Shape changes resulting from segmental flexibility are ubiquitous in molecular and biological systems [1–4], and are expected to affect both the diffusive motion and (biological) function of dispersed objects [3–6]. The recent development of colloidal structures with freely-jointed bonds [7–10], and flexible chains [11] have now made a direct experimental investigation of diffusive shape-changing objects possible. Here, we show the effect of segmental flexibility on the simplest possible model system: a freely-jointed cluster of three spherical particles. By combining experiments and modelling, we find a 3% enhancement of diffusivity compared to rigid clusters. Unique to flexible assemblies, we find that in addition to the rotational diffusion time, an analogous conformational diffusion time governs the relaxation of the diffusive motion. Additionally, we find a Brownian quasi-scallop mode, where diffusive motion is coupled to Brownian shape changes. Our findings could have implications for transport properties in molecular and biological systems, such as functional site availability in lock-and-key protein interactions [12].

Many (macro)molecular systems display segmental flexibility, e.g. bio-polymers such as transfer RNA [1], intrinsically disordered proteins [2], myosin [1], immunoglobulins [1], and other antibodies [3, 4]. For most of these systems, the flexibility not only affects the motion of the complex but also its (biological) function [3–6]. For example, proteins often function through shape-dependent lock-and-key interactions where active sites of enzymes are reshaped during the interaction, leading to an induced fit [12]. Additionally, enzymes like adenylyl kinase can accelerate biochemical reactions with remarkable specificity and efficacy thanks to a flexible “lid” that opens and closes at each reaction cycle. Because shape has a large effect on the diffusive motion of structures at the short timescales relevant to these reactions, it is expected that the diffusion of re-configurable objects is different from rigid ones [1, 13–15]. Therefore, a rigorous description of these types of processes requires quantitative knowledge of protein flexibility [16].

However, direct experimental measurements of flexibility in molecular systems are challenging because they require single-molecule measurement techniques with high spatial and temporal resolution. One way to circumvent this problem is to employ colloidal particles, which have been used as model systems for (macro)molecular structures [17], because of their unique combination of microscopic size and sensitivity to thermal fluctuations. Studies on the Brownian motion of rigid colloids of various shapes such as ellipsoids [18], boomerangs [19, 20], and clusters [21, 22] have revealed that shape affects the diffusive motion at short timescales. Additionally, displacements are larger in directions that correspond to smaller hydrodynamic drag [18–22] and different diffusive modes can be coupled, e.g. helical particles rotate as they translate and vice-versa [23]. At longer timescales, the influence of particle shape decreases because of rotational diffusion [18].

While rigid assemblies have been extensively studied, little is known about the effect of flexibility. In order to numerically and experimentally investigate the effect of segmental flexibility, we study a simple model system consisting of a freely-jointed chain of three spherical colloidal particles, called flexible trimers or “trumbbells” [24, 25]. Numerical models were proposed to capture the diffusion of segmentally flexible objects [24, 26, 27] and the long time diffusive motion was predicted to be determined by the shape average of the instantaneous diffusivities (so-called rigid-body approximation) [4, 28]. For the first time, we are able to test these models using direct experimental measurements of the diffusion of colloidal particles. These flexible trimers undergo translational and rotational diffusion while they are also free to change their shape (see Fig. 1a). The trimers are made by self-assembly of colloid supported lipid bilayers [7–9]. Briefly, spherical colloidal silica particles are coated with a fluid lipid bilayer. DNA linkers with complementary sticky ends are inserted into the bilayer using a hydrophobic bilayer. The particles are self-assembled by hybridization of the DNA sticky ends, which provide strong and specific interactions. The trimers are freely-jointed because the DNA linkers can diffuse on the fluid lipid bilayer that surrounds the particles (see Fig. 1b). For simplicity, we used heavy silica particles so that their mobility is confined to the bottom of the container by gravity, which leads to two-dimensional
Diffusion of flexible trimers

An overlay of bright-field microscopy images of a flexible trimer with the position of its center of mass as a function of time. Schematic (not to scale) of flexible trimers that are self-assembled from colloid supported lipid bilayers. We inserted DNA linkers into the fluid lipid bilayer surrounding the particle, resulting in bonded particles that can rearrange with respect to each other. Illustration of the co-moving coordinate system. The mean squared displacement of rigid and flexible trimers shown on a log-log plot. The translational mean squared displacement of flexible trimers in the y-direction is angle dependent for short lag times, at longer lag times this angle dependence is no longer present due to rotational and conformational relaxation, which happens on a shorter timescale than for rigid trimers (raw data, not scaled with friction coefficients).

For rigid objects in two dimensions, the diffusive motion can be described by a $3 \times 3$ diffusion tensor calculated from the linear increase of the mean squared displacements of the particle as function of lag time [29]. For flexible objects, this diffusion tensor has to be extended with an additional degree of freedom [24] for each internal deformation mode (here: one), and we therefore consider the $4 \times 4$ diffusion tensor $D[i,j]$. Here, $i,j \in [x,y,\alpha,\theta]$ are elements of a co-moving coordinate system (see Fig. 1c) at the center of mass. We chose the center of mass as reference point, because for flexible objects, it is more appropriate than either the center of diffusion or resistance of a rigid cluster of the same shape [24]. In this coordinate system, the y-axis is perpendicular to the end-to-end vector and points away from the central particle, the direction of the x-axis is chosen to form a right-handed coordinate system. We label the opening angle of the trimer $\theta$ and the (anti-clockwise) rotation angle of the x-axis with respect to the lab frame (x'-axis) $\alpha$.

Shape determines the diffusion tensor for rigid objects and therefore we expect it to be important for flexible objects as well, but due to its flexibility, the cluster shape is continuously changing. Therefore, we categorize the trajectories by their (initial) average opening angle $\bar{\theta}$ of the smallest lag time interval and we use angular bins to summarize the results. The short-time diffusion tensor is calculated from experimental measurements in the following way:

\[
D[i,j](\bar{\theta}) = \frac{1}{2} \phi_{ij} \frac{\partial (\Delta i \Delta j)}{\partial \tau},
\]

with $\tau$ the lag time between frames, $\langle \cdots \rangle_\tau$ denotes a time average over all pairs of frames $\tau$ apart and $\Delta i = i(t + \tau) - i(t)$, $\phi_{ij}$ is a correction factor that accounts for particle-particle and particle-substrate friction (see Methods section) which for translational diffusion agrees closely with predictions from hydrodynamic theory (see the Supplementary Information). We evaluated equation 1 at $\tau = 0.2s$, set by the frame rate of our camera.

Using equation (1), the resulting shape and time dependent translational diffusivity in the y-direction of twelve rigid and one flexible trimer are shown in Fig. 1d). Initially, at short timescales, there is a clear effect of cluster shape for both flexible and rigid trimers: translational diffusion in y is highest for compact shapes. In comparison to rigid trimers, the diffusivity of the flexible trimer is slightly enhanced. Two other features unique to flexible clusters are that using a measurement of only one cluster, all possible cluster shapes are sampled and the effect of shape vanishes on a much shorter timescale compared to the rigid clusters.

To study the diffusivity more carefully, we determined the average short time diffusion tensor of thirty flexible trimers. As shown in Fig. 2a, the diffusion tensor elements were obtained by fitting the slope of the mean squared displacement versus lag time. We find that for short times, translational diffusivity is higher along the longitudinal $x$ direction compared to the lateral $y$ direction (Fig. 2d). Additionally, the rotational diffusivity shown in Fig. 2b) is higher for compact trimers as opposed to fully extended trimers and we observe a coupling between translational diffusion and rotational diffusion in the $x$ di-
Fig. 2: Short-time translational, rotational, internal and coupled diffusivity of flexible trimers (up to 0.2 s). 

a) Mean squared rotational displacements for lag times up to $\tau = 0.25$ s, for two different instantaneous opening angles $\bar{\theta}$. b) The rotational diffusivity is highest for the most compact shapes. c) The joint flexibility increases as function of opening angle $\theta$. d) While equal for flexed trimers, the translational diffusivity along the long axis ($x$) is higher than along the short axis ($y$). e) We find a correlation between counter-clockwise rotation and positive $x$ displacements. f) There is a coupling between translational diffusion in the $y$-direction and shape changes: as the cluster diffuses in the positive $y$-direction, the angle $\theta$ increases, leading to a Brownian scallop-like motion at short timescales. In panels b-f, the scatter points show the experimental measurements and the lines show the numerical calculations based on [24].

rection (Fig. 2e). These findings are in line with previous studies on rigid trimers [21].

However, flexibility gives rise to other modes that are not present in rigid assemblies. We found that the flexibility itself, as shown in Fig. 2c, increases as function of the opening angle, leading to a four fold increase of flexibility for extended shapes compared to closed shapes. It is most likely caused by hydrodynamic interactions between the outer particles, as was predicted by earlier works [26].

Even more strikingly, the hydrodynamic drag on the outer particles leads to an increase in opening angle $\theta$ for positive displacements along the $y$ axis (Fig. 2f), which we call the Brownian quasi-scallop mode. We stress that this correlation does not lead to self-propulsion because it has time reversal symmetry. As the opening angle $\theta$ increases, the location of the center of mass moves in the negative $y$-direction of the original particle coordinate system. Therefore, this correlation is larger when the central particle is chosen as the origin of the coordinate center (see the Supplementary Information). This Brownian quasi-scallop mode may have implications for the accessibility of the functional side in induced fit lock-and-key interactions commonly observed in proteins [12].

To corroborate our experimental findings, we have modelled the diffusion of the flexible trimers using a segmentally flexible bead-shell model that includes hydrodynamic interactions [24] (see the Methods and the Supplementary Information for details). We find a good agreement between the numerical calculations and the experimental data. For large opening angles, the differences between model and data are moderately larger for translational diffusion, the Brownian quasi-scallop mode and the flexibility (see Fig. 2c, d and f). We hypothesize that these larger differences may arise because the numerical calculations do not take particle-particle and particle-substrate friction into account. Moreover, our model also does not account for some out-of-plane diffusive motions against gravity, that might occur in the experiments. Both effects are beyond the scope of our current work.

Next, we compared the diffusion of flexible trimers to rigid trimers that are frozen in a particular shape. We find that the average short time diffusion constant of rigid trimers is approximately 3% lower (15% lower without friction scaling) than that of flexible trimers (Fig. 3a, b). The ro-
tional diffusion constants for flexible and rigid trimers are equal (Fig. 3c, d), while the rotation-translation coupling mode between $x$ and $\alpha$ is slightly higher for flexible trimers at the shortest lag time (Fig. 3e, f). These findings are consistent with numerical predictions [13–15] for hinged chains of spheres of higher aspect ratio (20:1 instead of 3:1 for the trimers). For these hinged rods, a 10% increase in the translational diffusivity and a higher rotational diffusivity were found compared to rigid rods, which was attributed to hydrodynamic interactions between the sub-units [1]. The lower aspect ratio of our trimers results in larger hydrodynamic interactions between the two arms [24]. An increase in the hydrodynamic interactions between the arms was found to increase the translational diffusion because of shielding effects, but decrease the rotational diffusivity [24, 30]. This may explain why we have measured an increase in translational diffusion and translation-rotation coupling, but only a slight enhancement of the rotational diffusivity for the flexible trimers.

The last way in which flexibility affects the diffusivity of a cluster is through the timescales on which the effect of the initial cluster shape vanishes and translational diffusivity in the $x$ and $y$ directions become equal. For rigid clusters, this timescale is set by the rotational diffusion time $\gamma_r = (D[\alpha \alpha])^{-1}$, with $D[\alpha \alpha]$ in rad$^2$/s [18]. Using the values for the short time rotational diffusion coefficients for compact and extended trimers, we find that for both rigid and flexible trimers $30 \text{s} \leq \gamma_r \leq 60 \text{s}$. Indeed, by looking at the translational (Fig. 3b) diffusivity of rigid trimers, we see that the effect of shape on the diffusivity is preserved up to the maximum lag time we consider (10 s). Additionally, the rotational diffusivity (Fig. 3d) of the rigid trimers stays constant within error (up to at least 10 s).

However, for flexible trimers, the story is different. The effect of the initial opening angle is lost on a much shorter timescale than what one would expect from the rotational diffusion time. As can be seen from the translational diffusivity (Fig. 3b), the effect of the initial shape has vanished around 0.5 s while the diffusivity in $x$ and $y$ become equal after 1 s to 3 s. Similarly, for rotational diffusion (Fig. 3d), the effect of the initial shape is lost after 1 s to 2 s and this is also the timescale on which the translation-rotation coupli
The flexibility decreases as function of lag time because of hard-sphere repulsion between the two outer particles. c) Mean squared coupled displacements of $\theta$ for all flexible trimers. d) The Brownian quasi-scallop mode relaxes on a timescale of a few seconds because of conformational and rotational diffusion. In all panels, $\langle \cdot \cdot \cdot \rangle_c$ are the results for $\theta < 120^\circ$ (compact) and $\langle \cdot \cdot \cdot \rangle_e$ for $\theta \geq 120^\circ$ (extended). In panels a and c, the coloured points are the experimental data, the black points and lines represent the fitted slopes. In panels b and d, dotted lines indicate the minimum and maximum short-time diffusion coefficients obtained from the numerical models.

To explain this behavior, we define a timescale for the shape changes as $\gamma_s = (D[\theta \theta])^{-1}$, analogous to the definition of the rotational diffusion time. Using the values for the short time flexibility coefficients for compact and extended trimers, we find that for our flexible trimers $8 \, \text{s} \leq \gamma_s \leq 35 \, \text{s}$. Therefore, we hypothesize that for flexible trimers, internal deformations lead to faster relaxation of the shape-dependency we observe at short lag times and therefore also the relaxation of differences between translational diffusion in the $x$ and $y$ directions. We observe that for both translational and rotational diffusion, for lag times larger than 2 s, the effect of initial opening angle has vanished and the spread in diffusivities is at its lowest (see Fig. 3b, d). At longer lag times, due to continuous shape changes, the spread in the diffusivities increases again.

Short timescale relaxation of differences between clusters in extended and compact conformations exist also for the conformational diffusion tensor elements. The flexibility (shown in Fig. 4a, b) is smaller for trimers in flexed conformations than in extended conformations and the difference vanishes after approximately 2 s due to shape changes. Fig. 4b shows an overall decrease of flexibility with lag time, because the range wherein the joint angle can vary is bounded by the two outermost particles. Furthermore, the magnitude of $D[y \theta]$ (shown in Fig. 4c, d), which represents the Brownian quasi-scallop mode, vanishes on the same timescale of approximately 2 s, set by the conformational relaxation time $8 \, \text{s} \leq \gamma_s \leq 35 \, \text{s}$.

In conclusion, we studied the Brownian motion of flexible trimers and found features that are unique to flexible objects. We found a hydrodynamic coupling between conformational changes and translations perpendicular to the particle’s long axis ($y$-direction), which we call the Brownian quasi-scallop mode because of its resemblance to scallop propulsion at high Reynolds numbers. We found that this coupling persists over several seconds, a timescale relevant for biomolecular interactions, implying that it might affect the association of flexible proteins and other biomolecules. Secondly, we found that the long-time translational diffusion of the freely jointed trimers was three to fifteen per cent higher than that of their rigid counterparts, which may be important for experiments that rely on precise estimates of diffusion of flexible objects. Finally, we showed that the transition from short- to long-time diffusion depends not (only) on the rotational diffusion time but mainly on a timescale related to conformational changes of the particle. We were able to describe our experimental findings using a hydrodynamic modelling procedure that combines bead-shell modelling with the approach of Harvey and coworkers [24]. We hope this work inspires other researchers to more confidently apply this method in the context of the diffusion of segmentally flexible systems such as biopolymers and proteins.

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Author contributions

RWV and PM contributed equally to the work. PM, RWV, NL, LPPH performed the experiments. PM and RWV analysed the data. RWV performed the hydrodynamic modelling. PM, RWV, JG, WKK, AvB and DJK conceived of the experiments. PM, RWV, WKK, AvB
and DJK wrote the manuscript. All authors discussed the results and contributed to the final article.

Conflicts of interest
There are no conflicts to declare.

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Methods

Experimental Flexible clusters of three colloidal supported lipid bilayers (CSLBs) were prepared as described in previous work [7–9, i]. To test the generality of the results presented here, we used two particle sizes, namely 1.93 µm and 2.12 µm silica particles, with different methods of functionalization.

The CSLBs consisting of 2.12 µm silica particles were prepared as described in our recent work [9]. Briefly, the particles were coated with a fluid lipid bilayer by deposition of small unilamellar vesicles consisting of 98.8 mol% DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), 1 mol% DOPE-PEG(2000) (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N[(methoxy(polyethylene glycol))-2000]) and 0.2 mol% TopFluor-Cholesterol (3-(dipyrromethenonon difluoride)-24-norcholesterol) or DOPE-Rhodamine (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamerchondamine B sulfonyl)). The bilayer coating was performed in a buffer at pH 7.4 containing 50 mM sodium chloride (NaCl) and 10 mM 4-(2-Hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES). We added double-stranded DNA (of respectively strands DS-H-A and DS-H-B, see the Supplementary Information) with an 11 base pair long sticky end and a double stearyl anchor, which inserts itself into the bilayer via hydrophobic interactions (see Fig. 1b). When two particles with complementary DNA linkers come into contact, the sticky ends hybridize and a bond is formed. Self-assembly experiments were performed in a different buffer of pH 7.4, containing 200 mM NaCl and 10 mM HEPES. We imaged 21 trimers of 2.12 µm CSLBs, that were formed by self-assembly in a sample holder made of polyacrylamide (PAA) coated cover glass. The PAA functionalization was carried out using a protocol [ii] which we modified by adding 0.008 mol % bis-acrylamide and performing the coating under a nitrogen atmosphere, both of which resulted in a more stable coating. Using an optical microscope, we imaged the clusters for 5 min at frame rates between 5 fps to 10 fps. Particle positions were tracked using a custom algorithm [9] available in TrackPy by using the locate brightfield ring function [iii].

Additionally, we analysed 9 trimers of 1.93 µm CSLBs, with silica particles purchased from Microparticles GmbH (product code SiO2−R-B1072). For these particles, we used a similar protocol to form supported lipid bilayers with only 2 minor modifications: First, the lipid composition we used was 98.9 mol% DOPC, 1 mol% DOPE-PEG(2000) and 0.1 mol% DOPE-Rhodamine. Second, we added Cy3-labeled DNA with a self-complementary 12 base pair sticky end and a cholesterol anchor that inserts itself into the lipid bilayer due to hydrophobic interactions. We used the DNA sequence from Leunissen et al. [iv](see Supplementary Material, PA-A and PA-B).

To image the 1.93 µm CSLBs we used a flow cell produced as detailed in the Supplementary of Montanarella et al.[v] As the base of our flow cell we used a single capillary with dimensions 3 cm × 2 mm × 200 µm. To prevent the lipid coated clusters from sticking to the class capillary, we coated the inside of the capillary with polyhydroxyethylamine (pHEA) polymers. To this end, we first flushed the cell with consecutively 2 mL 2 mM NaOH solution, 2 mL water and 2 mL EtOH. We then functionalized the glass surface with the silane coupling agent 3-(methoxysilyl)propyl methacrylate (TPM) by filling the flow cell with a mixture of 1 mL EtOH, 25 µL TPM, and 5 µL 25 vol% NH3 in water and leaving it for 1 hour. We then washed and dried the flow cell by flushing with 2 mL ethanol and subsequently with Nitrogen. We grew pHEA brushes from the surface through a radical polymerization by filling the cell with a mixture of 2.5 mL EtOH, 500 µL HEA and 20 µL Darocur 1173 photoinitiator. We initiated the reaction by placing the cell under a UV lamp λ = 360 nm for 10 minutes. Finally, we flushed the cells with 10 mL EtOH or Millipore filtered water. We stored the coated cells filled with EtOH or Millipore filtered water and for no more than 1 day. Self-assembly experiments were performed in a buffer of pH 7.4, containing 50 mM NaCl and 10 mM HEPES. We imaged 9 freely-jointed trimers and 13 rigid trimers stuck in various opening angles shown in the Supplementary Information for 30 minutes with a frame rate of 5 frames per second. Particle positions were tracked using the 2007 Matlab implementation by Blair and Dufresne of the Crocker and Grier tracking code. [vi]

For all analysis, we only selected trimers that showed all bond angles during the measurement time, experienced no drift and were not stuck to the substrate. After the particle positions were tracked, we determined the short-time diffusivity of the trimers as described by equation (1).

The three friction correction factors that account for substrate friction were determined in the following way:

\[
\phi_{tt} = \langle D[tt]_t/(\sigma_e D[tt]_t,0) \rangle \\
\phi_{(\alpha\alpha,\theta\theta)} = \langle D[(\alpha\alpha,\theta\theta)]_t/(\sigma_e^2 D[(\alpha\alpha,\theta\theta)]_t,0) \rangle \\
\phi_{ij} = \sqrt{\phi_{it}\phi_{jt}} \quad \text{for} \quad i \neq j, \quad (2)
\]

where \( D[ij]_k \) denotes the theoretical \((k = t)\) or experimental \((k = e)\) diffusion tensor element and \( \sigma_e \) the experi-
mental particle radius. The subscript $tt$ denotes the translational component of the diffusivity. These factors were determined separately for each experiment, because differences in surface and particle functionalisations resulted in differences in substrate-particle and particle-particle friction, that in turn affect the diffusivity of the cluster. We separated the correction factors into these three factors because different modes of diffusion are expected to lead to different amounts of friction with the substrate [vii]. We calculated the elements of the diffusion tensor given in equation (1) separately for all trimers. For each pair of frames, we determined the initial average opening angle $\bar{\theta}$ of the trimer between $t$ and $t + \tau_{\text{short}}$, with $\tau_{\text{short}} = 0.25$ s. Then, we stored the diffusion tensor elements separately for each initial opening angle. For short times up to $\tau_{\text{short}} = 0.25$ s, we used a bin size of 15° while for longer times, we used two bins of 60° covering the range of [60°, 120°] and [120°, 180°]. We scaled each element with the friction factors we obtained for that measurement, based on the diffusion coefficient for lag times up to $\tau_{\text{short}}$. The average diffusion tensor elements were then obtained by fitting the overall slope of the mean (squared) displacements of all the individual diffusion tensor elements as a function of lag time (see Fig. 3a, c, e and Fig. 4a, c). We used a linear function (with zero intercept) divided into ten segments with slopes $2D_i$ (spaced evenly on a log scale), which correspond to the $i$th diffusion coefficient for those lag times. This resulted in the average diffusion tensor for all binned average opening angles $\bar{\theta}$ as a function of the lag time $\tau$. For fitting, we used a standard least squares method and we estimated the error using a Bayesian method to find an estimate of the posterior probability distribution, by using a Markov chain Monte Carlo (MCMC) approach as implemented in the Python packages lmfit [viii] and emcee [ix]. We estimated the autocorrelation time $\tau_{\text{acor}}$ of the chain using the built-in methods and ran the analysis for at least $100\tau_{\text{acor}}$ steps, where we discarded the first 2$\tau_{\text{acor}}$ steps (corresponding to a burnin phase) and subsequently used every other $\tau_{\text{acor}}/2$ steps (known as thinning). The reported values correspond to the maximum likelihood estimate of the resulting MCMC chain, the reported uncertainties correspond to the minimum and maximum of the obtained posterior probability distribution.

**Hydrodynamic modelling**  The diffusion of segmentally flexible objects can be described using hydrodynamic modelling [24, x]. To compare our experimental results to these predictions, we followed the procedure described by Harvey and coworkers. Of the seven degrees of freedom in three dimensions (three translational, three rotational, one internal degree of freedom) [24], we considered only the four degrees of freedom of interest for our quasi-two dimensional system of sedimented clusters. Briefly, following the method outlined by Harvey and coworkers [24], we determined the hydrodynamic resistance (or friction) tensor $R_0$ with respect to the central particle. Using this resistance tensor, we calculated the diffusion tensor $D_0 = kT R_0^{-1}$, to which we apply the appropriate coordinate transformation to obtain the $7 \times 7$ diffusion tensor $D_{\text{com}}$ relative to the center of mass of the cluster. We chose the center of mass as reference point because this is the best approximation of the center of diffusion of a flexible particle: in fact, it was found to be a better choice than either the center of diffusion or resistance of a rigid cluster of the same shape [24]. We have also calculated the diffusion tensor with respect to the central particle and these results are shown in the Supplementary Information. The diffusivity of flexible colloidal clusters can be modelled using bead or bead-shell models [xi] and we employed both methods. For the bead model, we modelled the trimer using three beads (diameter of 2 μm) and for the bead-shell model, we modelled the trimer using approximately 2500 to 9500 smaller beads with bead radii from 54 nm to 31 nm respectively, where the beads where placed to form three 2 μm shells. We followed existing methods [xii, xiii] for constructing the bead shell model: to summarize, the positions of the small beads were calculated by placing them on concentric circles, starting at the equator of an individual 2 μm sphere and continuing the process towards the poles of the sphere using circles of decreasing radius and finally putting one sphere at each of the poles. Three spherical bead-shell models were then put together to form a trimer and we removed overlapping beads at the contact points between the particles. Examples of the model are shown in the Supplementary Information.

Because drag forces act on the surface of the particles, the bead-shell model is more accurate in describing the diffusive properties of the clusters [28, xii, xiii]. The accurate consideration of hydrodynamic effects was found to be important for the segmentally flexible system we study: hydrodynamic interactions lead to a slower decay of the auto-correlation of the particle shape [xiv] and lead to an increase in the translational diffusivity [1, 4]. We compare our experimental data to such a bead-shell model because it describes our experimental data more accurately than the simple bead model, which is discussed in the Supplementary Information.

To calculate the diffusion tensor elements, we used the Rotne-Prager-Yamakawa (RPY) [xv, xvi] interaction tensor $T_{ij}$ to model hydrodynamic interactions between particles $i$ and $j$:

$$T_{ij} = \frac{1}{8\pi \eta_0 R_{ij}} \left[ I + \frac{R_{ij} R_{jj}}{R_{ij}^2} + \frac{2\sigma^2}{R_{ij}^2} \left( I - \frac{R_{ij} R_{ij}}{R_{ij}^2} \right) \right],$$

(3)

where $\sigma$ is the particle radius, $R_{ij}$ is the vector between particles $i$ and $j$, $I$ is the $3 \times 3$ identity matrix, $\eta_0$ is the viscosity of the medium. Using the RPY tensor prevents singularities that may lead to the large, non-physical numerical fluctuations [xvii] found when using lower order terms (Oseen tensor), higher order terms or multi-body effects [xviii].
We used the RPY tensor to model the hydrodynamic interactions between the beads and followed the procedure outlined by Harvey and coworkers [24] to obtain the diffusion tensor, as explained in the main text. This was done for all small bead radii and we used a linear extrapolation to zero bead size to obtain the final diffusion tensor elements [xii, xiii]. Additionally, we used HydroSub [28] to model the diffusivity of rigid trimers of the same opening angles.

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Supplementary information for: Brownian motion of freely jointed colloidal trimers

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A Modelling and analysis of the hydrodynamic properties of flexible trimers

We used three different models to describe the hydrodynamic properties of the flexible trimers: a bead model (Figure S1 a), a bead-shell model for a rigid trimer using HydroSub [3] (Figure S1 b) and a bead-shell model for flexible trimers (Figure S1 c). For the bead-shell models, the results were evaluated for multiple small bead sizes, so that the result could be linearly extrapolated [11, 12] to the limit where the small bead radius approaches zero (see the Methods in the main text for details).

In Figure S1 d, the calculated diffusivities are shown for all three models. The bead model predicts higher diffusivities compared to both bead-shell models for all different elements of the diffusion tensor. The bead-shell models agree qualitatively, but predict different magnitudes of the diffusivities due to differences in hydrodynamic interactions between the outer beads, which are higher for the flexibly linked clusters [5, 13–15]. We have used the bead-shell model of Figure S1 c (solid line) to model our experimental data, because it best describes our experimental data and because it can be used to model conformational changes, which are not yet implemented in the HydroSub model.

For our results described in the main text, we used the center of mass as reference point. For purely rotational and conformational terms, the diffusivity is expected to be independent of the chosen reference point, however, for terms that include translation, the location of the reference point has a large effect on the measured diffusivity [10, 16]. This can be seen in Figure S2: in panels a-c), we show the diffusivities calculated using the central particle as reference point. The results are remarkably different from the center of mass based results shown in Figure S2 d-f), where we have used the diffusivities relative to the central particle to calculate the diffusivities relative to the center of mass using the coordinate transformations determined by Harvey and coworkers:[10]

\[
D_{CM}^{tt} = D_{0}^{tt} + D^{(2)} \cdot W + W^{T} \cdot D_{0}^{tt} \\
- U \cdot D^{(2)} \cdot W + W^{T} \cdot D_{0}^{tt} \cdot U + W^{T} \cdot D_{0}^{tt} \cdot U \\
D_{CM}^{\alpha} = D_{0}^{\alpha} + D^{(2)} \cdot U + D_{0}^{(2)} \cdot W \\
D_{CM}^{\theta} = D_{0}^{\theta} + D^{(2)} \cdot U + D_{0}^{(2)} \cdot W
\]

We have made this comparison because the coupling terms are expected to be larger in the central particle frame. The results indeed show this larger coupling and exclude the possibility that the coupling modes we observed are artifacts of the coordinate system we used. Because the rotational and conformational diffusivities are independent of the reference point, localization uncertainties in the determination of the position of the reference point may have a larger effect on \(D_{xx, yy, xy, xo, yo, xo} \) than on \(D_{2, \theta^2, \alpha^2} \). Because of the uncertainties that are propagated when we first determine the diffusivity with respect to the central particle and then transform this to the diffusivity with respect to the center of mass (in Figure S2 d-f), the error is larger for this method compared to the direct calculation of the diffusivities with respect to the center of mass.
Supplementary Figure S1: **Comparison of the hydrodynamic models used.** Renderings made using FreeWRL [4] of **a)** the simple bead model, **b)** the bead-shell model (minimum radius of the small spheres $r = 55$ nm) used by HydroSub [3] for rigid trimers, **c)** the bead-shell model (radius of the small spheres $r = 31$ nm to 54 nm, $r = 45$ nm is shown) we used for calculating hydrodynamic properties of flexible trimers. For all models, the radius of the large particles is $R = 1$ µm. **d)** Top row, left to right: the translational diffusivity, rotational diffusivity and coupling between translational and rotational diffusivity for the bead model (a, dotted lines), the rigid bead-shell model generated with HydroSub (b, dashed lines) and the segmentally flexible bead-shell model (c, solid lines). Bottom row, left to right: the joint flexibility, coupling between shape changes and rotation and couplings between shape changes and translational diffusion.
Supplementary Figure S2: Influence of tracking point. a-c) The translational (a), translational-rotational (b) and translational-conformational (c) diffusivities with the reference point chosen in the center of the central particle. d-f) The translational (d), translational-rotational (e) and translational-conformational (f) diffusivities with the reference point at the center of mass of the cluster. For these graphs, we transformed the data from panels a-c using the coordinate transformation described in the text from the “center particle”-based to the “center of mass”-based diffusivity. Note that the combination of experimental errors of the $D[t\alpha]$, $D[\alpha^2]$, $D[\theta^2]$ and $D[\alpha\theta]$ terms lead to large uncertainties and deviations, especially for the translational diffusivities. In all panels, the points show the experimental data and the lines are the predictions of the bead-shell model.
B  Near-wall diffusion: friction factors

Supplementary Figure S3: Distribution of friction factors as given by equation (3) of the main text. The mean value for $\phi_{tt} = 0.29 \pm 0.04$ is close to the lower bound of 0.39 (indicated by the dotted line) as predicted by Equation 4. We find an average rotational friction factor $\phi_{\alpha\alpha} = 0.55 \pm 0.07$. The average flexibility friction factor $\phi_{\theta\theta} = 0.40 \pm 0.12$ shows a broader distribution, which we attribute to a spread in DNA linker concentration. The average friction factor of the rigid clusters is also indicated ($\phi_{tt,r} = 0.254 \pm 0.004, \phi_{\alpha\alpha,r} = 0.49 \pm 0.02$) and coincides with the friction factors we find for flexible clusters.

As a first approximation to compare the experimental diffusion of freely-jointed trimers above a substrate to models of trimers diffusing in the bulk, we use Faxen’s theorem: 

$$ \frac{D_w(h)}{D_0} = 1 - \frac{9}{16} \frac{R}{h + R} + \frac{1}{8} \left( \frac{R}{h + R} \right)^3 - \frac{45}{256} \left( \frac{R}{h + R} \right)^4 + O \left( \left( \frac{R}{h + R} \right)^5 \right), $$

(4)

with $D_0$ the translational diffusion coefficient in the bulk, $D_w(h)$ the in-plane translational diffusion coefficient near a wall at height $h$ and $R$ the particle radius. We calculate an effective particle radius $R_{eff} = \frac{k_B T}{6\pi \eta D} = 1.8 \mu m$ from the short-time translational diffusion coefficient [9], with $k_B$ Boltzmann’s constant, $T$ the temperature, $\eta$ the viscosity of the medium and $D = 0.136 \mu m^2 s^{-1}$ the lowest short-time translational diffusion coefficient of the trimer as predicted by the bead-shell model.

The expected Debye length of our medium (at $I = 200 \text{mM}$) is $\kappa^{-1} = \frac{0.304}{\sqrt{T}} \approx 0.7 \text{nm}$ [8], and so we neglect electrostatic interactions between the trimer and substrate. Therefore, the height of the particle above the substrate is set by balancing the effect of sedimentation and thermal fluctuations as expressed by the gravitational length $l_g = \frac{k_B T}{\eta \Delta \rho V}$, with $g$ the gravitational acceleration constant, $\Delta \rho$ the density difference between the particle and the medium and $V$ the volume of the particle.

Using the appropriate values for the trimer, we find $l_g = 20 \text{nm}$. By setting this as input for $h$ in Equation 4, we obtain a upper bound for $\frac{D_w(h)}{D_0}$, equal to 0.40. A lower bound is found at $h = 0$, which gives a value of 0.39. The translational friction coefficient $\frac{D_w(h)}{D_0}$ that we find has an average value of $0.29 \pm 0.04$, as shown in Figure S3, which is close to the lower bound we have calculated above. The experimental value is slightly lower than the predicted lower bound, because Equation 4 accounts for hydrodynamic interactions only and real experiments typically show lower diffusivities because of additional sources of friction [7], which in the present case could be explained by additional friction between the polymer coating and the particles.
Supplementary Figures

Supplementary Figure S4: **Freely-jointed trimers.** Probability and corresponding free energy of the opening angle of the flexible trimers used in this work (with the reference set at 180°). There is no preference for a specific opening angle within the experimental error, meaning the particles are freely-jointed, as was shown before. [1] Note that the slightly lower probability at angles smaller than $60^\circ + \sqrt{2J\tau} \approx 69^\circ$ (with $J$ the joint flexibility and $\tau$ the sampling interval) is caused by boundary effects inherent to our analysis method. [1]

Supplementary Figure S5: **Opening angles of rigid trimers.** The number of rigid clusters per opening angle used in this study. Six rigid trimers have a ‘compact’ opening angle (below 120°) while the other six are more extended.
Supplementary Figure S6: **Short-time diffusion of rigid trimers.** The (a) translational, (b) rotational and (b) translational-rotational diffusivities of rigid trimers with various opening angles (see Figure S5). In all panels, the points correspond to the experimental diffusivities (up to lag times of 0.25 s) and the solid lines correspond to the numerical calculations performed using HydroSub [3]. All points are scaled by the same average friction factor as shown in Figure S3 in order to compare the experiments to the numerical simulations.
### Table S1: Overview of all DNA strands used

Sticky ends are marked in cursive.

| Identifier | Sequence |
|------------|----------|
| DS-B       | 5′-TCG-TAA-GGC-AGG-GCT-CTC-TAG-ACA-GGG-CTC-TCT-GAA-TGT-GAC-TGT-GCG-AAG-GTG-ACT- |
|            | -GTG-CGA-AGG-GTA-GCG-ATT-TT-3′ |
| DS-S-A     | Double Stearyl-HEG-5′-TT-TAT-CGC-TAC-CCT-TCG-CAC-AGT-CAC-CTT-CGC-ACA-GTC-ACA-TTC- |
|            | -AGA-GAG-CCC-TGT-CTA-GAG-AGC-CCT-GGC-TTA-CGA-GTA-GAA-GTA-GG-3′-6FAM |
| DS-S-B     | Double Stearyl-HEG-5′-TT-TAT-CGC-TAC-CCT-TCG-CAC-AGT-CAC-CTT-CGC-ACA-GTC-ACA-TTC- |
|            | -AGA-GAG-CCC-TGT-CTA-GAG-AGC-CCT-GGC-TTA-CGA-CCT-ACT-TCT-AC-3′-Cy3 |
| PA-A       | Cholesterol-5′-TTT-ATC-GCT-CCC-TTC-GCA-CAG-TCA-ATC-TAG-AGA-GCC-CCT-TAC-GAT- |
|            | -ATT-GTA-CAA-TA-3′-Cy3 |
| PA-B       | Cholesterol-5′-CGT-AAG-GCA-GGG-CTC-TCT-AGA-TTG-ACT-GTG-AGA-GTA-GCG-ATT-TT-3′ |
| DS-H-A     | Obtained by hybridization of DS-B and DS-S-A |
| DS-H-B     | Obtained by hybridization of DS-B and DS-S-B |
| PA-C       | Obtained by hybridization of PA-A and PA-B |
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