Improved antireflection coated microspheres for biological applications of optical tweezers

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ABSTRACT

The success of optical tweezers in cellular biology[1] is in part due to the wide range of forces that can be applied, from femto- to hundreds of pico-Newtons; nevertheless extending the range of applicable forces to the nanoNewton regime opens access to a new set of phenomena that currently lie beyond optical manipulation.

A successful approach to overcome the conventional limits on trapping forces involves the optimization of the trapped probes. Jannasch *et al.*[2] demonstrated that an anti-reflective shell of nanoporous titanium dioxide ($aTiO_2$, $n_{shell} = 1.75$) on a core particle made out of titanium dioxide in the anatase phase ($cTiO_2$, $n_{core} = 2.3$) results in trappable microspheres capable to reach forces above 1 nN.

Here we present how the technique can be further improved by coating the high refractive index microspheres with an additional anti-reflective shell made out of silica (SiO_2). This external shell not only improves the trap stability for microspheres of different sizes, but also enables the use of functionalization techniques already established for commercial silica beads in biological experiments.

We are also investigating the use of these new microspheres as probes to measure adhesion forces between intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 1 (LFA-1) in effector T-Cells and will present preliminary results comparing standard and high-index beads.

Keywords: Optical Tweezers, Antireflection coating, Cellular Adhesion, T lymphocytes

1. INTRODUCTION

Optical tweezers represent a powerful tool in the field of cellular and molecular biology.[3,4] A multitude of experiments on single cells have been published in the years,[1] since the early stage of the technique.[5] This is justified by the versatility of optical tweezers, that allow the experimentalist to hold cells in specific positions,[6,7] to transport single cells to different locations in a sterile and not-contact condition,[8,9] to assemble cells into new structures,[10,11] and finally to perform force measurements on cells.[1] Measuring forces can provide fundamental information on cell movement mechanisms, on cell adhesion, and in general on cell cytoskeleton.[12]

Although the wide range of applicable forces by optical tweezers spans from femto- to hundreds of picoNewtons, the recent years have seen an increasing interest in the optimisation of such technique, with the aim to extend this force range to the nanoNewton regime. Optical tweezers with high stiffness could not only be used in those experiments where a strong trap is required without increasing the power to prevent the sample from heating, but it would also open access to a set of biological interactions taking places at the nN regime that have been overlooked until now.

The majority of the attempts to overcome the hundreds of picoNewtons limitation involves beam shaping techniques and set-up improvements.[13–16] With these approaches, it was not possible to achieve a quality factor, or Q-value (a measure of the laser light used to trapping, therefore a measure of trap efficiency), higher than 0.09.[13–15] Even when nanoNewton forces could be achieved,[16] it was only in one direction and at the price of highly demanding simulations.

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An alternative and more effective approach involves the optimization of the trapped probes. It was demonstrated that the use of an anti-reflective coating for optically trapped microspheres results in higher forces than for commercial probes.[17] More noticeably, anti-reflective coatings could be employed on high refractive index microspheres to enhance the gradient force in the optical tweezers while suppressing the destabilising scattering force acting on the microspheres. With this consideration, Jannasch *et al.*[2] reported forces above 1 nN with a Q-value of 0.25 for microspheres made in titanium dioxide in the anatase phase ($cTiO_2$, $n_{core} = 2.3$) coated with titanium dioxide in the amorphous phase ($aTiO_2$, $n_{shell} = 1.75$).

Although these findings are extremely exciting, the feasibility of biological experiments employing $cTiO_2$ @$aTiO_2$ core@shell microspheres is limited by the tight tolerance for microspheres size. Only microspheres in a small region of sizes can be stably trapped by optical tweezers; for the optimal case of cores of 500nm in diamter with a 200nm thick shell, a 10% change of the shell size would be sufficient for the scattering force to exceed the gradient force, destabilizing the trap, as shown in the simulations reported in the same paper.[2] Unfortunately microspheres samples produced by using the reported chemical synthesis protocols[2, 18] have low monodisperisity. Furthermore, the method is particularly sensitive on the experimental set-up being used, since the efficiency of trapping varies considerably using different filling factors and NA. As a result, beads that have been optimised in sizes for a certain optical set-up may not be trappable with other experimental conditions. These drawbacks limit the chances of using these beads for biological experiments, where due to the intrinsic high variance of the sample characteristics it is a priority to collect huge amount of data, and it would be then desirable for the majority of the beads to be trappable so to increase the throughput of the experiments.

Hereby we propose a solution to overcome the issues listed above by an additional silicon dioxide (SiO_2) coating on top of core@shell TiO_2 microspheres. This improvement results in a broad tolerance for the microsphere size; the whole set of sizes in the custom synthesised microspheres sample is stably trappable, at the price of a larger variance in the trap stiffness. Nevertheless, this should not represent a major concern, since calibration techniques that do not rely on prior knowledge of the microsphere size and refractive index are easy to implement,[19] so that each probe used for force measurement can be calibrated real-time before or after having interacted with the biological sample. The presence of the SiO_2 coating also results in better mechanical properties of the microsphere, as well as providing a suitable chemical surface for many functionalisation techniques that are already known to the scientific community for commercial SiO_2 beads.

As an example of the enhanced properties of $cTiO_2$@$aTiO_2$@SiO_2 core@shell@shell microspheres, we are investigating the advantages of these beads in an interesting case study: the adhesion properties of T-lymphocytes (referred to as *T-Cells*).

2. METHODS

2.1 Microsphere Synthesis

The synthesis of $cTiO_2$@$aTiO_2$@SiO_2 core@shell@shell particles is carried out in three steps: the synthesis of anatase $cTiO_2$ cores,[18, 20, 21] the growth of amorphous $aTiO_2$ shell on the cores,[18, 21] finally the diffusion of SiO_2 in the TiO_2 shell and the contemporary growth of SiO_2 on top using a variation on the Stöber process[22] optimised to obtain the required shell thickness (Fig. 1).
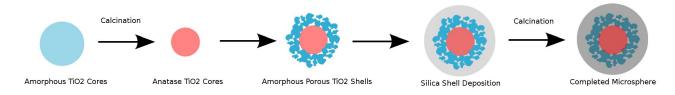

Figure 1. Cartoon of the chemical synthesis process for the production of $cTiO_2$@$aTiO_2$@SiO_2 core@shell@shell microspheres

The beads size and shapes have been characterised by use of a Scanning Electron Microscope Hitachi S-800 with back scattering detector. The samples are deposited on a metallic plate and coated by a thin film of 20-25 nm of Au by sputtering (Fig. 2).

Figure 2. SEM pictures of the synthesised microspheres. From left to right: calcinated cores particles in anatase $cTiO_2$; cores after porous amorphous $aTiO_2$ coating; $cTiO_2@aTiO_2$ core@shell after SiO_2 coating and calcination
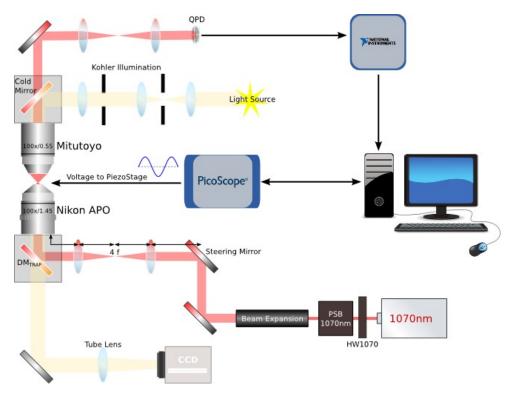
2.2 Experimental Set-Up

Figure 3. Optical tweezers set-up used for the trap stiffness evaluation.

Trap stiffness measurements were performed in a standard set-up of optical tweezers (Fig. 3), obtained by focusing a laser with 1.5W output power at 1064 nm (Laser Quantum Ventus 1064) by an oil-immersion objective (Nikon 100x Apochromat with NA=1.45.) to a diffraction-limited spot. The scattered light was collected by a long working distance objective (Mitutoyo NA=0.55 100x) onto a quadrant photodiode (QPD, Hamamatsu, G6849) to perform back-focal-plane interferometry. The sample was placed on a Thorlabs MAX302/M NanoMax piezoelectric stage, controlled by a Thorlabs MDT630A 3-Axis piezo controller. The piezo controller, in turn, can receive an external signal by the function generator channel of PicoScope (5000 Series).

2.3 Cell Culture

We extracted T-Cells collected from the spleen of control mice. Collected splenocytes are incubated with 2C11 peptide for activation at 37° C with 5% CO_2. Interleukin 2 and 12 (IL2 and IL12) are added to burst activation and to reproduce more physiological conditions. T-Cells are washed after 1-2 days, and then re-suspended in fresh media with IL2. They are counted daily and kept at a concentration of 0.5 milions/ml. They are used on days three, four and five after activation at a concentration of 1 milion/ml.

3. RESULTS

3.1 Trap Stiffness Measurements

The ideal size of core and shells for these photonically structured probes was calculated by simulations based on the Optical Tweezers Computational Toolbox by Nieminen et al.[23] For a core of 500 nm in diameter, the optimal shell thickness for the amorphous TiO_2 coating and for the SiO_2 coating resulted respectively in 200nm and 250nm.

Comparing the simulation results for the previously reported $cTiO_2@aTiO_2$ core@shell microspheres[2] and the same beads with an additional layer of 250nm of SiO_2 (Fig. 4), it is evident that the introduction of the additional SiO_2 antireflective coating solves the issue of the tight tolerance for the particles size.
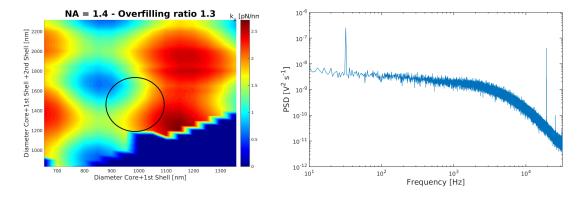

Figure 4. Left: Simulation of the trap stiffness for the x-direction, using effective NA=1.4, overfilling ratio 1.3 as per the set-up in use. The circle highlights the region of sizes that has been found through the calibration. The corresponding κ_x are in good agreement with what calculated. Right: Example of power spectrum for a cTiO2@aTiO2@SiO2 core@shell@shell bead. The laser power in the sample is 100mW.

To strengthen our results, we have measured the trap stiffness for these microspheres in the optical tweezers device described in Sec. 2.2, even if the beads were produced and optimised for the setup reported by Jannasch *et al.*[2] As the size of the beads is unknown, due to the high variance in dimensions after the chemical synthesis, we calibrated the trap by computing the power spectrum resulting from the thermal motion of the microspheres with the response to a sinusoidal movement of the stage[19] at a given frequency. For each bead, its size and the drag coefficient is calculated without any a priori assumptions. The spectra were analysed by a modified version of the *tweezerscal* Matlab toolbox.[24]

Even if the microspheres properties where optimised for a different experimental set-up, we found them to perform better than commercial beads. We measured an average trap stiffness of 1.15 ± 0.35 pN/(nm · W) for beads with diamters $1.3 < D < 1.7\mu m$ (Table 1), nearly 2.5 times higher than 0.45 ± 0.21 pN/(nm · W), as measured for commercial beads of comparable sizes ($1.86\mu m$ SiO_2 beads from *Bangs Laboratories*).

The measured stiffness is smaller than that predicted by simulations, but it is in good agreement taking into account the differences in the optical tweezers set-up used for the experiments (See Fig. 4).

In addition, we evaluated the percentage of beads that it was possible to trap in the case of $cTiO_2@aTiO_2$ composite core@shell microspheres and in the case of the same microspheres with the additional SiO_2 coating.

The measurements confirmed that only a few TiO_2 beads could be trapped for the set-up in use, while it was possible to trap all the probed $cTiO_2@aTiO_2@SiO_2$ beads.

Table 1. Trap Stiffness Evaluation for $cTiO_2@aTiO_2@SiO_2$ core@shell@shell probes: The laser power in the trap spot was of 100 mW for beads b01-04, 20 mW for beads b05-08 and 3 mW for beads b09-b10. The variation in the results is due to the variation in size of the beads.

Bead ID	k [pN/(nm.W)]		D [μm]
	k_x	k_y	
b01	1.2 ± 0.1	1.1 ± 0.1	1.6 ± 0.2
b02	1.3 ± 0.2	0.9 ± 0.2	1.5 ± 0.2
b03	0.9 ± 0.1	1.0 ± 0.1	1.6 ± 0.2
b04	1.9 ± 0.1	1.8 ± 0.1	1.4 ± 0.2
b05	1.5 ± 0.2	1.4 ± 0.2	1.3 ± 0.2
b06	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.2
b07	1.1 ± 0.1	1.0 ± 0.1	1.7 ± 0.2
b08	1.4 ± 0.2	1.2 ± 0.2	1.3 ± 0.2
b09	0.7 ± 0.1	0.7 ± 0.1	1.6 ± 0.3
b10	0.8 ± 0.1	0.7 ± 0.1	1.7 ± 0.3

3.2 Microspheres Functionalisation

Several studies have been already conducted within our group to observe the interaction between *Intercellular Adhesion Molecule 1* (ICAM-1) and *Lymphocyte function-associated antigen 1* (LFA-1) in leukocytes. Springer and collaborators proved that the ICAM-1 is a ligand for LFA-1,[25] and that their interaction represents one of the mechanisms for lymphocytes to adhere to endothelial cells.[26] Atomic force microscopy can be exploited to characterise and quantify ICAM-1 - integrins interaction.[27, 28] This interaction is particularly interesting in a shear flow environment, as it has been shown that the induction of stable integrin-dependent adhesiveness in T cells requires the application of shear forces.[28]

An alternative to AFM, optical tweezers can be used for the measurement of adhesion forces between ICAM-1 and T cells, allowing the observation of single molecules adhesion events, for example measuring the interaction between a trapped probed coated with ICAM-1 and an activated lymphocyte.[29] In that experiment, commercial beads coated with ICAM-1 are brought into contact by optical tweezers with cells that have settled on the glass surface of a custom made sample chamber; the beads are then pulled away from the cells, and if an adhesion event has occurred the force of such event is measured by back focal plane interferometry.[29]

In their study, the limited trap stiffness of commercial beads was not sufficient to repeat the study in shear flow environment.

We decided to carry on preliminary studies to confirm the feasibility of such experiments in shear flow environment with the use of the photonically structured beads hereby presented. The first requirement for the experiments to be feasible was to confirm that the $cTiO_2@aTiO_2@SiO_2$ could be functionalised, like commercial ones.

The ICAM-1 functionalisation protocol for commercial beads was extended to $cTiO_2@aTiO_2@SiO_2$ composite microspheres. The beads are washed twice in Phosphate-buffered saline solution (PBS) and then left in incubation at $4°$ C in 500μl of PBS with 2μg/ml ICAM-1. After collection they are washed three more time in PBS. When this protocol was used for the composite beads rather than the commercial ones, a longer centrifugation time was used for the washing, since it proved to be more effective for the sedimentation of the microspheres into a pellet.
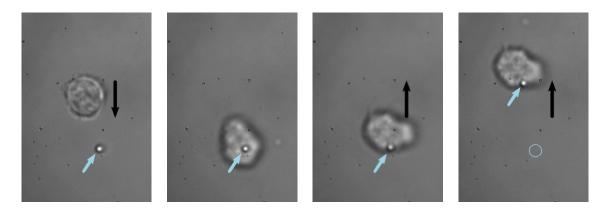
Figure 5. From left to right, a cell approaching a trapped functionalised $cTiO_2@aTiO_2@SiO_2$ bead, showing an ICAM-1 - LFA-1 binding. The blue arrow indicates the custom bead, the black arrow the direction of motion of the cell in regard to the bead. In the last panel on the right, the blue circle highlights the position of the trap, after the bead has left because attached to the cell. For more examples, see video at http://dx.doi.org/doi.number.goes.here

When beads coated with ICAM-1 were brought in contact with T-Cells, we could observe whether or not an interaction between ICAM-1 and LFA-1 had happened by observing if the bead would remain attached to the cell (see Fig. 5). Both ICAM-1 coated and uncoated microspheres, either commercial or photonically structured, have been observed (see Tab. 2).

Table 2. Percentage of observed beads that have shown to be interacting with T-Cells

	ICAM-1	Uncoated
Commercial beads	22.2%	$<1\%$
$cTiO_2@aTiO_2@SiO_2$ **beads**	26.3%	$<2\%$

4. CONCLUSION

NanoNewton forces represent an important milestone in optical trapping. Although they have first been reported more than four years ago[2] thanks to the development of an effective anti-reflective coating for high-refractive index probes, the synthesis of these photonically structured microspheres needed improvements prior to design biologically relevant experiments.

Recently a first application of titania microspheres to cell manipulation has been published.[30] Although the feasibility of experiments using $cTiO_2@aTiO_2$ core@shell beads has been proved, the mentioned limitations have played an important role in the conclusions drawn from the authors. While the authors showed how the microspheres could be internalised by different cell lines and how drag force measurements on the cells showed up to 220% increase in the Q-value compared to native cell lines, they also observed just a small number of trappable beads and an higher polydisperisty than what was previously reported. In addition because a direct calibration method was not implemented, the reported values need to be considered as an average results over different beads sizes.

The introduction of an additional shell of SiO_2 results in continuous trappable regions in size. For these beads we reported trap stiffness from 2 to 4 times higher than commercial SiO_2 beads of comparable sizes; the variance in stiffness directly reflects the presence of more sizes for which the beads can be trapped thanks to the outer SiO_2 layer. This fundamental improvement makes these $cTiO_2@aTiO_2@SiO_2$ composite microspheres more desirable for studies in biology, where it is important to increase the throughput of the experiments.

The preliminary results hereby presented for the case of ICAM-1 functionalisation have additionally proved that these beads behave as commercial ones in regard of surface treatments.

We are currently working on the evaluation of the interaction forces between ICAM-1 and LFA-1 by the use of the $cTiO_2@aTiO_2@SiO_2$ custom beads. For this T-Cells adhesion study, reproducing the experiments carried by McDonald *et al.* would guarantee a solid background before carrying a study in the more physiological shear flow environment. A measure of single molecules adhesion in shear flow environments would contribute in understanding the results previously observed regarding the role of shear flow on integrin-dependent adhesiveness in T cells.[28]

5. ACKNOWLEDGEMENT

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