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► **To cite this version:**

Jean Baudry, Emanuel Bertrand, Catherine Rouzeau, Olivier Greffier, Anne Koenig, et al.. Colloids for studying molecular recognition. *Annales de Chimie - Science des Matériaux*, 2004, 29 (1), pp.97-106. hal-00161909

**HAL Id: hal-00161909**

**<https://hal.science/hal-00161909>**

Submitted on 27 Dec 2022

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## COLLOIDS FOR STUDYING MOLECULAR RECOGNITION

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**Abstract** - During the past decades, organic and inorganic colloids have been widely used for biological or biotechnological applications. In the last decade, colloidal organic / inorganic composites, namely super-paramagnetic particles, have constituted a real improvement for some of these applications. Very recently, we have developed a new way of using such colloidal composites in the fundamental domain of molecular recognition and in the closely linked but more industrial field of immunological diagnosis.

**Résumé - Colloïdes pour l'étude de la reconnaissance moléculaire.** Au cours des décennies passées, les colloïdes organiques et inorganiques ont été largement mis à contribution pour des applications biologiques ou biotechnologiques. Lors des dix dernières années, les composites colloïdaux organique/inorganique, et précisément les particules super-paramagnétiques, ont constitué une véritable amélioration dans le cadre de certaines de ces applications. Très récemment, nous avons développé une nouvelle méthode d'utilisation de tels composites colloïdaux dans le domaine de la reconnaissance moléculaire et dans celui, étroitement lié mais plus industriel, du diagnostic immunologique.

### 1. INTRODUCTION

Organic and inorganic colloids have been used as biosensors for many years. A biosensor is aimed at determining various parameters associated to biological species such as single bio-molecules, molecular complexes, virus, cells and tissues. The understanding of mechanism from a molecular scale up to the cell function is certainly one of the most important scientific issues in molecular biology. It simultaneously requires information concerning concentrations, structures, conformations and dynamics, both at different length and time scales. The possibility of measuring such parameters requires the development of as much as possible sensitive, selective, real-time and multi-scale biosensors. After reviewing most of the biosensors that have already been used, we will show how the colloidal organic / inorganic composites that we use give access to new biophysical tools and are able to improve our quantitative knowledge of molecular recognition phenomena. In particular, we will explain their ability to improve considerably the sensitivity of immunological diagnosis agglutination tests.

One crucial aspect of bio-molecules concerns their ability to build reversible complexes through specific molecular recognition mechanisms. Indeed, a major property of bio-molecules is to bind a variety of ligands in order to fulfill a specific function such as mediating cell adhesion, triggering receptor mediated cell activation or regulating intracellular networks and immuno-response. Probing recognition phenomena has given rise to various types of sensors focusing on rupturing or binding, single as opposed to multi-molecular events as encountered in adhesion of mesoscopic objects (vesicles, cells or drops), and finally thermodynamic or kinetic aspects of bulk ligand-receptor reactions.

## 2. PROBING MOLECULAR RECOGNITION

Classical biochemistry methods give the thermodynamic and kinetic constants when both ligand and receptor are in solution, or when one of them is immobilized on a surface [1]. These constants can be readily determined with new tools, measuring for instance the variation of evanescent wave intensity upon binding of antigens on antibody-coated surface (Biacore S.A.). However, in lots of relevant situations, both macromolecules are supported by cells or tissues, so their fluctuations become strongly restricted, as a result of anchoring itself, crowding from neighbors and eventually compression due to surface forces... An important challenge in biophysics concerns the characterization of these effects, which certainly requires setting new tools.

Using red cell sensors and vesicles, both handled by micro-pipettes, rupturing of single molecular complexes has been precisely studied [2]. It was shown that the separation of a ligand from its receptor under force followed a unidimensionnal path, with an energy-distance curve determined by a so-called energy landscape, displaying sequential maxima or barriers. The plot of unbinding force versus the logarithm of the loading rate is found to be composed of sequential straight segments, the slope of which might be written as  $kT/x_i$ , where  $x_i$  represents the distance between the location of the energy minimum and the  $i^{\text{th}}$  barrier on the energy landscape. The surface force apparatus (SFA) is also a means to probe some collective binding processes. The measurement of macroscopic adhesion as a function of surface distance, after a reaction time of about one second allows the rate of binding to be inferred, which was attributed to the predicted molecular spacers dynamics [3]. Reflectance interference contrast microscopy (RICM) follows the formation of specific adhesive patches between a model solid grafted surface and a vesicle or a cell; the role of macromolecular repellers coupled to the presence of specific linkers has been largely addressed [4]. The laminar flow chamber operated at low shear rate can provide additional information on the fine properties of ligand-receptor association and rupture. Binding frequencies are obtained by pooling all colloidal trajectories in a given experiment and calculating the ratio of the number of steps followed by an arrest, divided by the total number of steps. In the same way, from the statistics of arrest duration, the unbinding frequency is computed. This technique gives valuable information about the role of shear rate, and can evidence the existence of transient states during the process of binding as in the case of streptavidin-coated micro-beads interacting with a biotin-derivatized surface [5].

## 3. BIOTECHNOLOGICAL IMPLICATIONS

Recognition has also many biotechnological implications. A major one concerns biological diagnosis or sorting of bio-molecules, virus and cells. In most cases, a targeted specie is probed using a complementary molecule which forms a complex, such as a ligand

and receptor, antigen and antibodies or complementary nucleic acids strands. The challenging aspect of diagnostic technologies is to transform the microscopic scale recognition process into a more macroscopic signal from a quantity of formed complexes as low as possible and within a biological mixture containing many different species. Today, developing simple, low cost, fast, and highly sensitive procedures to measure the concentrations of bio-molecules within serum, plasma, or in environmental conditions is certainly one of the major challenges of life science.

Early immuno-chemistry was based on precipitation of large complexes made of antibodies and antigens. Use of organic colloids, namely latex particles, did significantly improve the detection sensitivity because of the increase in scattered light when aggregation between grafted colloids takes place. Indeed, Brownian colloids will quickly find their target and at the same time optical change due to either agglutination or immobilization will be dramatically enhanced. As the most simple but very generic example, let us consider an antigen Ag having two epitopes for two monoclonal antibodies A and B. To reveal the presence of such an antigen, let us add a mixture of two types of colloids, each one grafted with A or B. The formation of small clusters is then expected, the formation rate depending on many factors. Change in light scattering due to the presence of these small clusters will reveal the existence of sandwich-like structures: A-Ag-B (Latex Agglutination Immunoassay). This homogeneous assay, as opposed to heterogeneous assays, which require washing steps prior to detection, is today by far the most simple and straightforward. Regarding heterogeneous assays, the most extensively used is the ELISA assay (Enzyme-Linked Immunosorbent Assay). In this assay, the latex particles have been commonly replaced by inorganic / organic composite magnetic particles, which are very useful since magnetic separation is rapid and convenient. The last part of this article will focus on the new biophysical and biotechnological applications of these magnetic particles and their exact chemical nature will thus be addressed later on. In an ELISA assay, the capture of antigens is operated through the recognition of only one type of antibody which is grafted onto the magnetic particles; note that there is no possible agglutination in that case. After removing and washing, a labeled-antibody is added to form the sandwich-structure; the label or the so-called tag of this second antibody may be either an enzyme (ELISA), a fluorescent, a radioactive or a chemi-luminescent molecule. After incubation, the sample is washed again to remove the labeled antibodies in excess, the signal being then detected. Because of these two washing steps, sensitivity as low as pmol/l are routinely obtained on automates. The main drawback of this approach concerns its complexity and therefore its associated cost as well as the required time compared to homogeneous assays. It should be noticed that the ELISA method is used in all commercial instruments once high sensibility is required, with test duration varying from 1 to 2 hours.

#### 4. IMPROVING THE SENSITIVITY OF HOMOGENEOUS ASSAYS

The challenge today for physicists, chemists, and immuno-chemists is to make homogeneous assays much more sensitive. In order to do so a detailed understanding of the different steps leading to specific sandwich-structures when starting with Brownian grafted colloids, should be addressed. "Agglutination assays" were introduced almost 50 years ago [6], and today several hundreds different tests based on this principle can be found on the market, mainly for infectious disease detection and protein quantification. These tests are rapid and simple: specific beads and samples to be analyzed are simply mixed, then absorbance after several minutes of incubation is measured. Today most of the ongoing

research work about these agglutination tests aims at improving their limited sensitivity ( $\sim$  nmol/l).

The more stringent limitation is related to colloidal stability in such a complex biological medium, having a very high ionic strength and containing a lot of very adherent proteins such as fibrinogen, which is found in plasma [7]. It is clear that aggregation must only occur in the presence of antigens, and never be due to any attractive colloidal force (van der Waals, hydrophobic or electrostatic). Therefore, some kinds of repulsive forces must exist between particles to ensure the absence of non-specific links, or so called “false positives”. However since antigen-antibody recognition involves quasi-contact forces (less than 1nm, see ref. [8]), increasing colloidal stability systematically decreases immuno-reactivity. Chemical and biochemical know-how has been developed [9] about the core and surface of particles (as responsible for colloidal stability and the possibility of having functional groups for further grafting with probes or ligands), about the antibody fixation (type and density, adsorption or covalent coupling, saturation with other protein) and about the medium which affects the optimization of the test (pH, ionic strength, surfactants, depleting agents). It turns out that all parameters are strongly coupled together and therefore conditions must be redefined for any change of antibodies, antigens or particles [10]. However, as long as this simple strategy is concerned, there is always some non-specific aggregation, which is superimposed to the specific one, imposing the limit of the sensitivity. Indeed, the measurable level of antigen in solution that can be detected, can never exceed roughly the number of non-specific links that form between beads during the test. Moreover the variability of biological fluids is adding some more complexity that limits even more the degree of reliability of the detection in the lower concentration limit.

The second limitation is related to the detection of aggregates. Since we are interested in determining low concentrations of antigens, the problem is essentially shifted to quantification of doublet formation. The most widely used technique is turbidimetry, because of its simplicity. As long as beads are small compared to the wavelength of the employed light (which is not exactly the case in practice), a doublet will scatter twice as much light than two separated beads would do. In the low concentration regime of antigens, which means that the bead concentration is larger than the antigen concentration, scattering of singulets (non aggregated single beads) will interfere with doublet signal, by contrast with ELISA-like assays where excess secondary labeled antibodies are washed out prior to detection. Therefore, the number of doublets that can be detected is limited by the total number of beads in solution. One possibility would be to decrease the particle volume fraction  $\phi$  in order to increase the sensitivity. Kinetics of doublet formation would then become too slow. Let us first assume an ideal system where the immune recognition is diffusion-limited, the average time  $t_b$  for a doublet to form is [11]:

$$t_b = a^2 / (6D\phi)$$

where  $a$  is the particle radius,  $\phi$  the volume fraction and  $D$  the diffusion coefficient. We find  $t_b$  of about 1s when typical values for  $a$  and for  $\phi$  are respectively 100 nm and 0.1%. But in fact, the specific link formation is most often reaction-limited, due to the intrinsic antibody-antigen association rate [1] or due to the repulsive colloidal forces, as considered previously. In this regime, several encounter events are necessary for a specific link to form. Therefore dilution will dramatically increase the assay duration, which precludes any optimization in that direction.

Several strategies have been proposed to overcome the noise caused by non-specific links. Single molecule studies are certainly inspiring these strategies. They are based on force

differentiation, originating from the fact that with well-designed colloidal surfaces, nonspecific links remain weaker than specific ones. Force differentiation may be applied through the following procedure: first, an immuno-chemical sandwich is expected between a grafted wall and a magnetic bead. This first step would take about 1-hour incubation, the necessary time to allow bead diffusion towards the wall. Second, a magnetic field is applied in a controlled manner, so that the applied force on the particles is enough to break up the nonspecific links only. The beads are then counted with a CCD camera [12], with a magneto-resistive transducer [13] or a CMOS Hall sensor [14].

Another method consists in using a SQUID (Superconducting Quantum Interference Device) “microscope” to measure the relaxation time of the particle magnetization at ambient temperature. Beads in solution undergo Brownian rotation, while for the bound particles only Néel relaxation is allowed. Since the time scales of these two relaxations are quite different [15] a precise differentiation is possible. Recently, this idea of force differentiation was extended, using DNA zipper as force probe [16], and better or comparable performance than “ELISA” assays was claimed. It should be noticed that the first washing step in ELISA assay may be considered as helping to remove part of non-specific capture as well and is also participating to a force differentiation strategy.

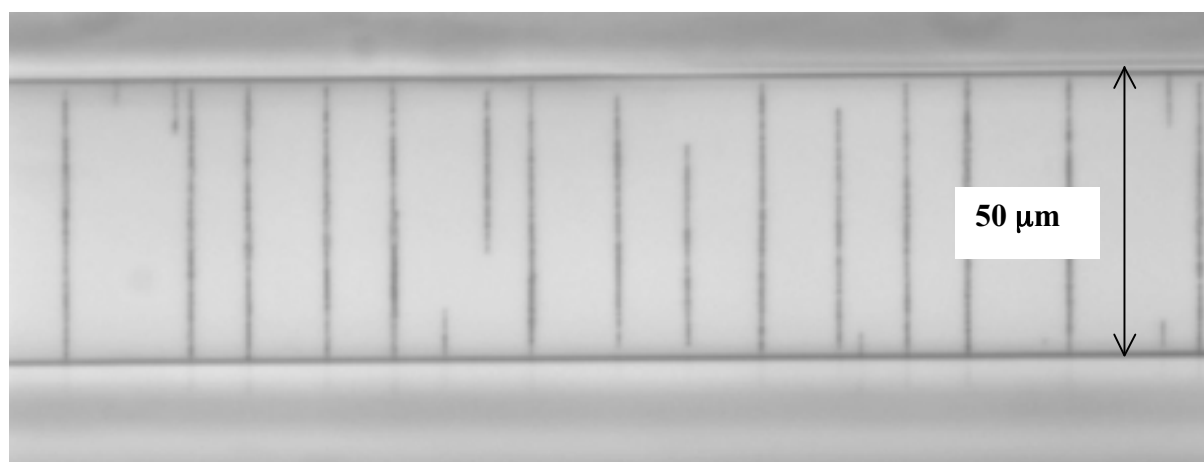
To tackle the immuno-reactivity/colloidal stability paradigm, a strategy using ultrasonic waves has been proposed [17]. It is still a classical agglutination format, but during the incubation time between beads and antigens, the sample is placed within an ultrasonic apparatus. Sensitivity is increased 10 to 1000 times depending on the nature of antigens, particle size and type. Two phenomena may be responsible for this enhancement. An ultrasonic standing wave-field subjects the particles to a radiation force and drives them towards pressure nodal regions. First, the local bead concentration increases, therefore increasing the collision probability. Second, the applied force can be enough (order of pN [18]), to overcome the repulsive colloidal force barrier.

Finally, several strategies have been proposed to improve the detection, all of them aiming at lowering as much as possible the background signal due to non aggregated beads. Colloids can be synthesized with a high level of refinement including controlled size, surface and content, which allows to implement new adjustable properties. Tiny gold particle will change color upon specific agglutination due to shifts in the plasma resonance [19]. Furthermore, when coupled to temperature change (melting studies), such shifts can reveal single base pair mismatch between complementary DNA strands [20]. In Luminescent Oxygen Channeling Immunoassay (LOCI [21]), “donor” and “acceptor” beads are used to form the sandwich: the donor particle produces excited singlet oxygen under 680 nm light exposition. This oxygen diffuses and initiates a chemi-luminescent reaction in the vicinity of the acceptor bead. Another strategy consists in tracking aggregates or particles one by one. In the Copalis™ technique, as in flow cytometry, a fluidic system aligns particles in a single file. They pass through a laser beam, and from their distinct scattering patterns it is possible to count the number of singulets, doublets etc. Related techniques are based on the formation of a sandwich type structure between a grafted bead and a secondary fluorescent antibody, which recognizes the desired antigen as in ELISA. Moreover, beads may be also tagged allowing multiplexed detection by using a flow cytometer (Luminex Corp.). Two-photon excitations have been also used for the same purpose, because the observation volume is so small that there is virtually no background fluorescence [22]. In both methods, the scattering signal comes from the tiny probed volume while the particles act as local concentrators of antigens, increasing the assay performance by orders of magnitude in terms of concentrations [23].

## 5. SELF-ASSEMBLING OF MAGNETIC COLLOIDS: MOLECULAR RECOGNITION AND APPLICATIONS

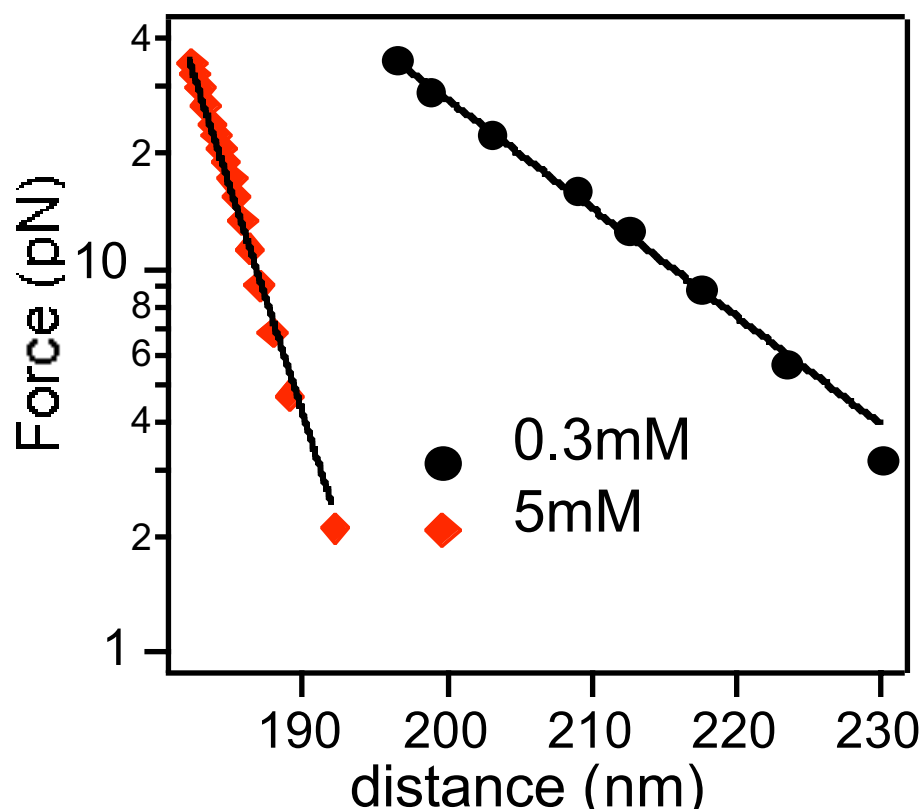
This part introduces the basics of using super-paramagnetic composite colloids and their spontaneous self-assembling ability under field, as new biosensors. The possibility to vary the magnetic interaction, and therefore the distance between particles within chain-like structures, opens new possibilities first as a new detection tool and second to resolve recognition at a molecular level.

The particles must fulfill some very specific requirements in order to be used for this strategy: beads must be spherical, mono-disperse, strongly magnetic and Brownian which imposes a maximum diameter of about 1  $\mu\text{m}$ . In addition, their surfaces must provide very strong colloidal stability, and possess organic functional groups to be further grafted by macromolecules of biological interest. Such particles are now commercially available and manufactured by Ademtech S.A. (<http://www.ademtech.com>). At the first stage of their preparation, they consist in calibrated emulsion droplets of an organic ferrofluid in water. The ferrofluid used is a suspension of  $\gamma\text{-Fe}_2\text{O}_3$  ferrimagnetic inorganic nano-particles of radii ranging from 5 to 10 nm in octane. The nano-particles are stabilized by oleic acid. Thanks to a controlled emulsification process [24], the obtained ferrofluid emulsion droplets are very mono-disperse with a diameter ranging from 150 nm to 1  $\mu\text{m}$ . This emulsion undergoes a thermal treatment in order to evaporate most of the octane within the droplets and to obtain very concentrated and thus strongly magnetic spherical beads. The calibrated beads then undergo a surface polymerization in order to avoid the presence of ferrimagnetic particles at their surface and to obtain a homogeneous controlled surface with a high density of carboxylic groups. Surface polymerization leads to highly charged particles at neutral pH, providing a strong colloidal stability through electrostatic repulsion. At this stage, one ends up with colloidal particles that are both inorganic inside and organic at their surfaces and that fulfill all the formerly exposed requirements. Finally, before being used for molecular recognition and its applications, biological molecules, such as antibodies, must be grafted onto carboxylic sites, which involves routine biochemical know-how.



**Figure 1.** Chains of super-paramagnetic particles aligned with the magnetic field in a square capillary tube of 50  $\mu\text{m}$  (optical microscopy, X100 objective)

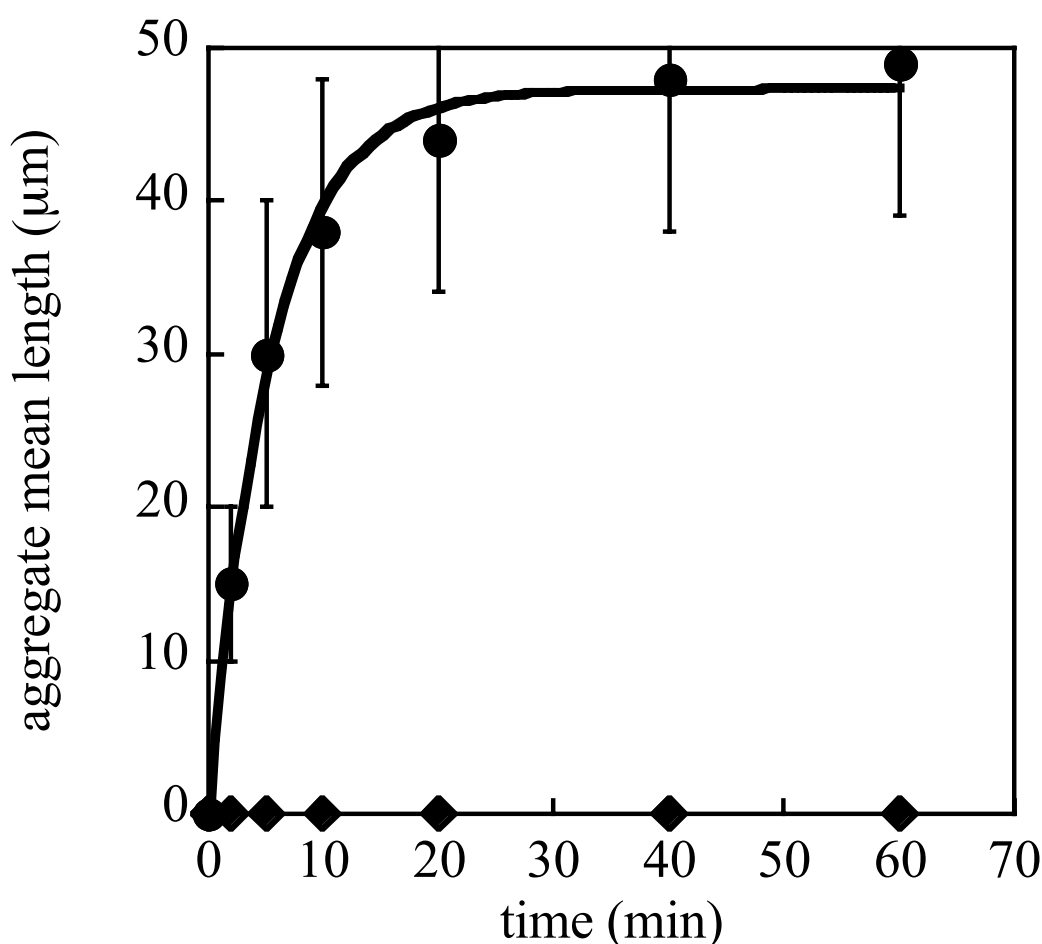
The application of a magnetic field to an aqueous suspension of such magnetic beads induces a magnetic dipole in each bead. Due to Brownian motion coupled to anisotropic dipolar interactions, the magnetic particles eventually form chains. These structures are reversible: by switching the external field off, magnetic moments vanish, and the Brownian agitation redisperses the suspension. At low bead concentrations, the chains are one particle thick, well separated and align along the field direction, as can be seen on *figure 1*. This one-dimensional order is giving rise to a very remarkable interference pattern which is used to measure the inter-particle distance from collecting the Bragg scattered light at  $180^\circ$  [25]. Moreover, the magnetic content of beads and their distance within the chain (deduced from Bragg scattering) enables to deduce the applied magnetic force. Since the colloidal repulsive force strictly balances this attractive magnetic force, the force distance profile is readily determined for various conditions. One example is given in *figure 2*, where electrostatic forces are characterized as a function of ionic strength. This technique originally offered the first direct measurement of electrostatic repulsion between colloids and the validation of the predicted exponential decay of the Debye-Hückel theory [25]. Steric repulsion induced by an adsorbed polymer was also probed with this technique. It was shown that the repulsion did follow an exponential decay with distance, the decay length being proportional to the radius of gyration of the adsorbed polymer [26].



**Figure 2.** Evolution of the colloidal force between adjacent magnetic beads with the centre-to-centre distance, for two ionic strengths. Points correspond to experimental values, the solid line to theoretical predictions (the surface potential is the only fitting parameter).



Another fundamental application of the self-assembling properties of these composite organic/inorganic colloidal materials lies in the determination of the micro-mechanical properties of biological complexes. Once the particles are grafted with ligands and put in the presence of receptors able to recognize two of them, the chains are no more reversible, and instead, specific recognition eventually leads to a sandwich-type molecular construct that can link adjacent particles together. Goubault *et al.* have just shown that the rigidity of the obtained magnetic chains is quantitatively linked with the bending rigidity of the molecular structure between the magnetic beads [27]. The authors therefore extend the exploration of biomechanics much further than the knowledge of rod-like biopolymers such as actin, DNA and tubulin. This technique should, on the basis of these first results, give a clearer understanding of the mechanical properties of most of the biomolecules involved in cell biology [27].



**Figure 3.** Kinetics of aggregation of streptavidin-coated magnetic particles (volume fraction: 0.02 %) in presence of biotinylated BSA (concentration: 5 nmol/l) in a 50 mmol/l phosphate buffer, after incubation under a 9 mT magnetic field (circles), and without magnetic field (diamonds); the solid line is an exponential fit.

In terms of biotechnological applications, agglutination assays can be greatly improved using these composite magnetic particles. The basic idea is to exploit chaining as a catalyzer for molecular recognition to occur. Nonetheless, the strategy remains very similar to that of classical agglutination tests except that a magnetic field is used. The antibody-grafted beads are first mixed with the sample to be probed, so the antigens are captured. Second, magnetic field is applied to form chains so that antigens will irreversibly link particles. Third, the field is relaxed, and the size or amount of the remaining chains, which reflects the antigen concentration, is detected using simple turbidimetry, as usually done for detecting aggregates. It has been shown that assay sensitivity can be easily improved by a factor of 1000 [28]. As an example, *figure 3* shows the kinetics of aggregation of streptavidin-coated magnetic particles under a magnetic field of 9 mT, in the presence of biotinylated BSA (Bovine Serum Albumin). The experiment is done in a square capillary tube of 50  $\mu\text{m}$  width and the aggregate mean length is determined by optical microscopy, as a function of the incubation time under the magnetic field. As can be seen on *figure 3*, the data points are well fitted by a mono-exponential, characteristic of a first-order like kinetics. The experimental characteristic time is approximately 5 minutes whereas no aggregation can be seen without field after one hour of observation. The origin of the enhancement of this sensitivity is still to be determined, and might be similar to the one responsible for the already mentioned “ultrasonic agglutination assay”: since a force is also applied on the beads, the repulsive colloidal barrier can be overcome. Moreover, they are also brought in close contact for quite a long time compared to what is expected from Brownian collisions, greatly enhancing the encountering probability between grafted macromolecules.

The main underlying idea is therefore to apply suitable mechanical and geometrical conditions, within a sufficient time, to induce specific molecular recognition. By contrast to bond rupturing, very little is known on binding, when the macromolecules are grafted on colloids, cells or drops. It seems that a “long” contact time, more than a strong applied force, is efficient [5]. The major goal, from a fundamental point of view, is to determine the rules which govern binding, both for specific (recognition) and non-specific (eventually protein mediated) routes.

## 6. CONCLUSION

To summarize, the use of colloidal organic / inorganic composites, namely surface-polymerized super-paramagnetic particles, offers new tools for studying molecular recognition. The organic part of these particles enables to graft them with all sorts of molecules of biological interest. Besides, their inorganic – and magnetic – part gives a way of manipulating these new biosensors with an external magnetic field. This novel approach has already proved to be applicable and successful in the domain of immunological diagnosis by considerably improving the sensitivity of agglutination assays.

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