Measuring the Kinetics of Biomolecular Recognition with Magnetic Colloids

L. Cohen-Tannoudji,[1,2] E. Bertrand,[1,2,*] J Baudry,[1,2] C. Robic,[1,2] C. Goubault,[1,2] M. Pellissier,[1,2] A. Johner,[3] F. Thalmann,[3] N. K Lee,[4] C. M. Marques,[3] and J. Bibette[1,2]

[1]ESPCI, UMR 7612, Laboratoire Colloïdes et Matériaux Divisés, ParisTech, 10 rue Vauquelin, Paris, F-75005 France
[2]Université Pierre et Marie Curie-Paris 6, UMR 7612, LI2C, 4 place Jussieu, Paris, F-75005 France";
CNRS, UMR 7612, LI2C, Paris, F-75005 France
[3]Institut Charles Sadron, UPR 22 CNRS ULP, 6 rue Boussingault, Strasbourg Cedex, F-67083, France
[4]Department of Physics, Sejong University, Seoul 143-743, South Korea
(Received 24 April 2007; published 10 March 2008)

We introduce a general methodology based on magnetic colloids to study the recognition kinetics of tethered biomolecules. Access to the full kinetics of the reaction is provided by an explicit measure of the time evolution of the reactant densities. Binding between a single ligand and its complementary receptor is here limited by the colloidal rotational diffusion. It occurs within a binding distance that can be extracted by a reaction-diffusion theory that properly accounts for the rotational Brownian dynamics. Our reaction geometry allows us to probe a large diversity of bioadhesive molecules and tethers, thus providing a quantitative guidance for designing more efficient reactive biomimetic surfaces, as required for diagnostic, therapeutic, and tissue engineering techniques.

DOI: 10.1103/PhysRevLett.100.108301 PACS numbers: 82.37.Np, 82.70.Dd

Successful strategies for bioadhesion require a combination of several tethered ligand-receptor pairs with distinct attraction ranges and binding kinetics [1,2]. For instance, the recruitment of leukocytes in response to tissue inflammation involves two different couples [3,4], one designed for rapid capture from the solution and the other for slow consolidation of adhesion and signalization. Such diversity being partly controlled by the structure of the spacer molecule, our understanding of the bioadhesion mechanisms requires the elucidation of the relationship between the range of interaction for a given pair and the time required to form a bond [5,6]. Here we introduce a new methodology to measure the time evolution of the ensemble-averaged probability to form a single-bond between ligands and receptors bound to opposing surfaces by a variety of tethers.

Our experimental method is based on the manipulation of Brownian superparamagnetic colloidal particles (Carboxyl-Adembeads, Ademtech) of 200 nm diameter under a magnetic field. These particles can be driven to organize into one-particle-thick chains in the direction of the external field, which also sets the interparticle distance [7]. The chains assemble within a few seconds and persist as long as the field is maintained. If the particles are functionalized with ligands and receptors, recognition bonds will form between adjacent particles and the chains may persist after the removal of the field. However, if no link between adjacent particles is formed, the particles redisperse instantly once the field is turned off. In our experiments, the particles are grafted with a controlled number n_R of streptavidins per particle, and are put in presence of a given number N of biotinylated ligands. That number N has to be small compared to the total number of particles. Under this so-called doublet condition, the removal of the field leads to a suspension consisting solely in individual particles and doublets, as shown on Fig. 1. Moreover, each persisting doublet of particles involves a single receptor-ligand-receptor sandwich. Since a doublet scatters more light than two individual particles, as given by Mie theory [8,9], the precise number of doublets $D(t)$ is determined from measuring the optical density difference (ΔDO) as a function of the time under the applied field [10].

In our first set of experiments, the particles are covalently grafted with streptavidins (Sigma Aldrich) as fol-

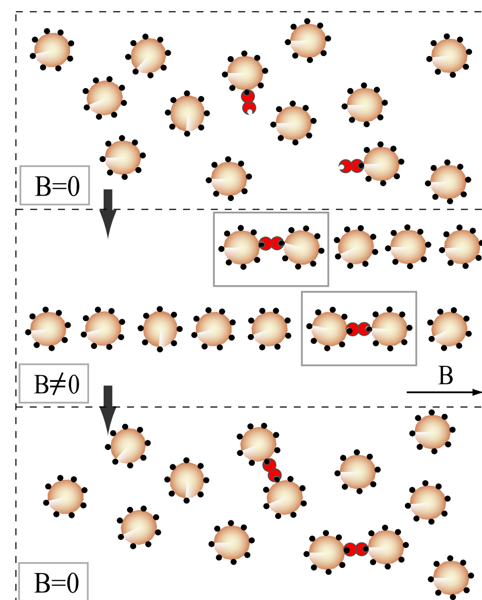

FIG. 1 (color online). Scenario for the formation of doublets of magnetic colloidal particles.

 © 2008 The American Physical Society

lows. 100 μL of particles at 1% wt are washed 3 times in a MES buffer (20 mM, pH $= 5.5$). Then, 50 μL of an EDC (N-(3-dimethylaminopropyl)-N-ethylcarbodiimide) solution (2 g $\cdot$ L^{-1}) and 50 μL of a NHSS (N-hydroxysulfusuccinimide) solution (2 g $\cdot$ L^{-1}) are added to the suspension. The suspension is incubated at 44 °C for 20 min. To end the activation step, the particles are washed 3 times in a phosphate buffer (10 mM, pH $= 7.2$). Coupling with streptavidins is obtained by mixing 50 μL of a streptavidin solution (4 g $\cdot$ L^{-1}) to the suspension. The concentration of streptavidin is adjusted to the intended coverage. The suspension is incubated at 44 °C for 30 min. The particles are washed 3 times in a glycin phosphate buffer (1.5 g $\cdot$ L^{-1} with 0.4% of a nonionic surfactant F-127) and, finally, after 30 min, with a phosphate buffer (10 mM, pH $= 7.2$, with 0.4% of F-127).

In Fig. 2, we plot $D(t)$ for two distinct experimental situations: one with no ligand at all and the other with 3×10^{-11} mol $\cdot$ L^{-1} of biotinylated Bovin Serum Albumine (biot-BSA). Particles and ligands are initially mixed and incubated for 5 min. The optical density of a sample, DO $= -\log(I/I_0)$, where I and I_0 are, respectively, the transmitted light and the incident light is measured with a spectrophometer (Perkin Elmer) at a wavelength of 700 nm. The intensity of the applied magnetic field is kept constant at a value of 30 mT. After each period under field, the DO is measured. The number of doublets and ΔDO are proportionally related. The error bars are obtained from the maximum fluctuation of the background signal. At zero ligand concentration, we detect a very small signal, which sets the background noise. In the presence of ligands, we systematically find an exponential shape for $D(t)$, as revealed by the fit to the experimental data shown in Fig. 2. $D(t)$ saturates at a value denoted N_{eff}, that corresponds to the actual maximum number of ligands that can contribute to the formation of doublets. Since all binding events are independent, the probability $p(t)$ that a bond occurs between two particles within a duration t is given by $p(t) = D(t)/N_{\mathrm{eff}}$. Our results show that this probability $p(t)$ follows a first order kinetics: $p(t) = 1 - \exp(-t/\tau_{\mathrm{exp}})$ where the experimental time τ_{exp} is independent of N. Here τ_{exp} refers to the binding time between one bead carrying one ligand and a second bead carrying n_R receptors. A linear variation of the binding rate $1/\tau_{\mathrm{exp}}$ with the number of receptors n_R is observed—see the inset of Fig. 2. The fundamental output of our experiments is therefore the association time $\tau = n_R \tau_{\mathrm{exp}}$, which represents the average time required for a bead carrying one ligand to bind a bead carrying one receptor. This also implies that the probability $p(t)$ follows the kinetic equation $dp(t)/dt = -p(t)n_R/\tau$. For this ligand-receptor pair the dissociation time is much larger than the experimental time, and it does not contribute to the kinetics. Note however that our method can easily be extended to the case of shorter-lived ligand-receptor pairs provided that their bond lifetimes, independently determined by one of the several available techniques [11], are larger than a fraction of a second.

We now focus on the consequences of changing the ligand tether and its number of specific reactive sites, as a manner of exploring a wide range of binding kinetics. Three different families of linker configurations have been designed, see Fig. 3. The first one, denoted as family (i), consists in streptavidin-coated beads with biotinylated

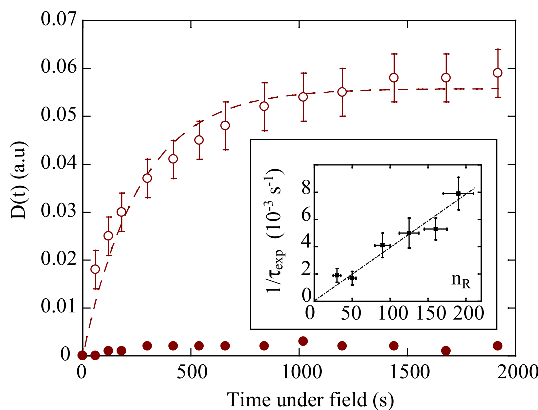

FIG. 2 (color online). Number of doublets $D(t)$ in arbitrary units as a function of the time under field. The mass fraction of particles is 0.04%, corresponding to a concentration of 10^{-10} mol $\cdot$ L^{-1}. The particles are grafted with approximately 90 streptavidins. The final concentration of ligands (BSA with 12 biotins) is 0 (●) or 3×10^{-11} mol $\cdot$ L^{-1} (○). Inset: plot of the association rate $1/\tau_{\mathrm{exp}}$ as a function of the number of receptors n_R.

(θ_1) bead 1 / (θ_2) bead 2	Molecular architecture	τ 10^2s	r_C Å
Family (i)	BSA 4biot	830	0.7
	BSA 12biot	250	1.3
Family (ii)	LCLC 6biot	100	2.0
	PEG 6biot	83	2.2
Family (iii)	LCLC 1biot	18	4.7
	PEG 1biot	3.4	11

FIG. 3 (color online). Schematic representation of the three distinct molecular architecture families. τ: corresponding measured times of recognition. r_C: capture radii deduced from theory.

BSA (grafted with either 4 or 12 biotins) as ligands. In this family, due to the globular structure of these two proteins (BSA and strepatvidin) and their relative stiffness, both ligands and receptors are rigidly linked to the particles surfaces. In the second family (ii), BSA is replaced by a Fc fragment on which 6 small biotinylated polymer chains are grafted, each chain consisting in a polyethylene glycol-like short polymer with a radius of gyration of either 1 or 2.5 nm. In this case, streptavidins still remain rigidly linked to the particles surfaces. In the third family (iii), both ligands and receptors are linked to the surfaces by the two types of biotinylated PEG spacers described above—see Fig. 3. For family (ii) and (iii), the polymer tethers consist in LC-LC (Pierce, spacer length 30.5 Å) and PEG_{3400} (Nektar, spacer length 189 Å). The Fc-fragments have been biotinylated by a protocol provided by Pierce (EZ-link Sulfo-NHS-Biotinylation-Kit) and the number of biotins per fragment has been determined by a HABA-Avidin method. For the measurement of the surface densities of receptors, the protocol consists in quantifying the number of excess fluorescent probes (biotin-FITC, or streptavidin alexa-554). In the case of streptavidin covered particles, families (i) and (ii), different amounts of grafted particles are added to a 1 mL solution of biotin-FITC (Sigma Aldrich) at 10^{-9} mol $\cdot$ L^{-1} in a borate buffer (10 mM, $p\mathrm{H} = 8.2$, with 0.4% of F-127). The samples are incubated at room temperature, under agitation, for 10 minutes. Magnetic particles are eliminated by sedimentation under a magnetic field. The concentration of excess biotin-FITC is determined by measuring the emission fluorescence intensity at 518 nm from an excitation at 492 nm (Xenius, Safas). In the case of biotin, family (iii), the fluorescent probe is streptavidin alexa-554 (Molecular Probes) and the same protocol is followed. For each studied configuration, we have always taken n_R sufficiently small to insure that there was neither multivalent binding between adjacent colloidal particles nor ''loop binding'' on the same particle.

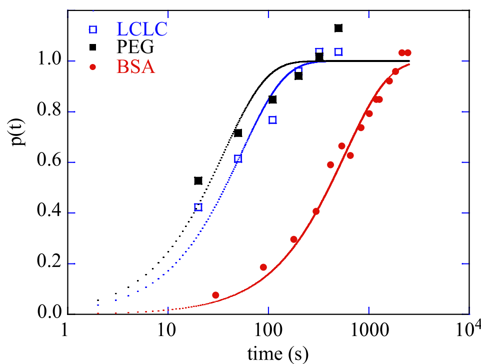

FIG. 4 (color online). Probability $p(t)$ that a bond occurs between two particles as a function of time under field for three distinct cases. (●): family (i), 50 streptavidins per particle, 12 biotins per BSA. (□): family (ii), 70 LC-LC-biotin per particle. (■): family (iii), 20 PEG-biotin per particle.

In Fig. 4, we show $p(t)$ for three selected situations within each family. By determining the surface densities of receptors, we obtain the value of the association time τ for each of the six designed cases shown in Fig. 3. The longest τ corresponds to the most constrained situation in family (i), where both the ligand and the receptor are firmly attached to the colloids. Any extra degree of freedom added to either the ligand or the receptor reduces the time required for the association to occur, the τ-value for the last and loosest configuration is 200 times smaller than the value of the first and most constrained one. We also note that the largest changes occur for the family (iii), when both receptors and ligands are carried by flexible spacers. We now show how the measured variations in the kinetics of the recognition events can be quantitatively associated with the reaction range imposed by the molecular structure of the spacer.

Smoluchowski [12] and Solk and Stockmayer [13], pioneered work recognizing the importance of Brownian dynamics as a key factor controlling the kinetics of reactions in solutions. Extension and refinement of their ideas by several authors [14,15] allows to understand the association kinetics of free ligand-receptor pairs in solutions on the basis of the translational and rotational diffusion that must bring the reactive species within a capture radius with the proper orientation [16–18]. We model our experiment by a closely related reaction-diffusion approach, where we consider reactions between bead 1 that carries one ligand and bead 2 holding one receptor. The distance between beads is governed by the intensity of the magnetic field. Experimentally we do not find any significant role of the magnetic field intensity above the so-called chaining threshold, which suggests that the rotation of the particles is the main control factor of the sampling dynamics. Since Neel relaxation is, for our magnetic beads, much faster than Brownian dynamics [19], we can assume that the characteristic rotation diffusion time τ_{rot} is independent of the magnetic field, and is given by the usual value $\tau_{\mathrm{rot}} = 8\pi\eta r^3/(k_B T)$, where k_B is Boltzmann's constant, T the absolute temperature, η the solvent viscosity and r the radius of the beads [20]. In order for the association to occur, the ligand and the receptor need to come into intimate contact. However, they possess a finite number of extra degrees of freedom, due to their own flexibility or to the nature of the spacers that tether them to the colloids. Since these local motions are much faster than colloidal dynamics, they can be accounted for by a capture cone θ_C, such that a reaction will occur provided that θ_1 and θ_2 are smaller than θ_C. This angle also defines a so-called capture patch with radius $r_C = r\sqrt{2(1 - \cos\theta_C)}$. By solving the reaction-diffusion equations for this situation [21,22], we find that the long-time behavior of $p(t)$ shows an exponential relaxation. The characteristic time is found to be

$$\tau = \tau_{\mathrm{rot}} 8 \ln 2 \frac{r^2}{r_C^2}, \tag{1}$$

provided that $r_C \ll r$. In Fig. 3, we assign to the six different ligand and receptor architectures the capture radii obtained from the above formula. The values of capture radii clearly fall into the expected molecular range, showing that Eq. (1) provides the reaction range for the tethered ligand-receptor pairs. In particular, for the most constrained situation in family (i) one extracts a value of $r_C \simeq 1$ Å, remarkably close to the geometric patch radius extracted from bulk solution measurements. Indeed, the solution association rate $k_{ab} = 7 \times 10^4\ \mathrm{m^3\,mol^{-1}\,s^{-1}}$ for avidin biotin, can be fully understood by requiring that the two species, with their known Stokes radii [23], are brought by diffusion into contact with their orientations restricted [24] within a reaction patch of radius ≃1 Å. Our results thus show that, for surface-bound ligands and receptors, the recognition mechanism is limited by diffusion, as in bulk solution. Furthermore, for the family (iii), we find capture radii of 4.7 and 11 Å, roughly proportional to the square root of the spacer length, and thus in line with the respective radii of gyration.

Time and length scales combine in this method to allow for extracting a molecular nanometric dimension from a kinetic measurement performed over many hundreds of seconds. The elementary time step in this setup is the exploration time of the rotational Brownian dynamics, $\tau_{rot} \sim 7$ ms for our beads, which is the time necessary for the exploration of a solid angle of 4π radians. However, finding a successful orientation between the receptor and the ligand requires many elementary attempts, because most of them are near misses of the target. For the most constrained situation that we have designed, 10^6 attempts are required on average before a connection can be made, while only roughly 10^4 attempts are necessary with the largest tethers.

As a summary we developed a method to study the recognition kinetics of tethered biomolecules that contrary to other available techniques [2,11] does not rely on mean-field assumptions to extract phenomenological association rates. Instead, it was designed (i) to limit the reactions to one ligand only; (ii) to give ensemble averages that are not tempered [11] by the intrinsic fluctuation effects of small size systems; (iii) to work within the linear regime of time dependence on the receptor number n_R so that one can measure a zero force [2] reaction time per ligand-receptor pair, independently from any model. A reaction-diffusion theory was then written to further deconvolute the reaction time from colloidal dynamics, to provide the capture radius, a fundamental quantity for the kinetics of diffusion-limited reactions. Beyond this case of diffusion-limited binding, we also foresee several experimental and theoretical developments for this simple and versatile technique, to tackle rate-limited biorecognition reactions.

We thank French Ministère de la Recherche, Université Pierre et Marie Curie-Paris 6, Ville de Paris and Centre National de la Recherche Scientifique for financial support. A. J., N. L., F. T., and C. M. benefited from the KOSEF/CNRS Program (No. F01-2005-000-10075-0).

*Corresponding author.
emanuel.bertrand@espci.fr

[1] P. Bongrand, Rep. Prog. Phys. **62**, 921 (1999).
[2] A. Pierres, A.M. Benoliel, C. Zhu, and P. Bongrand, Biophys. J. **81**, 25 (2001).
[3] U.H. von Andrian, J.D. Chambers, L.M. Mc Evoy, R.F. Bargatze, K.E. Arfors, and E.C. Butcher, Proc. Natl. Acad. Sci. U.S.A. **88**, 7538 (1991).
[4] M.B. Lawrence and T.A. Springer, Cell **65**, 859 (1991).
[5] C. Jeppesen, J.Y. Wong, T.L. Kuhl, J.N. Israelachvili, N. Mullah, S. Zalipsky, and C.M. Marques, Science **293**, 465 (2001).
[6] J. Huang, J. Chen, S.E. Chesla, T. Yago, P. Mehta, R.P. McEver, C. Zhu, and M. Long, J. Biol. Chem. **279**, 44 915 (2004).
[7] J. Bibette, J. Magn. Magn. Mater. **122**, 37 (1993).
[8] URL ftp://ftp.eng.auburn.edu/pub/dmckwski/scatcodes/index.html.
[9] D. W. Mackowski and M. I. Mishchenko, J. Opt. Soc. Am. A **13**, 2266 (1996).
[10] C.P. Price and D.J. Newman, *Principles and Practice of Immunoassay* (Macmillan, London, 1997).
[11] S.E. Chesla, P. Selvaraj, and C. Zhu, Biophys. J. **75**, 1553 (1998).
[12] M. von Smoluchowski, Z. Phys. Chem. **92**, 129 (1917).
[13] K. Solc and W.H.J. Stockmayer, J. Chem. Phys. **54** (1971).
[14] D. Shoup, G. Lipari, and A. Szabo, Biophys. J. **36**, 697 (1981).
[15] M. Schlosshauer and D. Baker, J. Phys. Chem. B **106**, 12 079 (2002).
[16] J. Janin, Proteins: Structure Function and Genetics **28**, 153 (1997).
[17] H.C. Berg and E.M. Purcell, Biophys. J. **20**, 193 (1977).
[18] S.H. Northrup and H.P. Erickson, Proc. Natl. Acad. Sci. U.S.A. **89**, 3338 (1992).
[19] P.C. Fannin, L. Cohen-Tannoudji, E. Bertrand, A.T. Giannitsis, C. Mac Oireachtaigh, and J. Bibette, J. Magn. Magn. Mater. **303**, 147 (2006).
[20] H. Stark, M. Reichert, and J. Bibette, J. Phys. Condens. Matter **17**, S3631 (2005).
[21] A. G. Moreira, C. Jeppesen, F. Tanaka, and C. M. Marques, Europhys. Lett. **62**, 876 (2003).
[22] N.-K. Lee, A. Johner, F. Thalmann, L. Cohen-Tannoudji, E. Bertrand, J. Baudry, J. Bibette, and C. Marques, Langmuir**24**, 1296 (2008).
[23] G. Balgi, D.E. Leckband, and J.M. Nitsche, Biophys. J. **68**, 2251 (1995).
[24] Y.Y. Kuttner, N. Kozer, E. Segal, G. Schreiber, and G. Haran, J. Am. Chem. Soc. **127**, 15 138 (2005).