right panel). However, a closer inspection
reveals that the average duration of rare, long residence
events increases with the linker length \( N \) in the presence
of an attractive interaction between the paratope/NB
system and the wall, even though with a much smaller
slope than for the increase of \( \tau_f \). Overall, we conclude
that the effect of a weak non-specific interaction with the
wall decreases the average duration of rare, long flights
and residence events. It is interesting to observe that the
SBCG model (with a repulsive wall) displays the shortest

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system and the wall, even though with a much smaller
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that the effect of a weak non-specific interaction with the
wall decreases the average duration of rare, long flights
and residence events. It is interesting to observe that the
SBCG model (with a repulsive wall) displays the shortest

duration of rare long flights (Fig. 8, bottom left), even shorter than for the spherical model in the presence of the most attractive wall \( \epsilon = 2.5k_BT \). Moreover, there seems to be an optimum (a pronounced dip) at a linker length that is approximately the same size of the attached NBs \( N = 20 \).

While rare, long flight and residence times are on the \( \mu s \) and tens of ns scales (exponential tails), respectively, the average values \( \langle t_f \rangle \), \( \langle t_r \rangle \) are dominated by the short-time power-law behaviour. Fig. 10 reveals that average flight times turn out to be of the order of about 10\(^2\) ns, while residence times are about 50 times shorter, of the order of 2 – 3 ns. Furthermore, one can appreciate that the SBCG model systematically displays shorter flight and residence times with respect to the spherical model. As for rare events, this feature should be attributed to the shape and intrinsic flexibility of the SBCG NBs as compared to equivalent rigid spheres of the same size.

It is interesting to observe that shorter linker (10-mers) correspond to rather unfavorable situations (large flight times). Remarkably, increasing the linker length from \( N = 10 \) results in a substantial reduction in the duration of the flight stretches, an effect that is more pronounced for the SBCG model (see again Fig. 10 A). A close inspection of the trajectories shows that for the 10-mer linker, for which the linker length is less than the size of the nanobodies, the tethered unit, due to its steric extension, introduces a steep entropic barrier for the free NB when it approaches the wall (see SI [40]). The consequence of such steric repulsion and its more pronounced effects in the case of the 10-mer linkers can also be seen from Fig. 7, where the peak of the \( z \)-distributions of the free paratope (bead P1) are farthest away from that of the simple equivalent Gaussian polymer for the 10-mer, and more so for the SBCG diabody. This effect is present for the SPH model too, albeit less marked. Again, also for average values, there seems to be an optimum length of the linker that minimizes the average time spent by the free NBs away from the tethering surface, that approximately matches the size of the binding units themselves. All the data for \( t_f \) and \( t_r \) for this set of simulations are reported in tabulated form in tables I to V in the SI.

In order to investigate further the details of the NB-wall interactions, we performed the simulations with repulsive tethering walls for two different values of the LJ repulsive length \( s_{\text{wall}} \) (4.5 \( \sigma \) and 3.0 \( \sigma \) for nbd-2 (spherical model)). We observe that for smaller linker lengths (10–30-mers), flight times are shorter for a smaller value.
of \( s_{\text{wall}} \). The effect is negligible for the longer linkers, and seems, in fact, inversely proportional to the linker length. The effect arises because the value of \( s_{\text{wall}} \) determines how nbd-2 (the tethered NB) would interact with the tethering wall and how much it is able to bend. This is expected to affect the value of \( \langle t_f \rangle \), more so in presence of the external harmonic potential that restrains the nanobody, as the shape would then determine the dynamics and effective interaction of the nanobody with the tethering wall.

The bending propensity of the tethered unit can be gauged by calculating the height distribution of CB2 (see Fig. 3) measured from the tethering wall surface for the SPH and the SBCG systems (see SI [40]). From the plot we see that the very low heights have a significant probability in case of the SBCG diabodies, which may contribute to the lower values of \( \langle t_f \rangle \). By contrast, for the SPH diabodies there is an abrupt lower cutoff depending on the value of \( s_{\text{wall}} \). From Fig. 10 we notice that the values of \( \langle t_f \rangle \) for the SBCG diabodies are substantially shorter as compared to the SPH diabodies. For the 30-mer linker, for example, the SPH system has \( \langle t_f \rangle \sim 80 \) ns while for the SBCG system \( \langle t_f \rangle \sim 50 \) ns. It thus seems that it is not an obvious task to reproduce the interaction with a wall within by a model that preserves the shape of the atomistic structure of the NB via a simple spherical representation. Thus the SBCG model seems more appropriate to calculate relevant dynamical parameters, such as the on-rate for second binding, that are expected to rely substantially on the details of the interactions with a wall/membrane.

A closer look at the average residence times reveals that, while \( \langle t_r \rangle \) shows negligible dependence on the linker length, the dependence on the model is noticeable. The residence time for the SPH diabodies is \( \sim 3.5 \) ns, while the values for the SBCG diabodies is close to \( 2 \) ns. The difference can be attributed to the fact that the SBCG nanobody likely generates larger reaction forces from the tethering walls on behalf of its fluctuating structure (see SI [40]), thus reducing the time it stays near the wall. By contrast, the SPH nanobody would face a smaller reaction from the wall, given its rigid and fixed surface. Here again one can appreciate the importance of the model being used.

Finally, to ascertain that the length of the flight-residence times simulations was sufficient to arrive at converged values of \( \langle t_f \rangle \) and \( \langle t_r \rangle \), we performed the calculation for different durations of the simulations, and checked how the calculated values changed as a function of the simulation length. For the 30-mer linker, for example, \( \langle t_f \rangle \) started from a value of \( 85 \pm 22 \) ns for a simulation length of 3.75 \( \mu \)s and slowly converged to the reported value of \( \sim 77 \) ns for a simulation length of 15 \( \mu \)s and stayed close to that for longer simulation lengths, suggesting that our simulation length of 30 \( \mu \)s is appropriate for producing converged results. The values of \( \langle t_f \rangle \) as a function of simulation length for the SPH systems with \( N = 30 \) and \( N = 60 \) linkers are shown in the SI [40].

### III. CONCLUSIONS AND PERSPECTIVES

In this work we have introduced two different coarse-grained models of polymer-linked, two-nanobody molecules, as a simple but paradigmatic example of novel immunotherapy agents that are increasingly being developed in a variety of contexts. More precisely, while the linker has been modeled invariably as a bead-and-spring system (stretching and bending), the nanobodies have been represented either as single large rigid spheres, or as collections of small spheres suitably connected by springs. Such representation was parameterized in a bottom-up philosophy directly from atomistic simulations in explicit solvent. The latter scheme led to binding units displaying the same shape and large-scale flexibility as the atomistic systems.

The aim of this work was to lay the bases of coarse-
grained, computationally aided drug design in this area from the firm standpoint of statistical and computational physics. In the spirit of the accepted model of sequential binding of bivalent molecules [41], whereby bivalent agents first bind to a target-covered surface from the bulk, and subsequently dynamically explore the surroundings for a second target within reach (either on the same or on a facing surface), our main focus was to elucidate the physics of the latter kinetic step. For this purpose, we have mainly focussed on the kinetic and equilibrium properties of a molecule tethered to a wall through one of its binding units (NB), in order to investigate the main dynamical and structural determinants of the second binding. In particular, we aimed at investigating (i) to what extent the degree of coarse-graining may impact the dynamics of the combined linker/free NB system and (ii) the interaction dynamics of the free paratope (the active binding site carried by the NB) with the surface.

In the first part of this work, we have shown that the calculation of potentials of mean force (PMF) along specific, one-dimensional collective coordinates can provide considerable insight into the nature, strength and range of the thermodynamics forces that govern the motion of the free paratope. Furthermore, such calculations may constitute a precious tool to investigate the role of such forces in the dynamics of the paratope-wall encounter, which, in turn, governs the kinetics of the second binding. We aim at illustrating this aspect in a forthcoming publication. For example, the PMF can be fruitfully used as an effective potential in approaches based on the Smoluchowski equation or on first-passage processes [50].

In the second part of the paper, we have delved into the kinetics of the paratope-wall encounters. To this end, we have developed a general strategy based on dissecting an equilibrium trajectory of the free paratope in flight and residence stretches, depending on whether the active site on the free NB was found above or below, respectively, a critical interaction threshold close to the wall in the vertical direction. We have shown that the encounter and escape kinetics with respect to the wall are simply related to the survival probability of the paratope in the flight and interaction domains, respectively, which can be simply computed from the series of flight and residence times observed over a long MD trajectory. We have illustrated how this method allows one to estimate the kinetic constants of the second binding, $k_{on}$ and $k_{off}$, in the presence of a purely repulsive wall and with weak, non-specific interactions between the free NB and the wall. Our simple method not only allows one to quantify the role of factors such as the linker length and flexibility (not considered in this study) and non-specific interactions on the average flight/residence times. It also makes clear and quantify the role and weight of rare, long-duration events that show up in the exponential tails of the survival probabilities.

It is worth stressing that the statistics of rare events is by no means a secondary issue in this context, as in many real-life situations the binding kinetics of such molecules is expected to be dominated by fluctuations, e.g. due to low copy numbers or tiny reaction volumes. For example, this is the case of novel bivalent and bispecific diabodies engineered to bind within the immune synapse, i.e. at the interface of two facing membranes, on the effector cell (NK or B-cell) on one side and on epitopes on a tumor cell on the other side. The synapse covering an area of the order of 100 $\mu$m$^2$ for a cell-cell separation of about 15 nm [24]–[25], the role of fluctuations in the number of bridging molecules is expected to be important, which likely makes the statistics of rare events a major determinant of the binding kinetics.

IV. ACKNOWLEDGEMENT

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[40] See Supplemental Material at [URL] for the details of the system simulated for calculating the flight/residence times, comparison of the interaction force between the tethering wall and nbd-1 for the two models, the radial distribution of the position of P1, Distribution of the normalized height of CB2 from the tethering wall for the 10-mer SPH system (repulsive wall, σwall = 4.5 for nbd-2) and the 10-mer SBCG system, Steric repulsion between nbd-1 and nbd-2 for the 10-mer linker preventing nbd-1 from reaching close to the tethering wall, convergence of the flight time values as a function with simulation length and flight/residence times for different systems in tabulated form.

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Appendix A: Supplementary Information (SI)

1. The angle-bending coefficient for the coarse-grained model of the linker

The bond-bond correlation function (BBCF) for a freely rotating chain is an exponentially decaying function of the monomer-monomer separation along the chain $^{\hbox{[52]}}$,

$$\langle \hat{t}_{i+m} \cdot \hat{t}_i \rangle = a^2 (\cos \theta)^m = a^2 e^{-ma/\ell_p} \quad \hbox{(A1)}$$

where $\ell_p$ is the chain persistence length,

$$\ell_p = -\frac{a}{\log(\cos \theta)} \quad \hbox{(A2)}$$

In eq. (A1), the average is taken over the free rotations about the segment axes (free torsions) and $\theta$ is the angle formed by two successive segments, $\cos \theta = a^{-2} \hat{t}_{i+1} \cdot \hat{t}_i$, $|\hat{t}_i| = a$ (see Fig. 12).

If the angle $\theta$ is no longer fixed but is controlled by a potential energy $V(\theta)$, in the hypothesis that free torsions along $\phi$ and bending are uncoupled degrees of freedom along the chain, the BBCF is still an exponentially decaying function and the persistence length can be computed as

$$\ell_p = -\frac{a}{\log \langle \cos \theta \rangle} \quad \hbox{(A3)}$$

where

$$\langle \cos \theta \rangle = \frac{\int_0^\pi \cos \theta \sin \theta e^{-V(\theta)/k_B T} d\theta}{\int_0^\pi \sin \theta e^{-V(\theta)/k_B T} d\theta} \quad \hbox{(A4)}$$

The calculation can be carried out explicitly for example in the case of the cosine angle potential, $V(\theta) = k_\theta (1 - \cos \theta)$, which reduces to an harmonic bending potential for a semiflexible polymer. From eq. (A4), one gets

$$\langle \cos \theta \rangle = \frac{\alpha - 1 + (\alpha + 1)e^{-2\alpha}}{\alpha(1 - e^{-2\alpha})} \quad \hbox{(A5)}$$

where $\alpha = k_\theta/k_B T$. For PEG the persistence length is $\ell_p = 3.8 \, \text{Å}$ while the monomer length is $a = 1.5 \, \text{Å} \hbox{[41]}$. In our coarse-grained representation the monomer size is 3.5 Å, which would set a lower bound on the angle bending energy $k_\theta \simeq k_B T$. In our simulations we fixed $k_\theta = 1.8 k_B T$, which we used as the bending coefficient for the linker (see Fig. 12 right panel).
FIG. 13. **Simulation for flight/residence time calculation:** Tethered 20-mer SPH diabodies in a $5 \times 5$ lattice. A similar lattice arrangement was used for all other systems (SBCG systems and SBCG systems with all other linker lengths). The distance between neighboring diabodies was increased as the linker length increased to avoid any interaction between neighbors.

FIG. 14. **Force:** Derivative of the PMF profiles as a function of $\rho_{z-proj}$ leading to the force on nbd-1 for a given value of the RC. The SBCG system experiences larger forces near the wall.

FIG. 15. The $xy$ distribution of the paratope position averaged over 25 diabodies with the condition that the paratope be within a distance of $5 \sigma$ from the lower wall of the box for linker lengths of (A) 10, (B) 20, (C) 30, (D) 40 and (E) 50 monomers.
FIG. 16. Distribution of the normalized height of CB2 from the tethering wall for the 10-mer SPH system (repulsive wall, $s_{\text{wall}} = 4.5$ for nbd-2) and the 10-mer SBCG system.

FIG. 17. Distribution of the $z$-coordinate of P1 for (A) 40-, (B) 50- and (C) 60-mer systems along with the fits made using Eq. (8) in the main text with $N_k$ as the free parameter.

| Linker length | Flight time (ns) | Residence time (ns) |
|---------------|------------------|---------------------|
| 10            | $69.50 \pm 1.92$ | $3.24 \pm 0.04$     |
| 20            | $68.88 \pm 1.10$ | $3.37 \pm 0.08$     |
| 30            | $79.18 \pm 3.05$ | $3.39 \pm 0.04$     |
| 40            | $88.26 \pm 2.22$ | $3.45 \pm 0.04$     |
| 50            | $89.38 \pm 6.45$ | $3.42 \pm 0.05$     |
| 60            | $97.45 \pm 6.90$ | $3.47 \pm 0.05$     |
FIG. 18. (Upper panel) Survival probability of the epitope in the flight ($z \geq z_{th}$) domain for the SPH (left) system and (right) SBCG system for different linker lengths. (Lower panel) Survival probability of the epitope in the residence ($z < z_{th}$) domain for the SPH (left) system and (right) SBCG system for different linker lengths. The dashed lines are plots of a power law of the kind $t^{-1/2}$.

FIG. 19. Steric repulsion between nbd-1 and nbd-2 for the 10-mer linker preventing nbd-1 from reaching close to the tethering wall.
TABLE III. Flight/Residence times SPH system, repulsive wall, $s_{wall}$ for nbd-2 = 3.0

| Linker length | Flight time (ns) | Residence time (ns) |
|---------------|-----------------|---------------------|
| 10            | 59.90 ± 0.67    | 2.93 ± 0.07         |
| 20            | 62.96 ± 0.75    | 3.21 ± 0.02         |
| 30            | 73.83 ± 2.50    | 3.34 ± 0.04         |
| 40            | 82.66 ± 3.38    | 3.44 ± 0.04         |
| 50            | 88.20 ± 6.12    | 3.38 ± 0.08         |
| 60            | 94.89 ± 5.30    | 3.49 ± 0.04         |

TABLE IV. Flight/Residence times SPH system, attractive wall, $\epsilon = 1.5k_B T$, $s_{wall}$ for nbd-2 = 4.5

| Linker length | Flight time (ns) | Residence time (ns) |
|---------------|-----------------|---------------------|
| 10            | 42.00 ± 1.53    | 4.76 ± 0.06         |
| 20            | 37.20 ± 1.67    | 4.76 ± 0.03         |
| 30            | 45.50 ± 1.61    | 4.83 ± 0.05         |
| 40            | 48.31 ± 1.24    | 4.81 ± 0.06         |
| 50            | 51.15 ± 0.92    | 4.82 ± 0.06         |
| 60            | 53.84 ± 5.89    | 4.85 ± 0.04         |

TABLE V. Flight/Residence times SPH system, attractive wall, $\epsilon = 2.5k_B T$, $s_{wall}$ for nbd-2 = 4.5

| Linker length | Flight time (ns) | Residence time (ns) |
|---------------|-----------------|---------------------|
| 10            | 26.27 ± 0.70    | 7.52 ± 0.06         |
| 20            | 23.27 ± 0.45    | 7.10 ± 0.06         |
| 30            | 27.89 ± 1.39    | 7.10 ± 0.11         |
| 40            | 29.54 ± 1.56    | 6.90 ± 0.03         |
| 50            | 32.71 ± 1.67    | 7.14 ± 0.14         |
| 60            | 32.06 ± 2.50    | 7.30 ± 0.13         |
TABLE VI. **Flight/Residence times** SBCG system, repulsive wall

| Linker length | Flight time (ns) | Residence time (ns) |
|---------------|------------------|---------------------|
| 10            | 53.72            | 1.90                |
| 20            | 45.03            | 1.83                |
| 30            | 49.50            | 1.86                |