

UNIVERSIDAD POLITÉCNICA DE MADRID

Escuela Técnica Superior de Ingenieros de Caminos, Canales y Puertos



**Developing affinity microscopy: Characterization and use of functionalized AFM tips to
measure intermolecular forces.**

DOCTORAL THESIS

Submitted for the degree of Doctor by:

Daniel Corregidor Ortiz

Graduated in Materials Engineering

Master's degree in Industrial Design Engineering.

Madrid, 2024



UNIVERSIDAD POLITÉCNICA DE MADRID
Escuela Técnica Superior de Ingenieros de Caminos
Canales y Puertos

PhD in Engineering of Structures, Foundations and Materials.

**Developing affinity microscopy:
Characterization and use of
functionalized AFM tips to measure
intermolecular forces.**

DOCTORAL THESIS

Submitted for the degree of Doctor by:

Daniel Corregidor Ortiz

Graduated in Materials Engineering.

Master's degree in Industrial Design Engineering.

Under the supervision of:

Dr. Gustavo Víctor Guinea Tortuero

Dr. José Pérez Rigueiro

Madrid, 2024

Title: Developing affinity microscopy: Characterization and use of functionalized AFM tips to measure intermolecular forces.

Author: Daniel Corregidor Ortiz.

Doctoral Programme: Doctoral Degree in Estructuras, Cimentaciones y Materiales

Thesis Supervision:

Dr. Gustavo Víctor Guinea Tortuero, Catedrático, Universidad Politécnica de Madrid (co-Supervisor).

Dr. José Pérez Rigueiro, Catedrático, Universidad Politécnica de Madrid (co-supervisor)

External Reviewers:

Thesis Defense Committee:

Thesis Defense Date:

This thesis has been partially supported by “Programa propio de I+D+I de la Universidad Politécnica de Madrid en colaboración con banco Santander universidades para la realización de doctorados con mención internacional.”

A mi abuela Aurora y a mi abuelo Manolo.

Acknowledgement

Desarrollar este trabajo ha sido una montaña rusa de emociones constante y no habría sido posible sacarlo adelante sin el apoyo de muchas personas que han estado ahí durante todo este tiempo. Me gustaría agradecer a mis padres todo el apoyo que me han dado siempre, porque he llegado hasta aquí en gran parte gracias a ellos. También, agradecer a Rubén el soporte que ha sido para mí durante este tiempo y el que sigue siendo. Por todos esos momentos en los que estaba derrotado y me has sacado de ahí, porque has aguantado los malos momentos y te has preocupado de que disfrutara de los buenos. Por todo ello, gracias y te quiero. Y por supuesto a Raquel, porque has sido un refugio, has sido calma, has sido acompañante, has sido apoyo, has sido muy buena consejera, has sido risas, has sido todo y has estado ahí siempre. Por todo eso, gracias.

Evidentemente, no puedo olvidarme de, probablemente, lo mejor que me ha dado este tiempo realizando este trabajo: las valquirias. Y no, no me refiero a los personajes de la mitología nórdica, me refiero a Aroa, Blanca, Raquel, Pablo, Paloma, Rocío, Aldo, María, Amira, Augusto, Cris D, Cris C y Javi. Son todas unas investigadoras e investigadores increíbles y aún mejores amigas y amigos.

También, quiero agradecer a Luis y a Rafa, porque me enseñaron todo lo que se sobre AFM y con su experiencia me ayudaron a sacar a delante algún que otro experimento. A dos alumnas excepcionales que tuve, Eva y Carla, que ayudaron en este trabajo. Al doctor Félix Rico, por aceptarme en su grupo de investigación para realizar la estancia de investigación y a todo su equipo. Esa estancia para mí fue un soplo de aire fresco donde aprendí muchísimo y creo crecí, no solo como investigador, sino también personalmente. À la Docteure Claire Valotteau, pour toutes les heures passées au microscope, et tout ce que j'ai appris auprès d'elle. Sa passion et son dévouement m'ont inspiré au moment où j'en avais le plus besoin et m'ont redonné vocation à la recherche que je pensais avoir perdue. Gracias también, en especial, a Natalia, Mar y Eider por la maravillosa acogida e inclusión que recibí nada más llegar. Sois increíbles chicas. Por último, agradecer a mis directores, el doctor José Pérez Rigueiro y el doctor Gustavo Guinea Tortuero por aceptarme y confiar en mí para desarrollar este trabajo. Agradecer también al programa propio de la Universidad Politécnica de Madrid con Santander universidades por el apoyo económico que ha hecho posible este trabajo.

Se que probablemente me olvidé de algunas personas, pero, para todas aquellas personas que me han ayudado en algún momento, gracias.

Me gustaría terminar haciendo algo poco ortodoxo. Quiero dar las gracias a una última persona. Esa persona soy yo. Un enorme gracias a todas mis versiones que han ido surgiendo durante este trabajo, por todo el esfuerzo, todas las horas dedicadas, toda la pasión, toda la vocación. Porque perseverasteis y al final nunca os rendisteis. Al final lo conseguimos.

Abstract

Atomic force microscopy is a near-field microscopy technique that has been used for the study of biological systems for several decades. This is because it is a technique that not only allows us to obtain images, but also allows us to measure other physical quantities such as forces, which allows the characterization of biological systems from different approaches. Furthermore, it is possible to carry out studies in environments that replicate the native physiological conditions of these systems. One of the most widespread applications is related to measuring the interaction forces between biomolecules that interact with one another. However, even though it is a powerful technique, it requires special preparation to be able to study this type of interactions, which means that the technique is not, procedurally, fast and straightforward. Thus, the usage of atomic force microscopy for the characterization of biological systems usually involves the chemical modification of the probes and the surfaces where the molecules to be studied are going to be fixed. For this reason, in this work, whose objective is to develop affinity atomic force microscopy, in order to detect interactions between biomolecules, novel probes and commercial surfaces already chemically modified with amino groups will be used. For all these reasons, this work is structured in three parts: the first part addresses a characterization process of commercial probes using various techniques to evaluate and understand their main features. The second part consists of developing a series of protocols to modify and fix, using different crosslinkers, the biomolecules of interest used in this work. And finally, a final part dedicated to the measurements of biomolecular interactions using the probes and surfaces prepared with some of the protocols developed, in addition to evaluating the performance of this type of commercial probes with respect to modified probes using alternative methods found in the bibliography.

From the entire experimental process, relevant information has been obtained after the characterization of the probes including, for example, the intrinsic parameters, the reactivity and temporal stability of the commercial probes or the initial state of the surfaces of these commercial products. Functionalized surfaces have also been chemically modified to switch the original amine groups to other reactive, such as stressed alkynes, azides or thiols, and with biomolecules such as biotin, streptavidin, as well as enzymes and antibodies. Biomolecular interactions have been measured with single-molecule resolution using two different model systems: biotin-streptavidin and antigen (lactate dehydrogenase, LDH)-antibody (anti-LDH).

Resumen

La microscopía de fuerza atómica es una técnica de microscopía de campo cercano utilizada para el estudio de sistemas biológicos desde hace varias décadas. Esto se debe a que es una técnica que no sólo permite obtener imágenes, sino que también permite medir otras magnitudes físicas tales como fuerzas, lo que permite la caracterización de sistemas biológicos desde diferentes perspectivas. Adicionalmente, es posible realizar estudios en entornos que replican las condiciones fisiológicas nativas de estos sistemas. Una de las aplicaciones más extendidas está relacionada con la medida de las fuerzas establecidas entre biomoléculas que interaccionan entre sí. Sin embargo, a pesar de ser una técnica potente, su uso implica una preparación laboriosa que permita el estudio de este tipo de interacciones. Esta característica hace que la técnica no sea, procedimentalmente, ni rápida ni sencilla. Así, la utilización de la microscopía de fuerza atómica para la caracterización de sistemas biológicos suele implicar la modificación química de las sondas y de las superficies donde se van a fijar las moléculas a estudiar, constituyendo procesos relativamente largos y complejos. Por ello, en este trabajo, cuyo objetivo es desarrollar la microscopía de fuerza atómica de afinidad para detectar interacciones entre biomoléculas, se utilizarán sondas y superficies comerciales funcionalizadas con grupos amino. Por todo ello, este trabajo se estructura en tres partes: la primera parte aborda la caracterización de las sondas comerciales utilizando diversas técnicas para evaluar y comprender sus características fundamentales y que hasta ahora no se han determinado con suficiente precisión. La segunda parte consiste en el desarrollo de una serie de protocolos para modificar y fijar, utilizando diferentes agentes entrecruzantes, las biomoléculas de interés utilizadas en este trabajo. Y finalmente, una última parte dedicada a las medidas de interacciones biomoleculares utilizando las sondas y superficies preparadas con algunos de los protocolos desarrollados, además de evaluar el rendimiento de este tipo de sondas comerciales respecto a sondas modificadas utilizando métodos alternativos más comunes encontrados en la bibliografía.

De todo el proceso experimental se ha obtenido información relevante tras la caracterización de las sondas incluidas, por ejemplo, los parámetros intrínsecos, la reactividad y estabilidad temporal de las sondas comerciales o el estado inicial de las superficies de dichos productos comerciales. Las superficies funcionalizadas también se han modificado químicamente para sustituir los grupos aminos originales por otros grupos reactivos tales como alquinos tensionados, azidas o tioles. A estos grupos se les han unido biomoléculas como biotina, estreptavidina, así como enzimas y anticuerpos,

demostrándose que estas biomoléculas siguen siendo funcionales tras el proceso de unión. Y, por último, en el tercer bloque, se han medido las interacciones biomoleculares con resolución de molécula única utilizando dos sistemas modelo diferentes: biotina-estreptavidina y antígeno (lactato deshidrogenasa, LDH)-anticuerpo (anti-LDH).

TABLE OF CONTENTS.

1. INTRODUCTION.	23
1.1. Atomic force microscopy.	23
1.1.1. Parts of an atomic force microscope.	23
1.1.2. The atomic force microscope probes. Resonance frequency and spring constant.	25
1.1.3. Operation modes on AFM	29
1.1.4. Measuring forces with an atomic force microscope.	30
1.2. Surface functionalization	31
1.3. Crosslinking chemistry.	34
1.3.1. Pyridyl disulfide crosslinkers (SPDP).	35
1.3.2. Click (azide/stressed alkyne) chemistry crosslinkers.	37
1.4. Atomic force microscopy for the study of molecular interactions	39
1.4.1. Cantilevers selection and functionalization for SMFS and affinity microscopy.	40
1.4.2. Distinguishing between specific and non-specific interactions.	43
1.4.3. Biotin-streptavidin complex as a model system for the study of biomolecular interactions.	49
2. MOTIVATION AND OBJECTIVES OF THIS WORK.	55
3. MATERIALS AND METHODS.	57
3.1. Materials used for developing this work.	57
3.2. Sample Cleaning.	59
3.3. Characterization of DeepTip™ AFM probes and MicroDeck™ substrates.	59
3.3.1. Functionality of the amino layer. Temporal stability of the layer.	59
3.3.2. Topographic analysis of the surface.	60
3.3.3. SEM observation of functionalized tips.	61
3.3.4. Characterization of resonance frequency and spring constant of functionalized cantilevers.	61
3.4. Chemical modification of DeepTip™ and MicroDeck™ functionalized samples...	62
3.4.1. Modification with thiols.	62
3.4.2. Modification with azide and alkyne.	63
3.4.3. Modification with biotin.	65
3.5. Decoration of the surfaces with biomolecules.	65
3.5.1. Decoration with antibodies. Temporal stability of the samples.	65

3.5.2. Decoration with proteins using SPDP crosslinkers.	66
3.6. Affinity microscopy and force spectroscopy measurements.	69
3.6.1. Biotin-streptavidin system.....	69
3.6.2. LDH/anti-LDH affinity measurements.....	72
3.6.3. SMFS of titin. Yields Comparison of functionalisation methods.....	74
4. RESULTS AND DISCUSSION.....	77
4.1. Characterization of DeepTip™ AFM probes and MicroDeck™ substrates.	77
4.1.1. Functionality and temporal stability of the amino layer.	77
4.1.2. Surface topography of functionalized samples	81
4.1.3. SEM observation of functionalized cantilevers.	85
4.1.4. Characterization of resonance frequency and spring constant of DeepTip™ cantilevers.....	86
4.2. Chemical modifications of DeepTip™ probes and MicroDeck™ substrates.	88
4.2.1. Chemical modification with thiols.....	88
4.2.2. Chemical modification with azide and alkyne (click chemistry).	91
4.3. Decoration of the modified surfaces with biomolecules.	93
4.3.1. Decoration with streptavidin-biotin.....	93
4.3.2. Decoration with antibodies. Temporal stability of the samples.	94
4.3.3. Decoration of thiol-modified surfaces.....	97
4.4. Affinity atomic force microscopy and force spectroscopy experiments.	102
4.4.1. Characterization of the biotin-streptavidin system.....	102
4.4.2. Characterization of the lactate dehydrogenase-anti-(lactate dehydrogenase) antibody system.....	115
4.4.3. SMFS of titin. Yield comparison of functionalisation methods.	123
5. CONCLUSIONS.	127
6. FUTURE WORK.....	129
REFERENCES.....	131
ANNEXES.....	141

LIST OF FIGURES.

Figure 1. Schematic representation of the parts of an atomic force microscope. Adapted from [2].	24
Figure 2. Example of an AFM chip showing two cantilevers.	24
Figure 3. Deflection-displacement approach curve (Left). Approach and retraction force-displacement curves are shown (Right).	30
Figure 4. Schematic structure of a self-assembled monolayer generated on gold from an alkanethiol. R represents a generic functional group.	32
Figure 5. Scheme of the conventional liquid silanization process with an aminosilane as a precursor.	33
Figure 6. Representation of SPDP crosslinker molecule and reactions that can be carried out.	36
Figure 7. The two types of click chemistry reactions based on alkynes and azides.	38
Figure 8. Various strategies for surface functionalization and immobilization of biomolecules for experiments studying molecular interactions with AFM [59].	42
Figure 9. Difference between specific and nonspecific interactions according to the shape of the F-Z curve. Adapted from [123]	44
Figure 10. Scheme of the typical patterns found in F-Z curves for various configurations of biomolecules and associated fingerprints [67].	45
Figure 11. (a) Generic plot of rupture or unbinding force vs $\ln(l_r)$. (b) Generic presentation of an energy landscape showing the transition between the bound state and the unbound state, as well as the parameters $x_{\square}$ and k_{off} . Extracted from [83].	48
Figure 12. Three-dimensional ribbon model of the tetrameric protein streptavidin (Left). Three-dimensional model of the streptavidin subunit (Center). Structural formula of the biotin molecule (Right).	49
Figure 13. Schematic representation of the AFM imaging process for surface roughness analysis.	60
Figure 14. Schematic diagram of the samples used during the AFM test. Modified from [111]	70
Figure 15. Schematic of the sample preparation process for affinity microscopy measurements in the LDH-anti-LDH system. [40].	73
Figure 16. Fluorescence micrographs corresponding to various DeepTip™ probes. (A) DeepTip™ SiN R67-600, (B) DeepTip™ SiN R67-300, (C) DeepTip™ SiN R67-150 and (D) non-functionalized control sample.	77

Figure 17. Bar chart comparing the average fluorescence intensity of DeepTip™ SiN R67-150, DeepTip™ SiN R67-300 and nonfunctionalized control probes.	78
Figure 18. Bar chart comparing the mean fluorescence intensity of DeepTip™ SiN R67-150 at increasing times.	79
Figure 19. Fluorescence micrograph of the DeepTip™ SiN R11 (A), DeepTip™ SiN R15 T125 (B) and DeepTip™ SiO R1200 (C) models.....	80
Figure 20. Fluorescence micrograph of MicroDeck-G-150 (A, B) and MicroDeck-m-300 (D, E) substrates. Control substrates, glass (C), mica (F).	81
Figure 21. Representative AFM topography images obtained using. Control sample (A), DeepTip™ SiN R67-150 (B) and DeepTip™ SiN R67-300 (C)	82
Figure 22. Representative AFM topography images at different positions along the cantilever in the DeepTip™ SiN R67-150 (top), DeepTip™ SiN R67-300 (middle) models and nonfunctionalized control sample (bottom).	83
Figure 23. Representative AFM topography images at two different scan sizes of nonfunctionalized control surfaces (top) and MicroDeck™-G-150 surfaces (bottom)	84
Figure 24. SEM micrographs of AFM tips (A)control sample, (B) DeepTip™ SiN R67-150, and (C) DeepTip™ SiN R67-300.....	86
Figure 25. Bar chart showing the average values of resonance frequency for cantilevers of two different geometries belonging to the DeepTip™ SiN R67-150 and DeepTip™ SiN R67-300 probes.	87
Figure 26. Bar chart showing the average values of spring constant for cantilevers of two different geometries belonging to the DeepTip™ SiN R67-150 and DeepTip™ SiN R67-300 probes.....	88
Figure 27. Representative fluorescence micrographs of samples modified with sulfo-LC-SPDP (A), control sample with amino groups (B) and sample modified with aldehyde groups (C). Bar chart (D) comparing the average fluorescence intensity between the different samples.	89
Figure 28. Representative fluorescence micrographs of MicroDeck™ surfaces with amino groups (A) and modified with aldehyde groups (B), OPSS (C) and sulfo-LC-SPDP (D).....	90
Figure 29. Bar chart comparing the average fluorescence intensity between the different probes modified with thiol groups.	90
Figure 30. Representative fluorescence micrographs of probes modified with DBCO (A), nonfunctionalized probe incubated with DBCO (B), and functionalized probe not modified with DBCO (C). Bar chart comparing the average fluorescence intensity between the different samples (D).....	92

Figure 31. Representative fluorescence micrographs of probes modified with azide (A), nonfunctionalized probe incubated with azide (B) and functionalized probe not modified with azide (C). Bar chart (D) comparing the average fluorescence intensity between the different samples.	93
Figure 32. Representative fluorescence micrographs of probes decorated with biotin-PEG-NHS (A) and control probe (B).....	94
Figure 33. Representative fluorescence micrographs of probes decorated with an anti-LDH antibody (A) and control probe (B) obtained with a secondary fluorescent antibody.	95
Figure 34. Bar chart comparing the average secondary antibody fluorescence intensity between the anti-LDH-decorated probe and the control.	95
Figure 35. Bar chart comparing the mean fluorescence intensity of DeepTip™ SiN R67-150 decorated with an anti-LDH antibody over time.	96
Figure 36. Biotin-HRP standard line for colorimetry assays.	99
Figure 37. Bar chart showing the absorbance variation of samples decorated with different amounts of LDH through different thiol-based cross-linkers.	101
Figure 38. Representative curves of each of the six types in which the F-Z curves of the streptavidin-biotin system were classified. A seventh type (not shown) is comprised by the discarded curves.	103
Figure 39. Histogram displaying the number of curves grouped by the maximum value of adhesion force value for (A) functional streptavidin and (B) streptavidin blocked with biotin-BSA. The type in which the curves are classified is indicated in the legend.	105
Figure 40. Difference in the percentage of curves corresponding to a given type as a function of the number of experiments for (A) pristine substrate decorated with streptavidin and (B) after incubation with biotin-BSA. The percentage difference is calculated by subtracting the percentage of curves of a given type in an interval of 50 curves (e.g., curves from 51 to 100) from the arithmetic mean obtained considering all the curves during the complete experiment	106
Figure 41. Chart showing the percentages of each group of curves under both experimental conditions: pristine streptavidin-decorated substrate (without BSA) and after incubation with BSA (blocked streptavidin). The groups are defined as follows: Type 1: no interaction; Type 2: nonspecific interaction; Type 3: single interaction; Type 4: multiple interactions with independent detachment; Type 5: multiple interactions with simultaneous detachment; Type 6: multiple interactions with combined independent and simultaneous detachment; Type 7: discarded F-Z curves.....	107
Figure 42 Representative F-Z curves of the streptavidin-biotin system for each retraction speed.	111

Figure 43. Top: Mean rupture force values obtained for each retraction speed used. Bottom: Representation of the values of mean rupture force vs Ln of the loading rate. The linear fit is shown in blue ($R^2=0.98$, Pearson's $r= 0.99$).	112
Figure 44. Comparison of the results obtained by applying the Bell-Evans model to the data presented in this Thesis with those found in previously published works [106]....	113
Figure 45. Representative curves of each of the groups in which the F-Z curves were classified.	114
Figure 46. Percentages obtained for each type of curve according to the functionalized AFM probe used to perform the test.	115
Figure 47. Representative force-displacement (F-z) curves obtained from the interaction between LDH and anti-LDH antibody for the types: (i) no-interaction; (ii) elastomeric; (iii) non-elastomeric; (iv) elastomeric with two peaks; (v) double-peak combined; and (vi) three- or four-peaks. Compression forces are positive, and positive displacement corresponds to the retraction of the AFM probe from the substrate.	117
Figure 48. Percentages of each type of F-Z curves: Type 1: 47%—no-interaction; Type 2: 10%—elastomeric curves; Type 3: 26%—non-elastomeric; Type 4: 2%—elastomeric two-peaks; Type 5: 7%—combined elastomeric/-non-elastomeric; Type 6: 1%— threefold or fourfold elastomeric peaks; Type 7: 7%—discarded curves.....	118
Figure 49. Distribution of adhesion forces of the LDH/anti-LDH system obtained from type 2 curves.....	119
Figure 50. Statistical distribution of the force-displacement curves corresponding to elastomeric and non-elastomeric peaks as a function of the measured adhesion force..	122

LIST OF TABLES.

Table 1. Summary of some of the strategies for the functionalization of AFM probes	41
Table 2. Summary of the parameters obtained in different studies in relation to the biotin-streptavidin interaction.	50
Table 3. Nominal resonance frequency and spring constant of the main models of DeepTip™ cantilevers used in this work. When more than one resonance frequency and spring constant is presented, each value corresponds to one of the cantilevers of the probe.....	57
Table 4 RMS and Ra surface roughness values of different models of DeepTip™ and nonfunctionalized control probes	82
Table 5. RMS roughness values of different samples belonging to MicroDeck-G150 surfaces.....	85
Table 6. Average values of resonance frequency and spring constant of cantilevers belonging to different DeepTip™ models.....	87
Table 7. Significance p-values for samples incubated with antibody vs. control samples for different days after the decoration process.	96
Table 8. Absorbance values obtained during the modification process of the Prup3 protein.....	97
Table 9. Absorbance values obtained during the modification process of the streptavidin protein.	97
Table 10. Absorbance values obtained from surfaces decorated with streptavidin after colorimetric assays.....	99
Table 11. Statistical significance of the differences obtained from the enzymatic test between substrates decorated with LDH under different conditions.	102
Table 12 Percentages obtained from each type of curve depending on the type of tip used to acquire them.	124

LIST OF ACRONIMS.

- A-AFM: Affinity Atomic Force Microscopy.
- AFM: Atomic Force Microscopy.
- APDMS/APDMES: 3-Aminopropyldimethylethoxysilane.
- APTES: 3-Aminopropyltriethoxysilane.
- ATP: Adenosine Triphosphate.
- AVS: Activated Vapor Silanization.
- BSA: Bovine Serum Albumin.
- CFM: Chemical Force Microscopy.
- CoA: Coenzyme A.
- CuAAC: Cu(I)-catalyzed azide–alkyne cycloaddition.
- DBCO: Dibenzocyclooctyne.
- DFS: Dynamic Force Spectroscopy.
- DMF: N N-Dimethylformamide.
- DMSO: Dimethyl Sulfoxide.
- DNA: Deoxyribonucleic acid.
- DPBS/PBS: Dulbecco's Phosphate Buffered Saline/ Phosphate Buffered Saline.
- DT: Deep Tip.
- EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide.
- EDTA: Ethylenediaminetetraacetic acid.
- EWLC: Extended Worm Like Chain.
- FESEM: Field Emission Scanning Electron Microscopy.

- FITC: Fluorescein Isotiocyanate.
- FJC: Freely Jointed Chain.
- GA: Glutaraldehyde.
- HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
- HRP: Horseradish peroxidase.
- IAF: Iodoacetamidofluorescein.
- LDH: Lactate dehydrogenase.
- MAL: Maleimide.
- MD: Micro Deck.
- MEMS: Micro-Electromechanical Systems.
- NADH: Nicotinamide adenine dinucleotide.
- NHS: N Hydroxysuccinimide.
- NTA: Nitrilotriacetic acid.
- OLS: Optical Lever Sensitivity.
- OPSS: Ortho-pyridyl disulfide.
- PEG: Polyethylene glycol.
- RNA: Ribonucleic acid.
- ROI: Region of Interest.
- RMS: Root Mean Square.
- SAM: Self-assembled monolayer.
- SDS: Sodium dodecyl sulfate.
- SFP: 4'-phosphopantetheinyl transferase.
- SMFS: Single Molecule Force Spectroscopy.

- SPDP/LC-SPDP: N-Succinimidyl 3-[2-pyridyldithio]propionate/ Long Chain N-Succinimidyl 3-[2-pyridyldithio]propionate.
- SPAAC: Strain-promoted alkyne-azide cycloaddition.
- TCEP-HCl: Tris(2 carboxyethyl)phosphine hydrochloride.
- TES: Triethylsilane.
- TMB: Tetramethylbenzidine.
- UV: Ultraviolet.
- WLC: Worm Like Chain.

1. INTRODUCTION.

1.1. Atomic force microscopy.

Atomic force microscopy (AFM) is a near-field microscopy technique invented and developed by G. Binning, C.F. Quate and Ch. Gerber in 1985 [1]. Like other near-field microscopy techniques, it is based on the fact that the information necessary for the acquisition of data that will form an image is obtained from the interaction that arises between a sharp tip and the surface of the sample of interest. However, the possibilities of atomic force microscopy are not currently limited to image acquisition, but it is possible, for example, to measure forces and obtain mechanical parameters or even perform nanomanipulation tasks on the surface of the samples, all at the nanoscale, with spatial resolution of tenths of nanometres and resolution in forces of piconewtons. Furthermore, this technique has a great advantage over its predecessor, tunnel effect microscopy, developed by H. Rohrer and G. Binning as well, and that is that it does not require the sample to be electrically conductive, which opens the possibility of studying an enormous range of samples.

1.1.1. Parts of an atomic force microscope.

Despite the great variety of configurations and models of AFMs that currently exist on the market, in all of them can be found three well differentiated parts, as can be seen in Figure 1: (i) the positioning system of the sample and of the probe, (ii) the detection system, and (iii) the control system. Each of these parts presented below.

i. Positioning system. This part consists of a surface located on a piezoelectric actuator that allows the movement of the sample in the three spatial directions with nanometric precision with respect to the sharp probe (AFM tip). This sharp probe is located at the end of a microcantilever that stems from a more massive piece of material ordinarily called “chip” as can be seen in Figure 2. The surface of the cantilever opposite to that of the tip is totally or partially covered by a thin sheet of reflective metal with a thickness of a few tens of nanometres. Microcantilevers are considered MEMS (microelectromechanical systems) and are manufactured using microfabrication technologies, such as photolithography, etching and deposition techniques. This fabrication process allows to produce these structures with sufficient precision. In most

microscopes, it is the sample that moves with respect to the probe, although there are microscopes that also allow the movement of the probe along the Z direction.

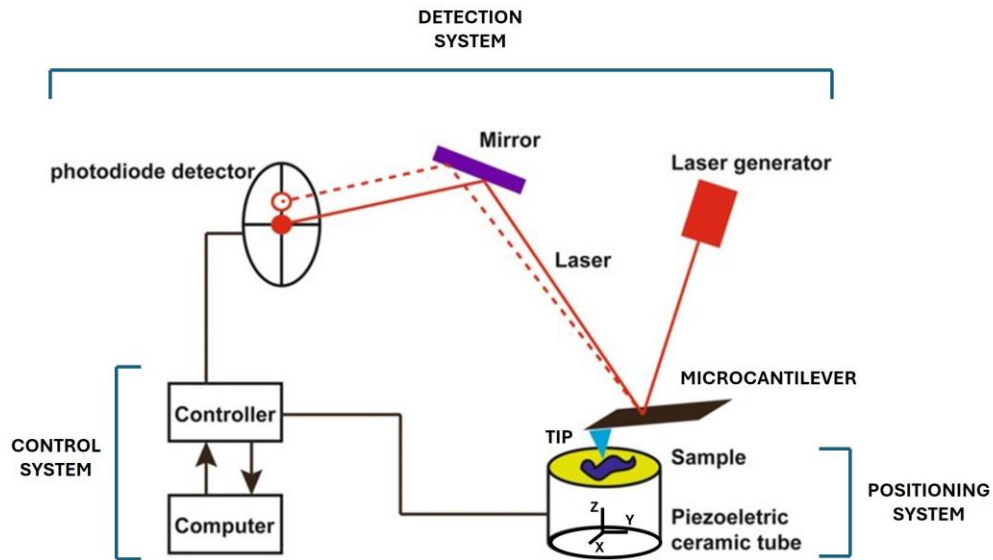


Figure 1. Schematic representation of the parts of an atomic force microscope. Adapted from [2].

(ii) The detection system consists of two main parts: a laser and a photodiode. Their operation goes as follows. When the sharp tip at the end of the microcantilever is close to the surface of the sample or directly in contact, a series of interaction forces arise between the tip and the sample that cause the deflection of the microcantilever. Meanwhile, on the reflective surface of the cantilever, a laser is focused and reflects directly onto a photodiode. As the cantilever is deflected, a change in position of the laser beam on the photodiode occurs. The photodiode detects this change in light intensity and transforms it into an electrical signal (voltage) that is measured and used for the feedback system.

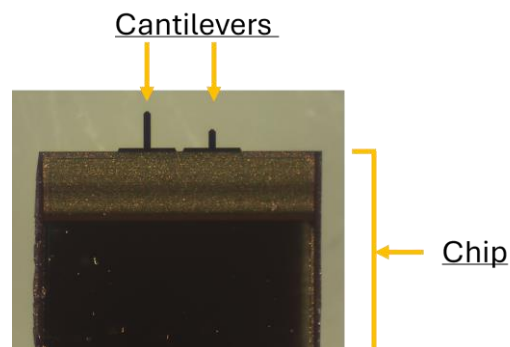


Figure 2. Example of an AFM chip showing two cantilevers.

(iii) Control system. Finally, the control system is made up of a series of electronic circuits that process electrical signals and a computer and associated software to control the operating parameters of the microscope.

Next, we are going to look in more detail at some of the characteristics of the microcantilevers, as they are of special relevance in this work.

1.1.2. The atomic force microscope probes. Resonance frequency and spring constant.

As we have already commented in the previous section, one of the basic elements of an atomic force microscope is the probe that will interact with the sample.

Currently, there is a great variety of probes on the market since, depending on the type of sample, on the experimental conditions or on the objective of the experiments, different probe models may be needed to get optimum results. To give us an idea, the probe is the tool that we are going to use to carry out our experiments in the same way that when we are going to join two pieces of material, we will use the most appropriate adhesive that meets the characteristics we need. Since there is a wide range of AFM commercial probes, these can be classified considering, among other features, the geometry of the tip or the geometry of the cantilever. Although we have mentioned that the tip is usually a sharp apex, this is not always the case, and different geometries such as pyramidal or spherical are possible. There are even tips that are made of carbon nanotubes and some probes are tipless for applications in experiments such as those performed for single cell force spectroscopy. The most common geometries of the cantilever are the rectangular and the triangular, although more peculiar geometries such as "T" or arrowhead shapes can also be found. With respect to the material, AFM probes are very often made up of silicon and silicon nitride. The combination of material and geometry of the probe yields the two most important parameters that define its performance: the resonance frequency and the spring constant. We are going to look at these two parameters in more depth, since they are decisive in most cases for the success of the experiments.

1.1.2.1. Resonance frequency of the cantilevers.

The resonance frequency corresponds to that frequency at which the oscillation amplitude at the end of the cantilever is maximum for a given excitation. Resonance frequency is especially important in those AFM experiments operating in a dynamic mode.

In cantilevers with a constant rectangular cross section, the resonance frequency (ν_0) can be defined mathematically in several ways, as it is shown in equations (1) and (2). Both equations are equivalent but expressed with different independent magnitudes.

$$\nu_0 = 0.1615 \frac{t_c}{L^2} \sqrt{\frac{E}{\rho}} \quad (1)$$

$$\nu_0 = \frac{1}{2\pi} \sqrt{\frac{k_c}{m^*}} \quad (2)$$

Where t_c is the thickness of the cantilever, L is the length, E refers to the Young's modulus and ρ is the density in equation (1) and k_c is the spring constant and m^* is the mass in equation (2). As can be seen in equation (2), the resonance frequency depends on the mass (m^*) of the cantilever which implies that if the mass of the cantilever varies, the resonance frequency will also vary.

This variation in the resonance frequency due to the change in mass in the cantilever can be used as a calibration method for the other intrinsic mechanical parameter, the spring constant.

In this work, as will be discussed in later chapters, this variation in the resonance frequency will be used as a method of comparing the pristine (non-functionalized) and functionalized cantilevers provided by Bioactive Surfaces S.L.

1.1.2.2. Spring constant of the cantilevers.

The other important intrinsic mechanical parameter for the interpretation of many AFM experiments, especially those whose objective is to measure and detect forces regardless of their origin, is the spring constant. For rectangular cantilevers of constant cross section, the spring constant can be calculated from the material properties and the geometric dimensions of the cantilever as shown in equation (3).

$$k_c = \frac{Ewt_c^3}{4L^3} \quad (3)$$

Where E is the Young's modulus, w is the width of the cantilever, t_c is the thickness of the cantilever and L is the length of the cantilever. Since the spring constant depends on the material and on the geometry, there are many combinations that give rise to commercial cantilevers available with a wide range of spring constants, typically from 0.01 N/m to 40 N/m.

However, in practice, the spring constant of a given cantilever is not usually calculated with equation (3), since the elastic constants calculated experimentally differ significantly from those calculated theoretically [3][4]. These differences may be due to different reasons. For example, the properties of a thin layer of a certain material may vary from the properties of the bulk material. In this specific case we are referring to the elastic modulus as can be seen in the study published by [5]. Furthermore, as these structures are the result of an industrial manufacturing process, there may be geometric variations between them. All of these variations can cause the spring constant of nominally identical cantilevers to vary significantly.

Therefore, there are a series of experimental calibration methods that allow us to know the spring constant of the cantilever that is being used for a given experiment in order to obtain accurate data. Specifically, we are going to present the three most used calibration methods: Cleveland, Sader and thermal noise:

Cleveland's Method

This calibration method [6] is based on measuring the variations in resonance frequency that occur in the same cantilever with a mass attached to the end and without that same mass. By measuring the resonance frequencies and by characterizing the value of the mass used, it is possible to calculate the spring constant by applying equation (4).

$$k_c = \frac{4\pi^2 M}{\frac{1}{v_0'^2} - \frac{1}{v_0^2}} \quad (4)$$

Where M is the added mass, v_0' is the resonance frequency of the loaded cantilever and v_0 is the resonance frequency of the unloaded cantilever.

Sader's method:

This method was proposed by Sader et al [7] and consists of determining the spring constant from the resonance frequency, the quality factor (Q) and the dimensions of the cantilever (for rectangular cantilevers) according to equation (5).

$$k_c = 0.1906\rho_f w^2 L Q \Gamma_i(Re) \omega_0^2 \quad (5)$$

Where ρ_f is the density of the fluid surrounding the cantilever, w is the width of the cantilever, L is the length of the cantilever, Γ_i is the imaginary part of the hydrodynamic function as a function of the Reynolds number (Re) and ω is the angular frequency.

This is the method that is mainly used in this work since it is the one implemented in the WSxM [8] AFM control software employed for the experiments.

Method of Hutter and Bechhofer or of the thermal noise

This method is based on measuring the thermal noise of the cantilever and modelling the behaviour of the cantilever as a harmonic oscillator where the mean square deflection of the fundamental mode of oscillation due to thermal fluctuations gives rise to equation (6) by equating this deflection to the equipartition theorem [9].

$$\frac{1}{2} k_c \langle \Delta Z_c^2 \rangle = \frac{1}{2} k_B T \Rightarrow k_c = \frac{k_B T}{\langle \Delta Z_c^2 \rangle} \quad (6)$$

Where k_B is the Boltzmann's constant, T is the absolute temperature and Z_c is the deflection of the cantilever. This method is the one implemented in most commercial microscopes today.

As can be seen, determining the elastic constant of cantilevers is not a trivial issue. In fact, present methods that allow optimizing the parameter determination time in different conditions are being developed [10] including methods that allow calibrating cantilevers regardless of their geometry [11].

1.1.3. Operation modes on AFM

As has been commented above, the acquisition of information in AFM experiments is carried out by monitoring the interaction that occurs between a sharp tip and the sample. Depending on the behaviour of the probe with respect to the sample several operating modes can be distinguished, such as contact, dynamic and F·Z mode. A brief discussion on each of these modes is presented below.

Contact mode.

Contact mode is the most basic way to operate an AFM. In this mode the tip is in physical contact with the surface of the sample during scanning. In this mode, the deflection of the cantilever is kept constant through a feedback loop that modifies the Z position of the sample while the surface is scanned. In this way, the image is formed from the displacements of the piezoelectric system along the z axis while maintaining a constant deflection of the cantilever.

Despite its relative simplicity, the contact mode has several disadvantages. The main and most important is associated with the intensive interaction between tip and sample that may lead, for instance, to the appearance of significant shear forces and prevents the study of soft samples, such as those found in biological systems.

Dynamic or tapping mode.

In the dynamic or tapping mode, the cantilever oscillates at its resonance frequency thanks to a small piezoelectric located in the AFM chip holder. Once the cantilever is oscillating, there are two main ways of working in the tapping mode, depending on the parameter that is kept constant through a feedback loop: amplitude modulation tapping mode (AM) and frequency modulation tapping mode (FM).

In the amplitude modulation mode, the amplitude of the cantilever oscillation is kept constant through a feedback loop. As the sample surface is scanned, when the tip detects a protrusion on the surface, its oscillation amplitude decreases, causing the system to move the sample in the normal direction (Z) to recover the previous value of the amplitude. Reversely, if the tip detects a "hole", and suffers an increase in the amplitude of oscillation, the piezoelectric system will tend to approach the tip to the sample to recover again the original amplitude.

The frequency modulation mode is more complicated both technically and in terms of the instrumentation required. This mode is based on maintaining the resonance frequency of the cantilever constant through the feedback loop. The resonance frequency will be affected by the forces generated between the tip and the sample, so that by measuring the difference between this resonance frequency when there is an interaction between tip and sample and when there is not such an interaction it is possible to generate an image of the surface.

Dynamic modes have certain advantages over the contact mode. In dynamic modes, ideally the tip does not touch the sample, and contact is occasional if the working parameters are appropriate. This implies that, since there is no contact, the sample will be better preserved, since the normal and friction forces generated by the contact of the tip with the sample are practically zero. Therefore, they are very interesting methods, especially when working with biological samples because these are normally very soft samples.

1.1.4. Measuring forces with an atomic force microscope

In addition to the previous methods, it is also possible to obtain very valuable information on the sample by recording the force-displacement curves that result from tip-sample interaction. These F-Z curves are illustrated in Figure 3.

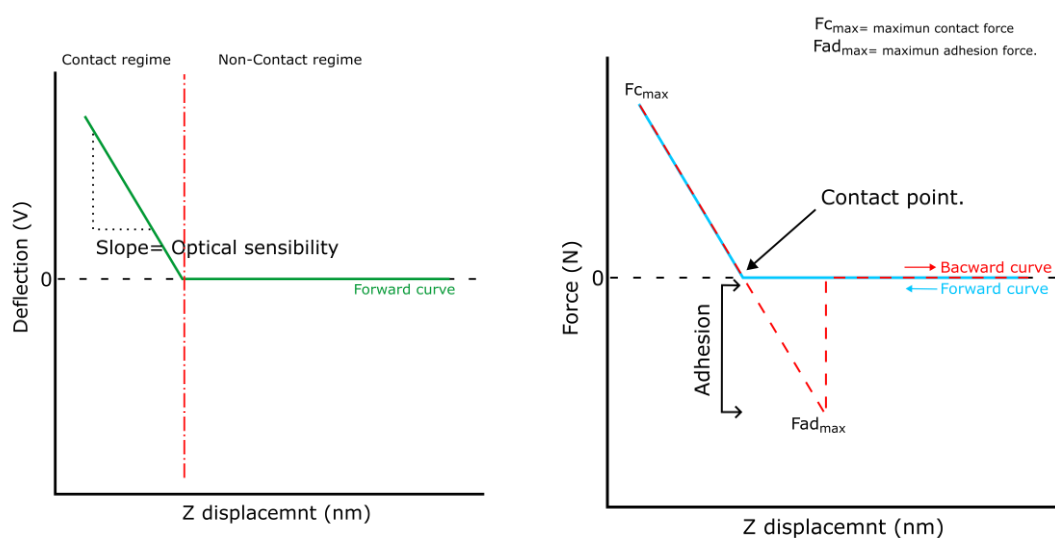


Figure 3. Deflection-displacement approach curve (Left). Approach and retraction force-displacement curves are shown (Right).

However, and as was already seen in the section on AFM instrumentation, the information on the deflection of the cantilever is obtained as voltage variations that result from the

different light intensity that impinges on the photodiode. So, how do we transform electrical magnitudes into units of force? To do this it is necessary to determine two experimental parameters: the optical lever sensitivity (OLS) and the spring constant of the cantilever. The 'optical lever sensitivity' relates the deflection of the cantilever with the voltage induced in the photodiode and can be obtained directly from the deflection-displacement curve, as the inverse value of the slope of the linear section in the contact regime (invers optical lever sensitivity (invOLS)) (Figure 3 (left)).

The spring constant can be calculated from one of the methods explained in section 1.1.2.1. Knowing these two parameters, and using the formula (7), it is possible to transform the deflection values of the curves obtained into force values in Newtons.

$$F[N] = deflection[V] * invOLS \left[\frac{m}{V} \right] * k_c \left[\frac{N}{m} \right] \quad (7)$$

Consequently, the F-Z curves are the final plot that is obtained after transforming the original voltage-displacement curves.

1.2. Surface functionalization

The term surface functionalization refers to the process of modifying a surface to impart it with new characteristics, either chemical, physical or biological, different from those of the original material [12]. Surface functionalization techniques have been used in various fields, however, one in which they have stood out greatly is in the field of biomedicine. In this specific field, the chemical modification of the surface with reactive functional groups that allow the formation of stable covalent bonds with biomolecules of interest is called biofunctionalization.

Biofunctionalization has been applied to various applications, such as the manufacture of biosensors, the improvement of the biocompatibility of biomaterials or the development of controlled drug release systems.

Depending on the nature of the modification process, it is possible to distinguish between physical and chemical methods. In physical methods, parameters such as temperature or

pressure are used to modify the surface of the material. These methods include, for example, laser ablation, polishing, or chemical vapor phase deposition.

The chemical methods are based on the use of chemical reagents that react with the surface of the material to modify it. By using chemical moieties that react covalently with the surface, functional groups such as thiols ($-SH$), carboxyls ($-COOH$), aminos ($-NH_2$), etc. can be introduced.

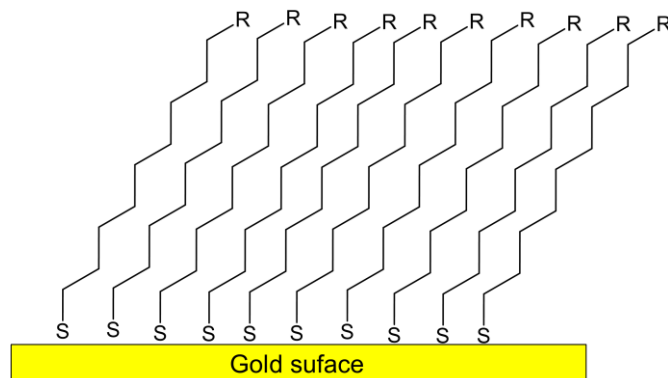


Figure 4. Schematic structure of a self-assembled monolayer generated on gold from an alkanethiol. R represents a generic functional group.

Among the most common chemical functionalization strategies may be mentioned those based on the formation of self-assembled monolayers (SAM's) and those broadly referred to as silanization processes. Self-assembled monolayers result from the spontaneous chemisorption of thiol groups present, for example, in aliphatic compounds on a gold surface, giving rise to stable S-Au covalent bonds (Figure 4). Conventional silanization consists of the covalent reaction between functional silane coupling organometallic agents and hydroxyl groups on the surface of the material, giving rise to siloxane-type structures (Figure 5).

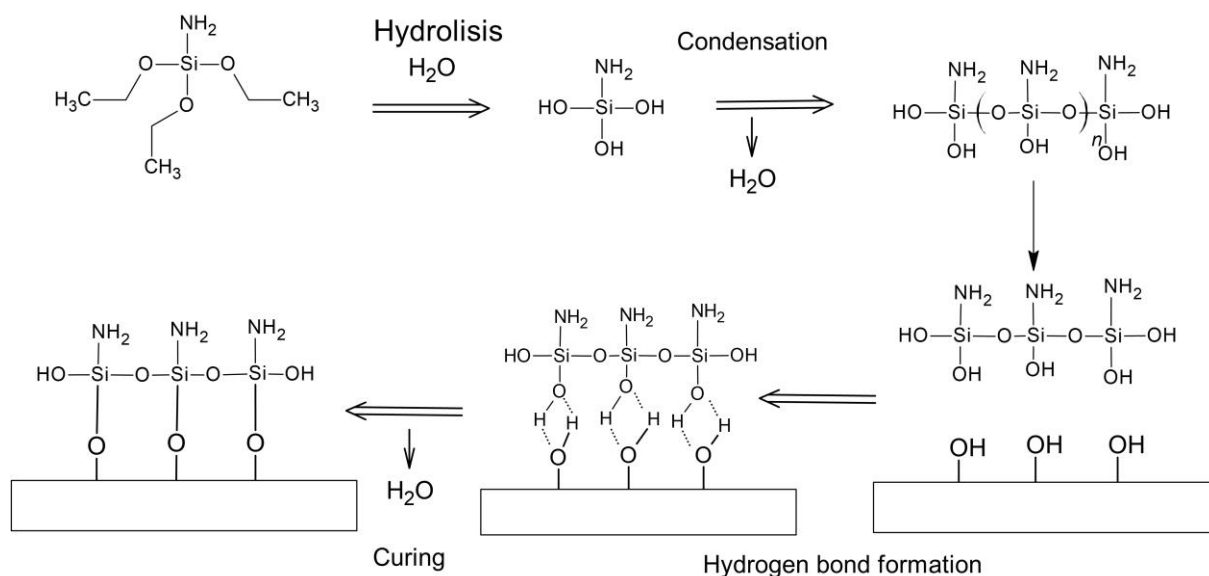


Figure 5. Scheme of the conventional liquid silanization process with an aminosilane as a precursor.

The silanization process can be carried out either in the liquid phase or in the vapor phase. In liquid phase silanization, the organosilane precursor is dissolved in an organic solvent (e.g. toluene, methanol, ethanol) and the samples to be functionalized are immersed in this solution. In vapor phase silanization the organosilane is evaporated and deposited on the surface of the material without the need to use an organic solvent. The usual setup used in vapor phase silanization consists of placing the samples in a desiccator along with a small amount of the organosilane of interest. Once prepared, a vacuum is generated inside the desiccator to remove water vapor and oxygen and is replaced by an atmosphere of an inert gas (usually argon or nitrogen). These low-pressure conditions make it easier for the organosilane to evaporate, generating an atmosphere of organosilane vapor and inert gas inside the desiccator. In this way the surfaces will be in contact with the generated gas, giving rise to the condensation of the compound on the material. This method, although efficient [13-16], is quite slow, since it may last up to 2 days [17]

An alternative to the conventional silanization methods was provided by the activated vapour silanization (AVS) process.

In this functionalization technique, 3-aminopropyltriethoxysilane (APTES) is used as an organic precursor to generate a thin film with a high density of amines at its surface and

is compatible with a wide variety of materials such as silicon, titanium, gold, silicon dioxide and silicon nitride[18-21].

1.3. Crosslinking chemistry.

Once the functionalized surfaces are obtained, it is usually necessary to modify the initial functional groups obtained to others that can bind the biomolecules of interest more efficiently. In this case, one of the preferred strategies to achieve this modification is the use of crosslinking agents or crosslinkers.

Crosslinkers are organic chemical compounds that have one or more reactive functional groups in their structure, so that allow the covalent binding of two or more molecules to each other. Due to their interest for a number of applications, there is a wide variety of crosslinkers, which can be classified according to: (i) the functional groups with which they react, especially whether a given crosslinker may bind to either just one or to several types of reactive groups, (ii) their linear or branched structure, and (iii) their molecular weight.

According to this last classification, crosslinkers are called heterofunctional if they have different functional groups or homofunctional if they have the same functional groups.

The main reactive groups to which crosslinkers can bind correspond to: thiol, aldehyde, amine, and carboxylic. In this regard, one of the most common reactive groups found in various crosslinkers is the N-hydroxysuccinimide ester (NHS ester). NHS reacts very efficiently with primary amines ($-NH_2$) at pH conditions between 7.2-8.5 with relatively short reaction times (30 min to 4 h) and over a wide range of temperatures, resulting in stable amide bonds between the crosslinker and the molecule that contains the amine group. However, during the reaction there is competition between the binding to primary amines and the hydrolysis of the functional group, a process that is favoured at increasing pH.

Additionally, crosslinkers with this functional group may show low solubility in aqueous medium, so that it is often necessary to dissolve first the compound in a polar organic solvent and resuspend it subsequently in an aqueous buffer. This low solubility in aqueous medium is solved with the used of sulfonated NHS-esters (sulfo NHS-esters). By including a sulfonated group in the molecule, the solubility of the compound in aqueous medium is increased without decreasing its ability to react with primary amines.

In the experimental work presented in later chapters, NHS ester-based crosslinkers have been used extensively to chemically modify the original amine-functionalized surfaces with other functional groups.

Additionally, heterobifunctional crosslinkers reactive towards thiol groups (pyridyl disulfide) and crosslinkers with functional groups of the “click” chemistry (azide/ stressed alkyne) have been used. These crosslinkers are commented on in greater detail below.

1.3.1. Pyridyl disulfide crosslinkers (SPDP).

N-succinimidyl 3-(2-pyridyldithio)propionate, known by its acronym SPDP, is a heterobifunctional cross-linker developed by J. Carlsson, H. Devin and R. Axén in 1978 [22]. Being a heterobifunctional cross-linker, it has two different reactive functional groups: an NHS group reactive towards amino groups, which has already been discussed in the previous section, and a pyridyl disulfide group reactive towards thiol groups (-SH) (Figure 6).

This reagent was formulated with the objective of creating protein conjugates without the concomitant formation of intramolecular crosslinks. Furthermore, it generated the possibility of introducing aliphatic thiols into molecules that contained amino groups, such as proteins. More recently, this cross-linker has been used for the conjugation of two different antibodies [23], an antibody and the F1-ATPase complex [24], or an antibody and an enzyme to generate an immunoconjugate to target solid tumour cells [25].

The synthesis method is relatively simple and does not involve many reactions since it is a two-stage procedure where 3-mercaptopropionic acid reacts with 2,2'-pyridyl disulfide and the resulting product is esterified using N-hydroxysuccinimide and NN'-diclohexylcarbodiimide.

When the pyridylthiol group reacts, a byproduct of the reaction, pyridine-2-thione, is released. Pyridine-2-thione presents optical activity at 343 nm, which allows to estimate the degree of reaction in a simple way. SPDP is soluble in polar solvent like DMSO or DMF, it can cross the plasmatic membrane and the reactions with the thiol groups can be carried out under very mild conditions of pH between 6.5-7.5.

Another advantage of this crosslinker is that there are pegylated forms. This means that aliphatic carbon chains can be inserted between the reactive functional groups with the aim of introducing a spacer between the functional groups. This allows, on the one hand, to enhance the solubility of the molecule directly in aqueous buffers, since pegylation

increases solubility in this environment and, on the other hand, allows the molecules conjugated with these variants to present greater mobility. In this case, the name given to the crosslinker is LC-SPDP (long chain SPDP), which, in turn, has a variant called sulfo-LC-SPDP that has greater solubility in water. Another variant has the abbreviated name OPSS-XKPEG-NHS, where XK refers to the molecular size of the peg introduced as a spacer (2K, 3K, 5K, 10K, ETC). These last two variants of the crosslinker are those used in some experimental parts of this work.

PEGylated forms have also been used in different studies, such as OPSS to functionalize silver nanocarriers with proteins to enhance the cytotoxic activity of an anticancer drug [26] or to conjugate peptides with chitosan with the aim of generating an RNA encapsulation system [27]. In the case of sulfo-LC-SPDP it has been used to modify surfaces with thiol groups with the objective of covalently joining proteins [28] or to functionalize carbon nanotubes with oligonucleotides as a vehicle in brain tumor models [29].

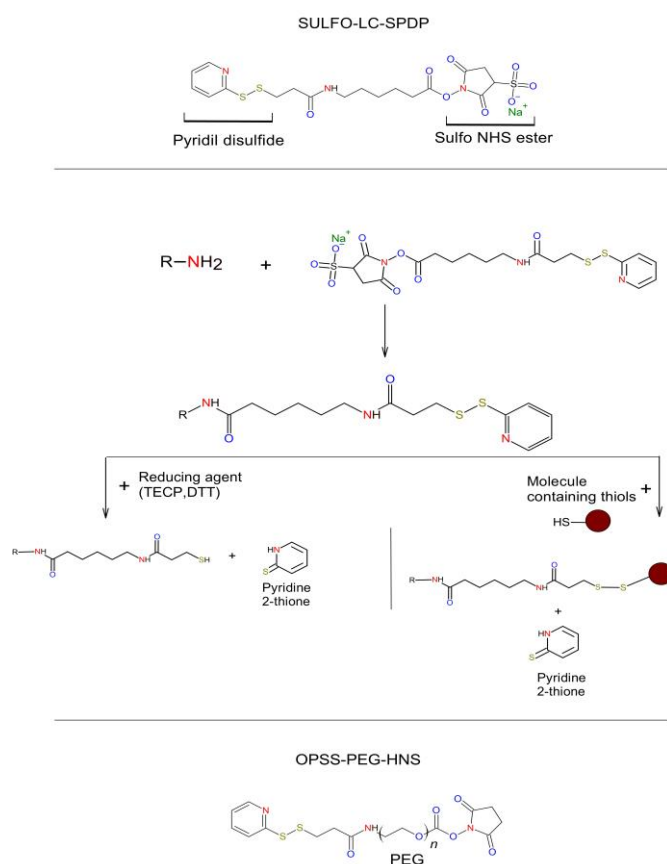


Figure 6. Representation of SPDP crosslinker molecule and reactions that can be carried out.

1.3.2. Click (azide/stressed alkyne) chemistry crosslinkers.

The term click chemistry was introduced by Sharpless et al. in 2001 [30]. The idea behind the click chemistry concept is based on the development of basic building blocks that react selectively and specifically with each other on both small and large scale to give rise to more complex hetero-atom linked (C-X-C) compounds. However, for a chemical reaction to be within the term click chemistry, it must meet a series of requirements:

- The reaction must be modular and high-performance. They typically have a high thermodynamic driving force, generally greater than 20 kcal mol⁻¹.
- The byproducts are not harmful or minimally harmful and can be easily eliminated using non-chromatographic methods.
- The byproducts must be stereospecific, but not necessarily enantioselective.
- The reaction conditions must be mild in terms of temperature and pH, as well as insensitive to water or air. In addition, they can be carried out without solvent or with non-harmful solvents.
- The starting compounds must be easy to obtain and easily available.
- The resulting compounds must be stable, covalent, and irreversible in general and, more specifically, under physiological conditions.

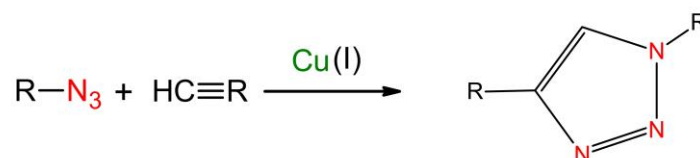
Due to the set of general characteristics that click chemistry reactions present, they have been used in a multitude of scientific fields [30-33] but in particular, they have a considerable impact on fields related to molecular biology, biochemistry, pharmacology, and other fields related to life sciences. In this regard, the bioorthogonality of many of click reactions is a desirable feature for their use in this field. The term orthogonal refers to a reaction that can occur in an aqueous environment, at physiological temperature and pH, and, in addition, the functional groups present in the precursor molecules of the reaction must be inert to other biomolecules, so they cannot be naturally found in biological systems.

Considering the characteristics that define click chemistry reactions, there are currently many types of reactions that meet these points. Among them are, for example, azide-alkyne cycloadditions, Diels-Alder addition, oxime/hydrazone formation, thiol-vinyl addition, thiol-en, thiol-isocyanate and 1,3-dipolar cycloaddition [33]

Of all these types of click chemistry reactions, we are going to comment on azide-alkyne cycloadditions, since they are one of the most used in life science fields because they also fulfil the bioorthogonality condition.

Within the azide-alkyne cycloadditions two types can be distinguished, copper-catalyzed reactions and copper-free reactions (Figure 7):

AZIDE-ALKYNE REACTIONS CATALYZED BY COPPER



COPPER FREE AZIDE-ALKYNE REACTIONS

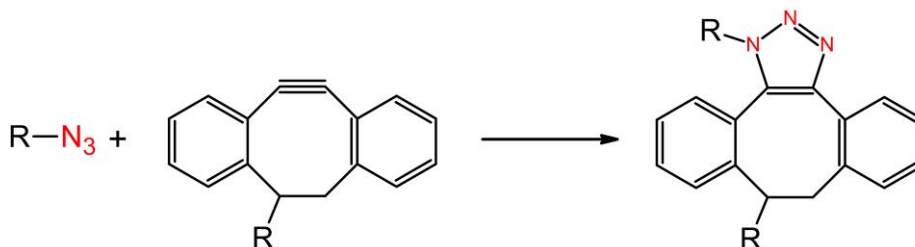


Figure 7. The two types of click chemistry reactions based on alkynes and azides.

As can be seen in Figure 7, in copper-catalysed cycloadditions (CuAAC) a compound containing an azide functional group and another compound containing a terminal alkyne are allowed to react in a medium with the presence of Cu(I), that acts as a catalyst, giving rise to a triazole derivative. On the other hand, in copper-free reactions [34], a compound with an azide functional group and a compound containing a strained-alkyne react (SPAAC), yielding again a triazole derivative.

Copper-catalysed reactions have been used in some studies, such as DNA labelling [35]. However, it has a great disadvantage, since the need for copper as a catalyst involves that the procedure is toxic for living systems [36]. Consequently, it can only be applied to isolated biomolecules.

In the case of copper-free reactions (SPAAC), this disadvantage disappears since the need to use copper is eliminated. In this way it can be used to crosslink not only isolated

biomolecules, but also those found in living organisms such as bacteria or cells, even under physiological conditions [37].

Due to the potential of azide/strained-alkyne reactions, nowadays, a wide variety of crosslinkers that include these functional groups have been developed. For this reason, they have been used in this work as crosslinkers to chemically modify the functionalized surfaces of AFM cantilevers.

1.4. Atomic force microscopy for the study of molecular interactions

Due to the unique characteristics of atomic force microscopy, which allow spatial resolutions of tenths of a nanometre and force measurements in the order of piconewtons, this technique has been used for several decades to study, not only the topography of a given surface, but also the interactions established between the tip and the sample. Special attention is devoted to the characterization of the interactions between different biomolecules or for the study of mechanical properties of these biomolecules, such as proteins or lipid membranes. Therefore, atomic force microscopy appears as an important tool for the study of biological systems at a molecular scale.

A bibliographical search allows finding references to various techniques that are used for the characterization of biological systems with an AFM, and that share some points in common. One of the most common approaches is the commonly named single molecule force spectroscopy (SMFS) [38], which is intended to measure the mechanical properties of a given biomolecule. Additionally, the terms chemical force microscopy (CFM) [39] or, alternatively, the term affinity microscopy (A-AFM) [40], is used to refer to those experiments in which a target molecule on a surface may be identified through its interaction with a sensor molecule immobilized on the AFM tip. Both SMFS and A-AFM share some common characteristics but will be presented separately below.

In single molecule force spectroscopy studies, two different types of experiments are usually performed: (i) the stretching of molecules, usually polymer chains, to measure intramolecular forces and other physical parameters, such as potential barriers between conformational states [41], and (ii) the detachment between two interacting molecules. This first type is very commonly termed as 'unfolding' studies [42, 43]. The second type of studies is concerned with the measurement of the interaction forces between biomolecules,

such as the interaction antigen-antibody [44] or receptor-ligand [45]. This last type of studies within the framework of SMFS are termed affinity studies [46, 47].

As indicated above, the identification of target molecules through their interaction with a sensor molecule was initially called chemical force microscopy (CFM). This term was coined by A. Noy et al. in 1997 and refers to its original use for measuring the interaction between functional groups at the nanoscale. These measurements, in turn, allowed to extract values of chemical magnitudes, such as local pK values of acid-base groups on surfaces, or to obtain the spatial distribution of various functional groups at different ionization states [48-50]. The usage of this procedure in systems in which the interaction between sensor and target molecules depends on the assembly processes characteristic of the biological systems led to the usage of the alternative term Affinity atomic force microscopy [51].

According to the objectives proposed in this work and the studies performed, we are going to study model systems both within the context of SMFS and A-AFM, dealing in more depth with some relevant topics in these techniques in what follows.

1.4.1. Cantilevers selection and functionalization for SMFS and affinity microscopy.

The forces involved in the interactions between biomolecules, or those characteristics of intramolecular forces are normally found in the range between 5-1000 pN, so that the cantilevers have to be sensitive enough to measure them with sufficient resolution. Consequently, the cantilevers must be as compliant as possible, so that the lowest forces of interest must be able to deflect them and be detected. This implies that the spring constants of these cantilevers are typically in the range of 0.01-0.5 N/m.

But not only the mechanical properties of the cantilevers are important, but also the material or materials from which they are made, since a fundamental step in this type of experiments is the functionalization of the AFM tip and, in many experiments, of the substrate. Functionalization is necessary to decorate the surfaces with the sensor molecule and, if necessary, also with the target molecule.

Very often, cantilevers are made up of silicon-based materials (silicon, silicon nitride or silicon dioxide) or gold (or, possibly, gold-coated surfaces). The selection of these materials is largely dependent on the possibility to functionalize the surfaces through a conventional silanization process (silicon-based materials) or through the formation of self-assembled monolayers (gold, and gold-coated surfaces).

Starting from the functionalized material, it tends to be necessary to modify its chemistry with the usage of a crosslinker in order to allow the decoration of the surface with the molecule of interest. Table 1 illustrates some specific cases extracted from some relevant references.

Table 1. Summary of some of the strategies for the functionalization of AFM probes

Material	k (N/m)	Cleaning process	Functionalization reagent	Exposed group or molecule	crosslinker	Ref
Si_3N_4	0.03	UV/O ₃	APDMES (silane)	Amino	NHS-PEG-MAL	[52]
Si_3N_4	0.01-0.03	Chloroform	APTES (silane)	amino	NHS-PEG-ACETAL or NHS-PEG-Aldehyde	[53]
Si_3N_4	0.01-0.03	Chloroform	APTES	amino	NHS PEG-PDP	[17]
Si_3N_4 (gold plated)	0.01-0.03	----	NTA-terminated alkanethiols	NTA	non	[54]
Si_3N_4	0.01	Piranha solution	APTES	amino	NHS-PEG-MAL	[55]
Si_3N_4	0.01-0.1	UV/O ₃	TES (SILANE)-PEG-CARBOXYL	Carboxyl	EDC/NHS	[56]
Si_3N_4 / Silicon	0.09	ACETONE/ O ₂ PLASMA	SILANE-PEG-BIOTIN	BIOTIN	--	[57]
Si_3N_4	0.07	Chloroform/ UV-O ₃	APTES	amino	Fmoc-photo-NHS	[58]

In recent years more complex functionalization strategies have emerged that involve not only the use of crosslinkers but may also rely on enzymatic reactions or on genetically engineered biomolecules to bind the biomolecules of interest to the surfaces with high specificity. A selection of some of these strategies is presented below (Figure 8) (taken from [59])

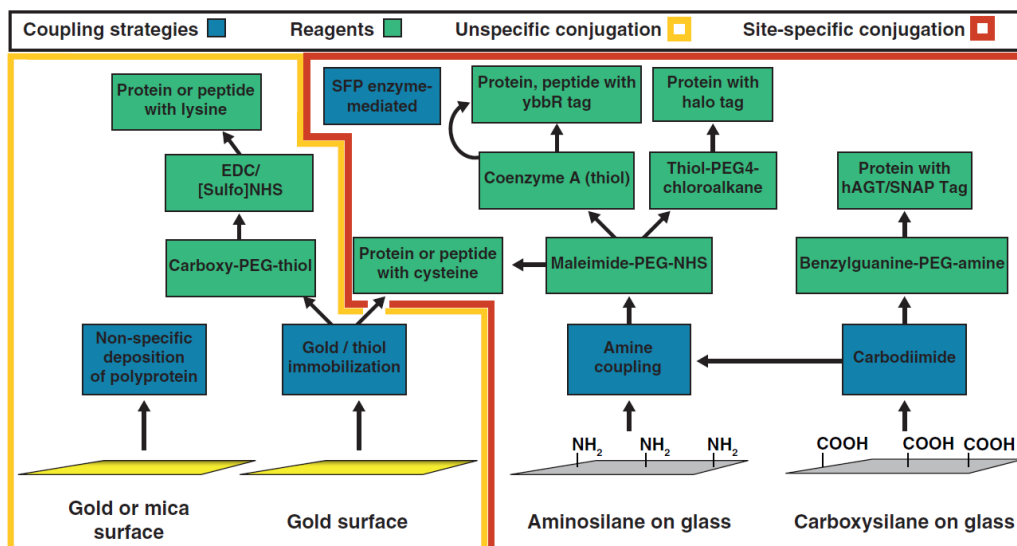


Figure 8. Various strategies for surface functionalization and immobilization of biomolecules for experiments studying molecular interactions with AFM [59].

(i) HaloTag

HaloTag is a modular protein tag that consists of a modified bacterial enzyme, haloalkane dehalogenase. This enzyme reacts with halides in aliphatic chains through nucleophilic displacement, giving rise to an ester-type covalent bond on the enzymatic active site [60]. This type of immobilization method is very specific and has been widely employed to carry out SMFS tests [61].

(ii) SpyTag/Catcher

This attachment method is based on the mechanism that *Streptococcus pyogenes* presents naturally to stabilize the structure of its proteins since, due to its living conditions, it is not easy for the bacteria to generate disulfide bonds as a method to stabilize the tertiary structure of the proteins.

The mechanism consists of the spontaneous reaction between a small peptide and a larger protein component, both obtained from a domain of the FbaB protein of the bacteria. This reaction gives rise to an irreversible isopeptide bond between both parts that occurs in a wide range of temperatures and pH. Spy tag is the term that refers to the peptide and spyCatcher to the larger protein. As the peptide and the protein are composed of amino acids, these can be introduced into the sequence of a protein of interest through genetic engineering, making this a very versatile linking method for many different proteins [62].

(iii) ybbr/SFP

This binding method consists of the reaction catalysed by 4'-phosphopantetheinyl transferase (SFP) between a peptide formed by 11 residues with the sequence DSLEFIASKLA (ybbr-tag) and coenzyme A (CoA) [63]. After the reaction, ybbr and CoA remain covalently linked through a phosphopantetheinyl bond. Since ybbr is a peptide, it is possible to incorporate it in the sequence of a protein of interest, either at the C terminal, at the N terminal or in an intermediate region of the sequence, as long as this does not affect the final structure of the protein. This makes the union based on this method very versatile and robust to specifically bind molecules of interest [64] [65]

1.4.2. Distinguishing between specific and non-specific interactions.

When carrying out the measurements in the system of interest, adhesion events will appear between the tip and the sample. These adhesion events may be due either to non-specific interactions, which are of no interest, or to the specific interactions of the system of interest (Figure 9). The latter are the main objective of the whole experimental setup. Consequently, distinguishing specific from non-specific events is crucial, as well as one of the major challenges of the application of AFM to biological systems. Over the years, strategies have been developed to classify the interactions detected into specific and non-specific.

One of the first strategies developed is based on the usage of the so-called spacers. Spacers are polymer chains, very often polyethylene glycol (PEG), that are inserted at some stage during the functionalization step of the tip and/or substrate to increase the distance between tip and substrate at which the interaction is effective. This effective distance is easily controlled through the length of the spacer [66]. In addition, these polymer chains

increase the mobility of the molecules, making the interaction of interest occur more easily. In many of the functionalization protocols that can be found in the literature, crosslinkers that already include these spacers between the reactive functional groups are used, as can be seen in Table 1

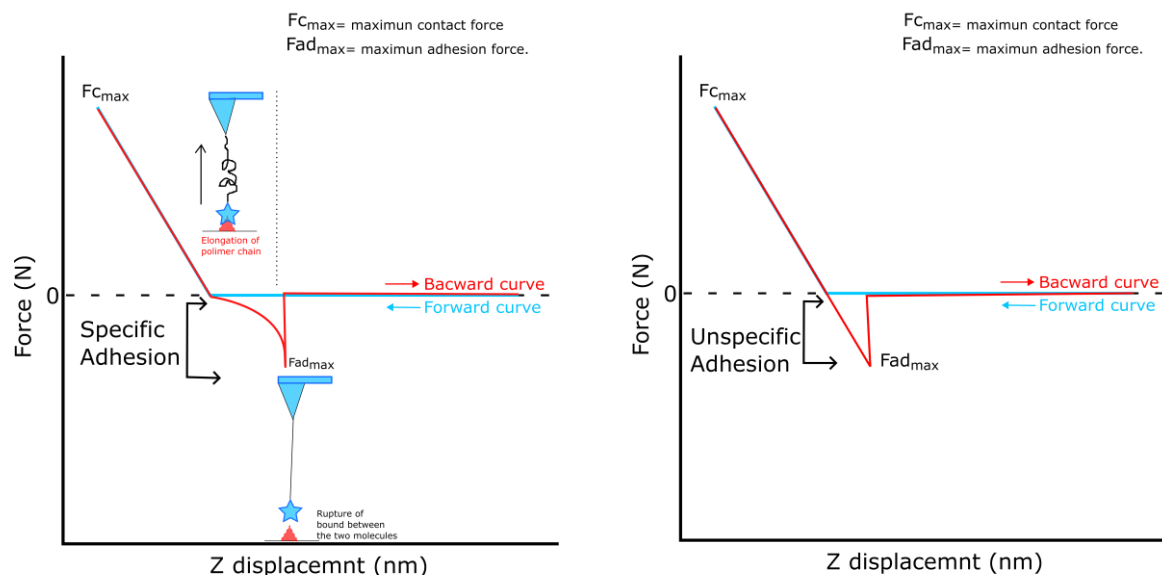


Figure 9. Difference between specific and nonspecific interactions according to the shape of the F-Z curve. Adapted from [123]

The other method used, commonly in combination with the previous one, is the use of what is known as mechanical fingerprints (Figure 10). Mechanical fingerprints are characteristic patterns that appear in F-Z curves due to the stretching of specific regions (these molecules are usually proteins or peptides). A mechanical fingerprint is easily recognizable in a F-Z curve, since corresponds to a given pattern characterized by specific values of forces and extensions. What is common in this case is to join the molecule of interest to one of these mechanical fingerprint molecules, so that upon stretching, the pattern will appear in the F-Z curve, identifying clearly the specific interaction of interest. However, it must be considered that the characteristic pattern must correspond to forces smaller than the interaction of interest, otherwise the unbinding between the molecules of interest would occur first at a lower force, causing the fingerprint not to appear in the F-Z curve, so that it would not be possible to assure the specificity of the interaction.

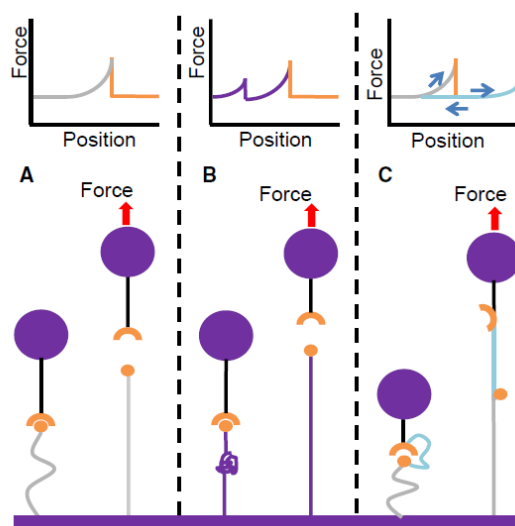


Figure 10. Scheme of the typical patterns found in F-Z curves for various configurations of biomolecules and associated fingerprints [67].

Next, we are going to review some of the most commonly used fingerprints [68].

One of the most frequent fingerprints found in the literature is derived from the I27 protein. The I27 protein is a component of the I-band region of the protein titin, which is made up of 86 aa giving rise to seven β -sheets. The most common way in which I27 is used as a mechanical fingerprint consists of creating a tandem of three, five or eight units, thus generating an I27 trimer, pentamer or octamer. In this case, the characteristic pattern of I27 in the F-Z curves corresponds to a number n of consecutive peaks ($n=3$ for the trimer, $n=5$ for the pentamer and $n=8$ for the octamer) at a force around 200 pN [69] [70]. Another commonly used fingerprint is ddFLN4, which corresponds to the domain 4 of filamin obtained from *Dictyostelium discoideum* [71]. Finally, two proteins with an analogous structure that are used as fingerprints are the proteins L and GB1. The L protein corresponds to the B1 domain of the multidomain virulence factor of the bacteria *Peptostreptococcus magnus*. It is composed by 64 aa and presents a secondary structure with four β -sheets and an α -helix [72]. The GB1 protein is a structural analogue of the B1 domain of the streptococcal G protein [73].

Although the recognition of these characteristic patterns in many cases can be done qualitatively in the F-Z curves, it is usual to analyse the curves by adjusting them to a mathematical model that describes the stretching of the polymeric spacer or of the molecular fingerprint. Among the most used models are the freely jointed chain (FJC) and the Wormlike chain (WLC) models with their possible variants.

Freely jointed chain model (FJC)

The FJC model was first proposed by Kuhn in 1936. In this model it is assumed that the polymer chain is made up of N links. Each link has a fixed length called the Kuhn length. The orientation of each link is independent of the previous and subsequent links. The total length of the chain is called the contour length (L_c) and is defined by the equation (8).

$$L_c = \sum_{i=1}^N b = N * b \quad (8)$$

$$\langle x \rangle = L_c \left(\coth \frac{Fb}{k_B T} - \frac{k_B T}{Fb} \right) \quad (9)$$

Where N is the number of segments, b is called the Kuhn length, F is the force, T the absolute temperature, x is the extension of the chain and k_B is the Boltzmann's constant. The relationship between the extension of a polymer chain and the force applied to that polymer chain is determined by (9) [42, 74].

Worm-like chain model (WLC).

The WLC model [75] assumes that the polymer chain is continuous and divided into N segments. Each segment measures a distance d and is oriented at an angle θ with respect to the adjacent segment. In this model, the segments are not independent of each other, but the θ angle of each segment affects the orientation of the adjacent segments. In this model there are two fundamental parameters, which are the contour length (L_c), defined as the total length of the polymer chain and the persistence length (p), defined as the length of the smallest rigid component of the chain (and therefore it is a measure of the rigidity of the polymer). In this case, the force necessary to elongate the polymer chain is defined by equation (10) [42]

$$F(x) = \frac{k_B T}{p} \left[\frac{1}{4} \left(1 - \frac{x}{L_c} \right)^{-2} - \frac{1}{4} + \frac{x}{L_c} \right] \quad (10)$$

Where k_B is the Boltzmann's constant, T the absolute temperature, p refers to the persistence length, x is the extension and L_c is the contour length.

FJC and WLC are the main models used for the fitting of the F-Z curves. However, both models work for extensions of polymer chains that occur in force ranges of up to 200 pN. For higher forces, sometimes these models do not define the behaviour precisely [76]. Because of this, there are other models derived from the original ones, such as the EFJC (Extended Freely Jointed Chain) model [77], which includes in the formulation the elasticity of the link that forms the chain or the EWLC (Extended Worm-Like Chain) [78] which also includes the elasticity of the segment.

The adjustment to these models is usually carried out either with software routines developed by the research groups themselves, from free software programs created by the research community in this field [79, 80] or with the analysis software included in many commercial microscopes.

Finally, there is a procedure to determine the specificity of an interaction that, although not direct, allows to extract more information on the interaction itself, especially on parameters such as dissociation rates, unbinding energies, and the energy landscapes of the system.

This procedure is based on the application of the Bell model which assumes that the force-induced dissociation of a biomolecular complex decreases the activation energy barrier [81] and, additionally, on the Evans and Ritchie model, that theoretically explains the linear relationship that exists between the unbinding force and the natural logarithm of the loading rate (increase in force per unit of time) [82]:

$$F_u = \frac{k_B T}{x_\beta} \ln \left(\frac{l_R x_\beta}{k_{off} k_B T} \right) \quad (11)$$

Where k_B is the Boltzmann constant, T is the absolute temperature, l_R is the loading rate, x_β is the distance between the bound state and the transition state in the direction of the pulling force and k_{off} is the dissociation rate at zero force [83].

In order to apply these models, it is necessary to record the F-Z curves at different retraction speeds (which implies applying different loading rates), in addition to requiring a large amount of data for a statically significant analysis. This method of recording the F-Z curves at different retractions speeds is called dynamic force spectroscopy (DFS) [84].

Once the experimental data is obtained through DFS, calculating the parameters x_β and k_{off} is relatively simple. The process begins by calculating the most probable breaking forces obtained for each loading rate used and, subsequently, the data is represented by an x-y plot of breaking force (y axis) vs natural logarithm of the loading rate (F_u vs $\ln(I_R)$) (x -axis). Once the data are represented, these are adjusted using linear regression to obtain the parameters of the slope (m) and the x-intercept (b) of the fit line obtained.

Knowing the values of the slope (m) and the y-intercept (b) and applying equation (11), the following expressions are obtained that allow calculating both x_β and k_{off} [85]:

$$x_\beta = \frac{k_B T}{m} \quad (12)$$

$$b = \frac{k_B T}{x_\beta} * \ln\left(\frac{x_\beta}{k_{off} k_B T}\right) \quad (13)$$

Figure 11 shows in a generic way the representation of data obtained from DFS and also the representation in an energy landscape diagram where the graphical representation of the parameters x_β and k_{off} .

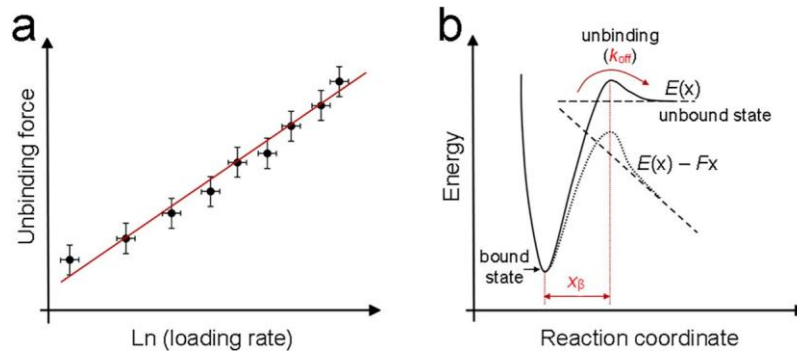


Figure 11. (a) Generic plot of rupture or unbinding force vs $\ln(I_r)$. (b) Generic presentation of an energy landscape showing the transition between the bound state and the unbound state, as well as the parameters x_β and k_{off} . Extracted from [83]

1.4.3. Biotin-streptavidin complex as a model system for the study of biomolecular interactions.

Streptavidin is a homotetrameric protein of approximately 54 kDa (PDB 3RY1) purified from the organism *Streptomyces avidinii* (Figure 12, left). This protein has high thermal stability (between 73°C and 112°C) in addition it is also stable to enzymatic degradation, to denaturing agents and within a wide pH range [86].

Biotin [87] is a biomolecule (MW 244.31 g/mol) classified as a vitamin (vitamin H) that acts as a cofactor of carboxylic acid enzymes involved in the catalysis of fatty acid, carbohydrates and amino acids. Structurally, it is composed of a ureido ring linked to a tetrahydrothiophene ring with a valeric acid side chain [88] (Figure 12, right).

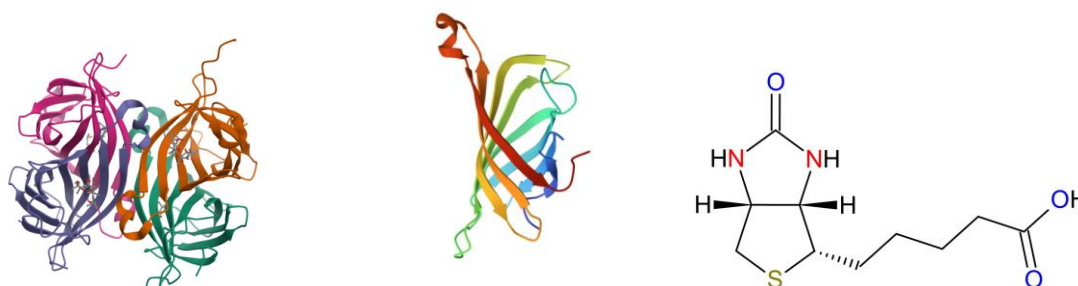


Figure 12. Three-dimensional ribbon model of the tetrameric protein streptavidin (Left). Three-dimensional model of the streptavidin subunit (Center). Structural formula of the biotin molecule (Right).

Biotin binds to streptavidin reversibly forming a biotin-streptavidin complex in which up to 4 biotin molecules can bind to a streptavidin tetramer, one biotin to each subunit of the protein. The formation of the complex is very fast, and it is not significantly affected by denaturing agents, extreme pH ranges or by the presence of organic solvents.

The biotin-streptavidin complex has a dissociation constant $K_d = 10^{-14}$ M, making it one of the strongest non-covalent interactions in nature [89]. This is probably due to fact that the interaction is mediated by the formation of a large number of hydrogen bonds and intense van de Waals interactions at the binding site, in addition to the contribution produced by three tryptophan residues of one subunit together with a fourth tryptophan residue of the adjacent subunit [90-92].

Due to the strength of this interaction, as well as the high stability of the molecules themselves and of the complex they form to different environmental conditions, the biotin-streptavidin system has been frequently used as a model system to study the application of AFM for the characterization of intermolecular interactions.

The initial studies on this system date back to 1994, when Gil U. Lee, et al. published a study where discrete interactions between biotin and streptavidin were detected using surfaces functionalized with biotin-BSA and streptavidin [93]. Also in 1994, Ernst Ludwig Florin et al. published a study in which the interaction forces between avidin and different types of biotins were measured [94]. In 1999, Joyce Wong et al. published another study in which, they measured the unbinding force of biotin-streptavidin complexes using AFM [95].

The biotin-streptavidin model continued to be characterized during the first and second decades of the 21st century, especially from the point of view of dynamic force spectroscopy. This way the Bell-Evans model was applied to obtain kinetic parameters of the interaction. A summary of the parameters obtained in different studies can be seen in Table 2.

Table 2. Summary of the parameters obtained in different studies in relation to the biotin-streptavidin interaction.

Molecules (substrate-tip)	Average unbinding forces F_u (pN)	Loading rates l_r (pN/s)	x_β (nm)	Reference
Streptavidin- biotin	126±2.3 207±5.8	198 2300	--	Yuan et al 2000 [96]
Streptavidin- biotin	--	2x10 ⁸ - 55x10 ⁹	0.56 0.48 0.23 0.12	Galligan et al 2001 [97]
Streptavidin- biotinylated BSA	167±20 236±26 289±13	39000 390000 1950000	---	Lo et al 2001 [98]

	350±30 442±17	3740000 6280000		
Streptavidin- biotin	150-500	10 ³ -10 ⁴		Zhan and Moy 2003 [99]
Streptavidin- biotin	200	1267 300-1700 1700-9600	0.081±0.002 0.024±0.03	Odrowaz piramowicz et al 2006 [100]
Streptavidin- biotin	263±36 378±42 494±39	3900 39000 1950000		Zhou et al [101]
Streptavidin- biotin		200-113000	0.64 0.072(inner) 0.54	Thorman et al [102]
Streptavidin- biotin	(17°C) 72±2 95±3 177±4 (37°C) 27±1 66±2 121±4	338±7 597±65 65602±758 387±9 6997±94 69457±1055	0.35±0.27 (OUTER) 0.09±0.02 (INNER) 0.38±0.21 (OUTER) 0.09±0.03 (INNER) 0.031±0.07(OUTER) 0.15±0.05(INNER)	Rico and Moy 2007 [103]
Streptavidin- biotin			0.40±0.05 0.44±0.06	Guo et al 2008 [104]
Streptavidin- biotin	46	301	0.11	Walton et all 2008 [105]
Streptavidin- biotin	68(F1) 120(F2)	1153		Teulon et all 2011 [106]

	260(F3)	67-2208 67-2208 2208-162755 2208-162755 2208-162755	0.40±0.08(F1 OUTER) 0.37±0.14(F2 OUTER) 0.08±0.004(F1 INNER) 0.05±0.002(F2 INNER) 0.04±0.004(F3 INNER)	
mStreptavidin-biotin	201 212 217 222 230	1736 7469 20680 52390 111900	0.38±0.02	Sedlak et al 2017 [107]

In 2011, Teulon et al [106] published a study where they provided an interpretation for the discrepancies that existed in previous studies [105]. In this study, the energy landscape of the biotin-streptavidin complex was determined by assuming that, due to the tetrameric nature of streptavidin, multiple parallel bonds could be formed that lead to multiple unbinding events. Such multiple unbinding events had not been clearly considered in previous studies. Under this hypothesis and for values of loading rates in the range of 100-150000 pN/s, it was found that the most probable rupture force of the interaction between a single biotin molecule and single unit of streptavidin (F1) was 68 pN. Considering the hypothesis of multiple bonds that they proposed, the breaking force for double (F2) and triple (F3) bonds was 120 pN and 260 pN respectively. As mentioned, there was a discrepancy in previously published studies on the data obtained from the biotin-streptavidin interaction that, with the interpretation of multiple parallel bonds might be accounted for. Thus, regarding the most probable rupture forces, the results obtained by Thorman et al (2006) coincide with those obtained by Teulon et al. when considering single-molecule interaction. Alternatively, the values of the dissociation force

obtained by Yuan et al (2000) may be interpreted as resulting from double or triple events. Finally, those obtained by Odrowaz Píramowicz et al. (2006) coincide with what Teulon interprets as triple and quadruple bonds.

The application of the Bell-Evans model yields to the identification of a non-linear trend with differentiated regimes. These differentiated regimes were also described by Teulon, so that the data corresponding to each regime can be fitted to a straight line.

The application of the Williams formalism [108] to these experimental data allowed considering the possible formation of multiple links, and to establish the expected results at different loading rates. Teulon et al concluded that the dissociation of the biotin-streptavidin complex implies two energy barriers: one internal, related to the union state close to the bond conformation, and another external, related to the state close to the breakage of the bond.

Due to what has been stated about the formation of, not only single, but also multiple bonds, more advanced strategies have been developed to try to avoid this complication. In this regard, non-tetrameric streptavidin (monovalent, divalent or trivalent) was used by Sedlak et al [107, 109, 110]. The use of incomplete streptavidin allows the interaction between the ligand and the active site of the protein to be studied in more detail and in a more isolated way.

2. MOTIVATION AND OBJECTIVES OF THIS WORK.

The development of this work is motivated by the collaboration between the company Bioactive Surfaces S.L. and the Biomaterials and Regenerative Engineering Laboratory of the Biomedical Technology Center (CTB, Madrid, Spain). The main interest of the research group was to continue the development of affinity atomic force microscopy, while the interest of the company was the characterization, in various aspects, of the functionalized AFM tips and substrates they manufacture. In this context, materials manufactured by the company are essential for the procedures involved in affinity microscopy described below.

Taking this into account, the main objective of this work is to develop affinity microscopy in order to detect intermolecular interactions with single-molecule resolution using commercial functionalized AFM tips and substrates.

To achieve the main objective the following subobjectives were established:

1. Characterization of the topography and resonance frequency of functionalized AFM tips.
2. Development of protocols of crosslinking chemistries applicable to the amino functionalized surfaces based on the NHS ester chemistry.
3. Decoration of the surfaces with biomolecules of interest, biotin, streptavidin, antibodies and LDH) and evaluation of their functionality after the binding process
4. Measurement of the molecular interaction between a sensor and a target molecule (biotin-streptavidin and antiLDH-LDH) within the context of affinity microscopy.

3. MATERIALS AND METHODS.

3.1. Materials used for developing this work.

AFM tips and substrates.

To carry out this work, AFM DeepTip™ tips functionalized with amino groups supplied by the company Bioactive surfaces S.L. were used (Table 3). Specifically, the models DeepTip™ SiN R67-150, DeepTip™ SiN R67-300, and DeepTip™ SiN R67-600 were acquired. These models differ in the thickness of the functionalized layer with amino groups according to the information provided by the company, going from the minimum thickness for the 150 model to the maximum thickness for the 600 model; DeepTip™ SiN R11, model with reduced thermal drift; DeepTip™ SiN R15 T125, that presented multiple cantilevers of triangular and rectangular geometry with different elastic constants and resonance frequencies; and DeepTip™ SiO R1200 designed for high-speed applications. Besides that, the company supplied nonfunctionalized DeepTip™ tips to be used as controls for the various characterization techniques.

Table 3. Nominal resonance frequency and spring constant of the main models of DeepTip™ cantilevers used in this work. When more than one resonance frequency and spring constant is presented, each value corresponds to one of the cantilevers of the probe.

Model	Nominal resonance frequency (kHz)	Nominal spring constant (N/m)
DeepTip™ SiN R67-150	67	0.48
DeepTip™ SiN R67-300	17	0.06
DeepTip™ SiN R67-600		
DeepTip™ SiN R11	11	0.01
DeepTip™ SiN R15	7	0.01
T125	15	0.02
	15	0.03

	22	0.07
	38	0.1
	125	0.6

Nonfunctionalized AFM probes of the models PNP-DB (Nanoworld) and PPP-NCHR-20 (Nanosensors) were used for the acquisition of AFM topography images.

MicroDeck™ substrates functionalized with amino groups were used for the immobilization of the target molecules. Specifically, Microdeck™-G-150, (functionalized glass with a diameter of 5 mm), MicroDeck™-SiO-900, (functionalized silicon substrate with dimensions 5 x 5 mm) and Microdeck™-m-300 (functionalized mica substrate with a diameter of 4 mm) substrates were employed.

Chemical reagents.

For the development of the experimental part of this work, the following specific chemical reagents have been used. Sulfo-LC-SPDP (sulfosuccinimidyl 6-(3-(2-pyridyldithio)propionamido)hexanoate, Thermo fisher scientific 21650), , 5-iodoacetamidofluorescein (5-IAF; Thermo fisher scientific 62246), Zeba™ Spin Desalting Columns, 7K MWCO (Thermo Fisher scientific, 89882), 1-Step™ Turbo TMB-ELISA (Thermo Fisher scientific, 34022), β -nicotinamide adenine dinucleotide (NADH; Fisher scientific N8129-500 mg), Biotin-HRP (biotinylated peroxidase, thermo fisher scientific 432040), Streptavidin-FITC (Thermo Fisher scientific, INVITROGEN™ SA1001) Sodium dodecyl sulphate (SDS, Fisher Scientific 15450685), NHS-PEG4-azide (thermo fisher scientific 26130), streptavidin agarose resin (Thermo fisher scientific 20347), TCEP-HCl (Tris(2-carboxyethyl)phosphine hydrochloride, Sigma-Aldrich C4706), EDTA (ethylenediaminetetraacetic acid; Sigma Aldrich E9884), lactate dehydrogenase (LDH, Sigma-Aldrich Merk L1006-12.5KU), sodium pyruvate (Sigma-Aldrich P2256), poly(ethylene glycol)-(N-hydroxysuccinimide 5-pentanoate) ether 2-(biotinylamino)ethane (NHS-PEG-biotin, Sigma Aldrich 757799), streptavidin (Sigma Aldrich, S4762), glutaraldehyde (GA, 50 wt. % in H₂O, Sigma Aldrich 340855), Fluorescein 5(6)-isothiocyanate (Sigma-Aldrich 46950-250MG-F), albumin biotinamidocaproyl labelled bovine (biotin-BSA, lyophilized powder, Sigma-Aldrich A6043). Dulbecco's Phosphate

Buffered Saline (DPBS, Sigma-Aldrich D8537), Biotinylated anti-lactate dehydrogenase (anti-LDH) antibody (IgG polyclonal antibody, Origene AP21329BT-N), Goat Anti-Rabbit IgG H&L Alexa Fluor® 488 (Abcam ab150077), OPSS-PEG5K-SCM (JemKem USA A5109-1), NHS-PEG24-DBCO (BroadPharma BP-25726), azide-FITC (FAM azide, 6-isomer, Lumiprobe C5130), DBCO-Cy3 (Cyanine3 DBCO, Lumiprobe B10F0).

3.2. Sample Cleaning.

Before using any model of MicroDeck™ substrates for any subsequent process, they were cleaned by sonication in isopropanol at 60°C for 15 min, sonication in acetone at 60°C for 15 min and finally dried with argon.

All models of DeepTip™ AFM tips were rinsed in isopropanol and acetone, for 1 min each one and dried with argon before using in any experimental procedure.

3.3. Characterization of DeepTip™ AFM probes and MicroDeck™ substrates.

3.3.1. Functionality of the amino layer. Temporal stability of the layer.

To check the initial state and corroborate the presence of amino groups on the functionalized surfaces of the models: DeepTip™ SiN R67-150, DeepTip™ SiN R67-300, DeepTip™ SiN R67-600, DeepTip™ SiN R11, DeepTip™ SiN R15 T125, DeepTip™ SiO R1200 and Microdeck™-G-150) fluorescence microscopy was used. For this, the fluorescein isothiocyanate (FITC excitation 498 nm/ emission 517 nm) fluorophore was used, which will react covalently with the primary amines present on the surface.

Samples were incubated in a 0.25 mg/ml solution of FITC in PBS pH 7.4 (8 mM Na₂HPO₄ and 2 mM KH₂PO₄, 137 mM NaCl and 2.7 mM KCl) for 20 min at room temperature. The fluorophore solution was then removed, and the samples were transferred to a clean, empty well. Now, the samples were incubated with a solution of 10% SDS in PBS pH 7.4 three times for 5 min each time. Finally, the samples were rinsed abundantly with PBS pH 7.4 and mQ distilled water. Afterwards, the excess liquid was removed with optical paper, and the samples were mounted on a glass slide, fixing them with a small amount of vacuum grease.

For observation, a LEICA DFC340FX inverted fluorescence microscope was used with the following observation parameters: exposure 1s, gain 2.1, gamma 0.83, magnification 20x.

The ImageJ/FIJI software was used to analyse the images. The sample analysis process consisted of measuring the fluorescence intensity in a region of interest (ROI). In the case of the cantilevers, the ROI was the area of the cantilevers and in the case of glass coverslips, the ROI was the total area of the image.

With the fluorescence intensity values of different micrographs (13 images per functionalized DeepTip™ model), the mean fluorescence was calculated for each functionalization condition. Finally, the difference between samples was evaluated by applying the Student's statistical test (t-test).

To evaluate the temporal stability of the functionalized samples after reception, the same fluorescence labelling protocol as described above was used, but only with AFM probes. In this case, the samples were labelled with FITC at 3, 7, 14 and 365 days after reception. For the fluorescence analysis, the same procedure as described above was followed.

3.3.2. Topographic analysis of the surface.

Considering that the functionalization process results on a functionalized thin layer on the corresponding surface, according to the information provided by the manufacturer, there was special interest in measuring the roughness of this thin layer. The surface roughness was measured in both AFM probes (DeepTip™ SiN R67-150, DeepTip™ SiN R67-300) and substrates (MicroDeck™-G-150).

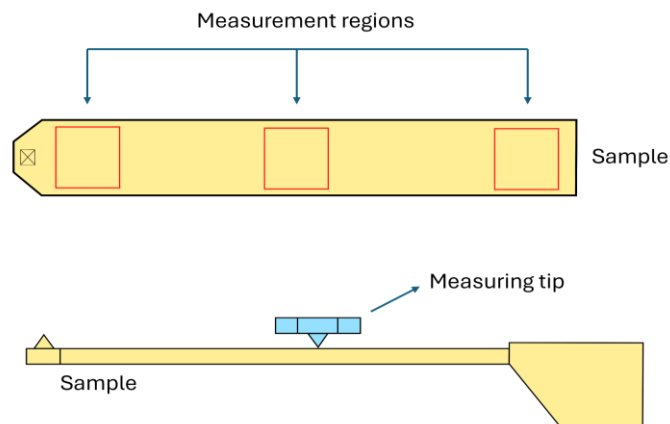


Figure 13. Schematic representation of the AFM imaging process for surface roughness analysis.

In the case of the AFM probes, the roughness was measured by atomic force microscopy using a Cervantes model AFM with a Dulcinea control unit (Nanotec SL) and operated by the WSxM software. Images were taken in contact mode in air, on three different areas along the AFM cantilever. Images were taken using nonfunctionalized PNP-DB cantilevers ($k=0.03$ N/m nominal value). Figure 13 shows a diagram of the image acquisition process.

For the substrate, the same AFM equipment was used to take images. In this case, images were taken in the tapping mode in air, using nonfunctionalized cantilevers (PPP-NCHR-20 $k=42$ N/m). Images were taken on $2 \times 2 \mu\text{m}$ and $0.5 \times 0.5 \mu\text{m}$ areas.

To calculate the roughness of any sample, the topography images were processed using the roughness analysis tool of the WSxM software. Non-functionalized surfaces were used as control.

3.3.3. SEM observation of functionalized tips.

DeepTip™ SiN R67-150 and DeepTip™ SiN R67-300 were observed by scanning electron microscopy using a FESEM Auriga Zeiss microscope operated at 5.0 kV. The samples were mounted on electron microscopy holders and fixed to the holder using a fast-curing cyanoacrylate-based adhesive.

As a prior step to the observation, samples were metallized with Au/Cr using an EMITEC SC7620 metallizer (Quorum Tech, Laughton, East Sussex, U.K.). The metallization parameters were 18 mA for 60 s. As a control, non-functionalized AFM cantilevers were used.

3.3.4. Characterization of resonance frequency and spring constant of functionalized cantilevers.

The resonance frequency and spring constant of DeepTip™ SiN R67-150 cantilevers were characterized using a Cervantes AFM with a Dulcinea control unit from the company Nanotec SL (Madrid, Spain). The resonance frequency was experimentally estimated from the thermal noise oscillation of the cantilevers in air. The spring constant was calculated in air by the Sader method implemented in the AFM WSxM control software. Both parameters were measured 5 times for each cantilever and the average value was obtained

as the final value of the parameter. A total of 12 cantilevers of each model were used to carry out the analysis and nonfunctionalized cantilevers were used as control.

3.4. Chemical modification of DeepTip™ and MicroDeck™ functionalized samples.

As a previous step to modify the amino-functionalized surfaces with NHS containing chemical reagents, the surfaces were treated as follows:

Both the substrates and the AFM probes were incubated for at least 30 min in 0.1 M carbonate-bicarbonate buffer pH 9.1 at room temperature with the aim of deprotonating the amino groups on the surface and favouring the subsequent reaction with the corresponding reagent.

3.4.1. Modification with thiols.

AFM cantilevers

DeepTip™ SiN R67-150 were incubated in 200 µl of a 2.5 mg/ml solution of sulfo-LC-SPDP in PBS-EDTA (1mM) pH 7.4 for 2h at room temperature. In parallel, control samples without sulfo-LC-SPDP were prepared and incubated in PBS-EDTA.

Once the incubation was completed, samples were washed in the same well with 200 µl of PBS-EDTA three times for 2 min each time.

Consecutively, the samples were incubated in 200 µl of a 3 mg/ml solution of TCEP in PBS-EDTA for 15 min at room temperature with the objective of reducing the disulfide bonds of the LC-SPDP reagent and leaving exposed thiol groups. Control samples were incubated with 200 µl of 50% glutaraldehyde in distilled water for 15 min at room temperature.

Once the 15 min of incubation was completed, all samples were washed 4 times with PBS-EDTA, pipetting exhaustively each time.

When the modification of the samples is completed, they are transferred to a clean well.

To verify that the modification has been carried out correctly, fluorescent microscopy was employed using a specific reagent for the detection of thiol groups (5IAF excitation=494

nm / emission=518 nm). All samples were incubated with 200 μ l of a 400 μ g/ml solution of 5IAF in DMSO for 10 min at room temperature protected from light.

At the end of the incubation time, all samples were washed with 10% SDS in PBS for 5 min at room temperature. They are then washed 3 times for 5 min each time in PBS and finally rinsed with mQ water. Samples are then mounted in a glass slide to be observed with the LEICA DFC340FX microscope.

For the observation in the microscope the following observation parameters were used: exposure 1s, gain 2.1, gamma 0.83, magnification 20x.

For the analysis of the images, the same method that was described in section 2.3.1 was used.

SUBSTRATES

For the modification of MicroDeck™-G-150 surfaces, the same protocol described above was followed with only slight modifications:

In the surface modification step, with the reagents of interest, substrates were incubated with 200 μ l of a solution of 2.5 mg/ml of sulfo-LC-SPDP in PBS-EDTA pH 7.4 for 2 h at room temperature. Alternatively, other substrates were incubated with 200 μ l of a 2.5 mg/ml solution of OPSS-PEG5K-SCM in PBS-EDTA pH 7.4 for 2 h at room temperature.

Although subsequent chemical modification steps for the substrates are essentially the same as those discussed above, in this case incubation with the fluorophore is carried out using 200 μ l per sample, of a solution of 5IAF in DMSO at a concentration of 500 μ g/ml for 30 min at RT and protected from light. The washing procedure after incubation with the fluorophore is the same as that used for the AFM chips.

To observe the samples, they were mounted on glass slides, fixing them with a small amount of vacuum grease. A LEICA DFC340FX fluorescence microscope was used with the following parameters: exposure 1s, gain 2.1, gamma 0.83, magnification 20x. For the analysis of fluorescence intensity, the same procedure as set out in section 2.3.1 was used.

3.4.2. Modification with azide and alkyne.

The modification with click (azide/alkyne) crosslinkers was only assessed on DeepTip™ SiN R67-150 AFM chips.

Azide modification

AFM probes were incubated in a p96 multiwell plate with 110 μ l of a 0.25 mg/ml solution of azide-PEG4-NHS in PBS pH 7.4 for 2 h at room temperature. As a negative control, two types of samples were used: functionalized probes not modified with azide, and nonfunctionalized probes incubated with azide. After incubating with azide-PEG4-NHS, all samples were washed with PBS pH 7.4, pipetting 5 times exhaustively each time.

Next, all samples (controls included) were incubated in a 0.25 mg/ml solution of DBCO-Cy3 (Excitation=555 nm / Emission=570 nm) in DMSO for 45 min at room temperature and protected from light.

Once the reaction time was over, samples were washed with DMSO 3 times by gently pipetting each time and, consecutively, 3 more times by pipetting each time with 10% SDS in PBS or 0.1% SDS/ 0.2% tween20 in PBS. Finally, samples were rinsed with PBS and mQ water.

Once the modification process was completed, the samples were mounted in a glass slide in the same way as mentioned in section 2.4.1 for observation by fluorescence microscopy. A LEICA DFC340FX fluorescence microscope was used with the following observation parameters: exposure 40.9 ms, gain 1, gamma 0.84, magnification 20x. The analysis of the samples was carried out as described in section 2.3.1.

DBCO modification.

For the modification of the surface with DBCO-PEG24-NHS, similar steps were employed as those described in the azide modification section, with slight modifications according to the type of chemical reagents used in this case.

The first modification was that the samples were incubated in a 0.25 mg/ml solution of DBCO-PEG24-NHS in PBS pH 7.4 for 2 h at room temperature.

The second modification consisted of incubating the samples in 150 μ l of a 0.25 mg/ml solution of azide-FITC (Excitation= 492 nm / Emission= 517nm) in DMSO for 1 h at room temperature protected from light. All other steps parallel those described in the previous section.

Observation by fluorescence microscopy was carried out following the same procedure as for the samples modified with azide, but using the following parameters: exposure 1.4 s, gain 3, gamma 0.80, magnification 20x. The analysis of the images was carried out following the same procedure as well.

3.4.3. Modification with biotin.

DeepTip™ SiN R67-150 were incubated in 100 µl of a 5.5 mg/ml solution of biotin-PEG-NHS (Mw=3800) in PBS pH 7.4 for 2 h at room temperature.

Consecutively, the samples were rinsed by pipetting exhaustively with 0.1% SDS/0.2% tween20 and then with PBS pH 7.4.

In order to corroborate the presence of biotin bound to the surface, the modified samples were transferred to a new well and incubated in 200 µl of a 250 µg/ml solution of streptavidin-FITC in PBS pH 7.4 for 2 h at room temperature, and protected from light.

Finally, samples were washed thoroughly by pipetting with 0.1% SDS/0.2% Tween20 and then with PBS.

For the characterization by fluorescence microscopy, samples were mounted on a glass slide, fixing them with a minimum amount of vacuum grease and observed in a LEICA DFC340FX microscope. The following observation parameters were used: light colour: blue, exposure 1.5s, gain 3, gamma 0.83, magnification 20x. The analysis of the images was carried out following the same procedure as that discussed in section 2.3.1.

3.5. Decoration of the surfaces with biomolecules.

3.5.1. Decoration with antibodies. Temporal stability of the samples.

DeepTip™ SiN R67-150 were decorated with anti-LDH antibody through a binding chemistry based on the biotin-streptavidin system as explained in the following protocol.

Samples were first decorated with biotin according to the protocol explained in section 3.4.3, using in this specific case a 1 mg/ml solution of biotin-PEG-NHS in PBS pH 7.4 for 30 min.

After that, samples were washed with PBS pH 7.4 by thorough pipetting and transferred to a new well, where they were incubated with 100 µl of a 500 µg/ml solution of streptavidin in PBS pH 7.4 for 30 min at room temperature.

Once the incubation with streptavidin was completed, the samples were washed again as mentioned in the previous step and transferred to a new well where they were incubated with 100 μ l of a biotinylated anti-LDH antibody at a concentration of 0.25 mg/ml for 1 h at room temperature. Samples were washed as mentioned above and stored in PBS pH 7.4 at 4°C until their characterization by fluorescence microscopy or their use for affinity microscopy measurements.

To corroborate the presence of anti-LDH fixed to the surface, an Alexa fluor 488 anti-rabbit secondary antibody was used. Both the samples decorated with anti-LDH and control samples without the anti-LDH antibody were incubated in 100 μ l of a 1:800 secondary antibody solution in 0.1%BSA/PBS pH 7.4 for 1h at room temperature and protected from light. Subsequently, the samples were washed exhaustively with PBS and mounted on a glass slide for observation by fluorescence microscopy. The observation parameters were light color: blue, exposure 1s, gain 2.1, gamma 0.83, magnification 20x.

For the temporal stability study, 12 samples were decorated with anti-LDH on the same day. Subsequently, on the first day of the modification, 3 samples were observed with fluorescence. This observation was repeated 3, 7 and 14 days after decoration.

The analysis of the images was carried out following the same procedure as that discussed in section 2.3.1.

3.5.2. Decoration with proteins using SPDP crosslinkers.

3.5.2.1. Protein modification

Proteins without free cysteines were modified with sulfo-LC-SPDP to be able to bind them to surfaces with thiol groups on the surface. The main protein of interest to be modified was streptavidin and as a control of the modification process, Prup3 was used alternatively to validate the protocol. The developed protocol is a modification of the protocol published in [28].

100 μ l of protein solutions of concentration 1 μ g/ μ l-5 μ g/ μ l were prepared in amine-free buffer (in this specific case, PBS-EDTA (1 Mm) pH 7.4 was used).

The protein solutions were mixed with 25 μ l of a 20 mM solution of sulfo-LC-SPDP in mQ water and incubated for 1 h at room temperature.

Once the incubation was over, the protein solution was filtered using Zeba Spin desalting columns 7K MWCO centrifugation columns. Filtering ensures recovery of the modified protein without excess of free unreacted sulfo-LC-SPDP.

Filtration was carried out by centrifugation at 2800 rpm for 2 min at room temperature.

The modified protein (stv-pdp) was stored at -20°C for until use (< 3months).

To verify that the protein had been modified correctly, the absorbance of the solution at different stages of the modification process was measured by spectrophotometry at 280nm and 343 nm using a NANODROP 1000 (Thermo scientific).

3.5.2.2. Decoration with proteins without free cysteines

MicroDeck™-G-150 surfaces modified with reduced thiol groups (reduction to thiol groups is described in section 2.4.1) were used as substrate for the binding of modified streptavidin (stv-spdp).

The surfaces with exposed thiol groups were incubated with 200 µl solutions of stv-spdp at concentrations of 0.5 µg/ml, 1 µg/ml, 2 µg/ml and 4 µg/ml. Incubation was carried out overnight for at least 18 h at 4°C.

Next, the substrates were washed with 200 µl PBS/0.2% tween20/0.1% SDS for 30 min, at room temperature and a shaking at 300 rpm to remove unbound protein from the surface. The washing solution was then removed, and the samples were transferred to a new well where they were rinsed with 400 µl of PBS pH 7.4.

To verify that the protein was bound to the surface, a colorimetry assay was performed using biotin-HRP as a reagent, as adapted from [28].

To do this, the surfaces with bound protein were incubated for 30 min at room temperature and shaking (300 rpm) with 200 µl of biotin-HRP at a concentration of 100 ng/ml in PBS-EDTA. Afterwards, the biotin-HRP solution was removed and washed with PBS/0.2% tween 20/0.1% SDS for 30 min while shaking (350 rpm) and washed again with PBS-EDTA exhaustively.

Next, 200 µl of TMB was added and allowed to incubate for 5 min with shaking (300 rpm) at room temperature and protected from light. Once the incubation time was over, 17 µl of 2M sulphuric acid was added and the absorbance of the supernatant was measured at 450 nm in a BioTek ELx800.

3.5.2.3. Decoration of proteins with free cysteines.

MicroDeck™-G-150 surfaces modified with nonreduced sulfo-LC-SPDP and OPSS (described in section 2.4.1) were used as substrate for the binding of the enzyme lactate dehydrogenase (LDH).

Nonreduced OPSS and sulfo-LC-SPDP modified surfaces were incubated with 200 µl of LDH in PBS pH 7.4 at concentrations of 125 µg/ml and 400 µg/ml overnight at 4°C. After incubation, the surfaces were washed 5 times with PBS-Tween 20 0.05% pH 7.4 under shaking at 300 rpm, changing the washing solution every minute.

Once the surfaces were washed, they were used to carry out enzymatic assays or to carry out affinity measurements using AFM as explained in section 2.6.2.

Carrying out enzymatic assays was the method used to verify that the LDH molecules had been immobilized on the surface and remained active.

To carry out the enzymatic assays, a solution of 2.9 mM sodium pyruvate and 180 µM NADH in PBS pH 7.4 was prepared (test solution).

Once the test solution was prepared, the surfaces decorated with LDH at different concentrations were placed in wells of the p24 multiwell plate. Aliquots of 1 ml of test solution were added and the initial absorbance of each aliquot was measured at 340 nm. Once the value was measured, each surface was incubated with 1 ml of test solution for 8 h at room temperature.

After the incubation time was over, the supernatant was removed from each well and the absorbance was measured again at 340 nm.

As a negative control of the assay, non-functionalized bare glass cover slips were incubated with 200 µl of a 400 µg/ml LDH solution at the same time as the functionalized samples.

3.6. Affinity microscopy and force spectroscopy measurements.

3.6.1. Biotin-streptavidin system

3.6.1.1. Affinity microscopy studies with DeepTip™ tips: First approach.

To carry out affinity measurements using AFM probes, DeepTip™ SiN R67-150 and MicroDeck™ -G-150 were decorated with the molecules of interest.

Decoration of the AFM probes with biotin.

DeepTip™ SiN R67-150 were modified with NHS-PEG-biotin (Mw 3800) following the protocol described above (section 2.4.3), except for decreasing the incubation time with biotin solution to 1 h. Once modified with biotin-PEG, the probes were stored in PBS pH 7.4 at 4°C until use, for no more than 24 h.

Decoration of the substrates with streptavidin.

MicroDeck™-SiO-900 were incubated for 30 min in 500 µl of glutaraldehyde solution (25% of GA in mQ water). Samples were then washed exhaustively with PBS pH 7.4 and incubated again at room temperature, in 500 µl of a 0.5 mg/ml solution of streptavidin in PBS pH 7.4, for 30 min. Lastly, the samples were rinsed several times with PBS to remove unbound protein. The samples were used to carry out affinity measurements as soon as they were prepared.

As a control sample, streptavidin-blocked surfaces were used. To do this, the streptavidin-decorated surfaces were incubated with 1 ml of a 0.5 mg/ml solution of biotin-BSA for 30 min at room temperature.

Acquisition of force-displacement curves.

Prior to the acquisition of the curves, the spring constant of the cantilevers was calculated experimentally using the Sader's method in air. For the acquisition process, an AFM model Nanolife (Nanotec Electronica S.L, Madrid, Spain) was used. The acquisition of F-Z curves was carried out in PBS pH 7.4. The operating parameters used were: approach speed 1000 nm/s, retraction speed 2600 nm/s, contact force 500 pN, contact time 1s and rest time between curves 2s. In each experiment, 250 curves were recorded on the streptavidin surface and 250 additional curves on the blocked substrate. Once the acquisition was completed, no less than 10 F-Z curves were taken, in this case using a contact force of 800-1000 pN in order to calculate the optical sensitivity. The data were transformed and processed using a MATLAB routine developed in the research group.

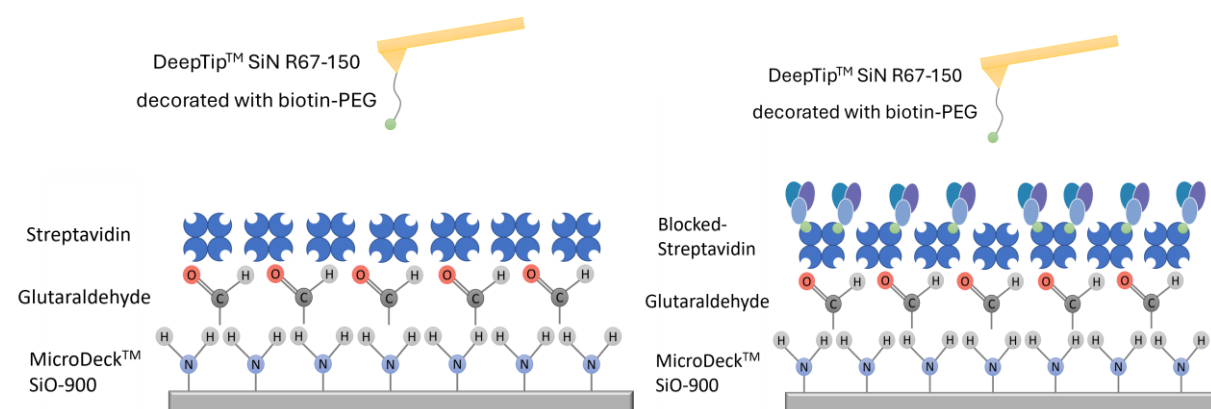


Figure 14. Schematic diagram of the samples used during the AFM test. Modified from [111]

3.6.1.2. Affinity microscopy studies with DeepTip™ tips: Dynamic Force Spectroscopy on biotin-streptavidin model.

The entire experimental part presented in this section and in section 2.5.1.3 was developed in the laboratory of Dr. Felix Rico, from the Aix-Marseille Université-Inserm-CNRS, as part of the research stay carried out in Marseille between September and December 2023.

Preparation of tips and surfaces.

For this series of experiments, DeepTip™ SiN R11 were used. NHS-PEG-biotin was immobilized on the tips following the protocol of section 2.4.3 with the following modification: Tips were incubated in drops of 50 µl of NHS-PEG-biotin solution at a concentration of 1 mg/ml for 1 h.

As a test surface, agarose beads functionalized with streptavidin (Thermo Scientific 20347) immobilized in a 4% agarose gel matrix were used as indicated in the reference [57]

Acquisition of curves.

The DFS measurements were carried out using a JPK AFM model Nanowizard V4. The F-Z curves were carried out in liquid medium using DPBS. The parameters used to acquire the curves were the following: contact force 200-300 pN, contact time 100 ms, Z amplitude 200 nm, approach speed 2000 nm/s, retraction speeds: 10 nm/s, 100 nm/s, 1000 nm/s and 10000 nm/s. Two tips of the model DeepTip™ SiN R11 were used and with each of them 200 curves were made with the slowest speed and 300 curves with the rest of the speeds. The spring constant of the cantilevers was calculated experimentally in liquid medium using the thermal noise method implemented in the microscope software before proceeding with the acquisition of F-Z curves.

Data analysis.

Data analysis was carried out using the microscope built-in software. The retraction curves were fitted using the freely jointed chain (FJC) model to identify the stretching of the PEG chain. For the statistical analysis of the loading rate and rupture forces values, only curves that presented a single specific adhesion peak were used.

3.6.1.3. Affinity microscopy in the biotin-streptavidin system: yields comparison of different functionalized tips.

Preparation of tips and surfaces.

To carry out this comparison between tip functionalization methods and their performance when performing interaction measures, DeepTip™ SiN R15 T125 and MLCT-BIO-DC-B chips were used (nominal value $k = 0.02$ N/m $f = 15$ kHz in both cases). DeepTip™ SiN R15 T125 decorated with biotin were used following the protocol presented in the previous section (2.4.3). MLCT-BIO-DC tips were functionalized by immersion silanization using APDMS as silane coupling agent as described below. First, tips were cleaned using O₂

plasma (Diener Electronics, Zepto equipment) for 20 min at 80% power. Next, tips were immersed in a 1:1:0.005-APDMS: EtOH:H₂O mQ solution for 5 min at room temperature. Once the incubation was completed, the tips were rinsed thoroughly in absolute grade ethanol and mQ water and dried with N₂. To finish, the tips were baked for 30 min at 80°C. Once baked, they were stored at 4°C in 0.1 M borate buffer pH 8.5 overnight. For the modification with biotin, the same protocol as for the DeepTip™ tips was followed.

The same surfaces as those discussed in the previous section were used as streptavidin source (agarose beads functionalized with streptavidin embedded in a 2% agarose gel matrix).

Acquisition of curves.

The F-Z curves were carried out in liquid medium using DPBS in JPK AFM model Nanowizard V4. The parameters used to acquire the curves were the following: contact force 200-300 pN, contact time 100 ms, Z amplitude 500 nm, approach speed 2000 nm/s, retraction speeds 2000 nm/s. Two functionalized tips were used for each method and 1000 curves were acquired with each tip. The spring constant of the cantilevers was calculated experimentally in liquid medium using the thermal noise method implemented in the microscope software before proceeding with the acquisition of F-Z curves.

Data analysis.

Data analysis was carried out using the microscope built in software. The retraction curves were fitted using the FJC model to identify the stretching of the PEG chain. For performance analysis, the curves obtained were classified into four groups depending on the type of interaction detected (multiple, single, non-interaction, non-specific) and the percentages were calculated to compare the two types of AFM probes.

3.6.2. LDH/anti-LDH affinity measurements.

As an additional model, the antigen-antibody LDH/anti-LDH system was used to measure the forces between these two molecules with high affinity (Figure 15).

Preparation of AFM probes and substrates

DeepTip™ SiN R11 tips were decorated with the anti-LDH antibody following the protocol described in section 2.5.1.

MicroDeck™-150-G decorated with LDH were used as a surface using the protocol described in section 2.5.2.3. This protocol employs sulfo-LC-SPDP as cross-linker and an LDH solution at a concentration of 500 µg/ml.

Acquisition of curves and data analysis.

To measure the force between sensor (anti-LDH) and target (LDH) molecules, a Nanolife atomic force microscope (Nanotec Electronica S.L., Madrid, Spain) was used. The F-Z curves were taken at different points on the surface, acquiring 10 curves for each point. Measurements were carried out in PBS pH of 7.4 and a total of 1040 F-Z curves were acquired using the following parameters: contact force: 600–800 pN, contact time 1 s, approach speed 1000 nm/s, retraction speed 500 nm/s. The elastic constant of the cantilevers was previously calibrated in air using the Sader method and the optical sensitivity was calculated from the slope of no less than 10 approach curves. The curves

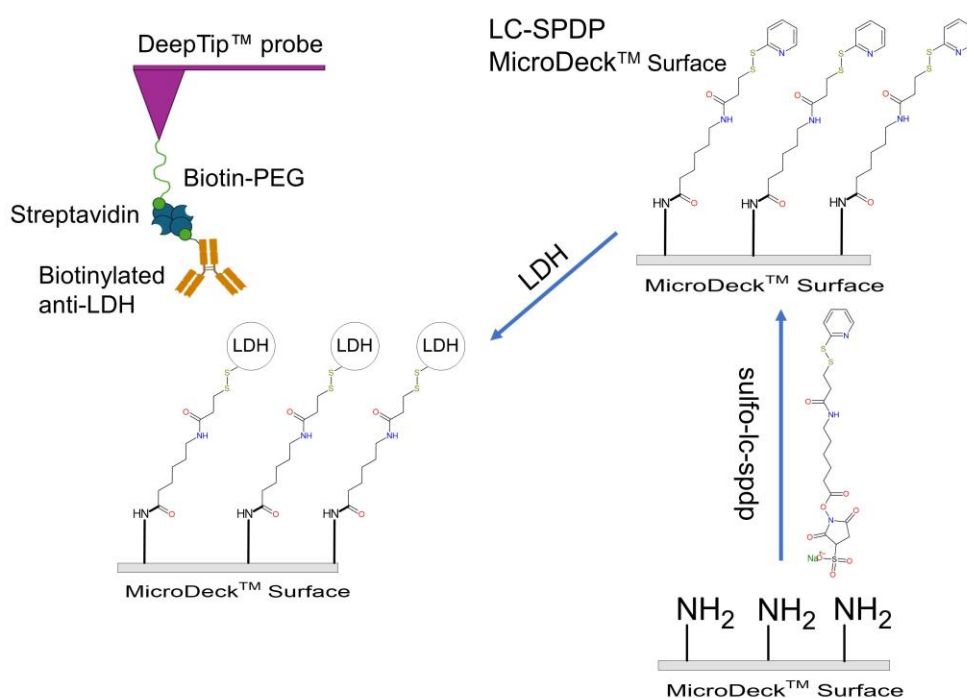


Figure 15. Schematic of the sample preparation process for affinity microscopy measurements in the LDH-anti-LDH system. [40]

obtained were analysed using WSxM 5.0 software and a Matlab routine developed in the group.

3.6.3. SMFS of titin. Yields Comparison of functionalisation methods.

These experiments were carried out in collaboration with the group led by Doctor Jorge Alegre-Cebollada of the National Institute for Cardiovascular Research (CNIC).

The objective of these experiments was to compare the performance for the detection of specific unfolding events of the Halo-I27₈ protein using DeepTip™ probes and probes functionalized with the immersion silanization protocol used in the group of Dr. Jorge Alegre-Cebollada (from now on they will be called JAC probes)

Functionalization of JAC probes.

A set of probes of the model qp-scont (Nanosensors) were functionalized by immersion silanization. In this case the organosilane used was (3-aminopropyl) trimethoxysilane (APTMS). Briefly, cantilevers were cleaned for 5 min in chloroform. Next, they were air dried and subsequently washed for 5 min in HPLC grade ethanol. To finish, they were air dried again. They were then treated with O₂ plasma for 15 min using a Harrick Plasma model PDC-32G-2 plasma cleaner.

Next, the tips were incubated at room temperature for 20 min and shaking at 50-80 rpm in a 1% solution of APTMS in HPLC grade ethanol. Once the incubation time was over, the tips were washed 5 times with HPLC grade ethanol and placed in a Petri dish. They were then baked for 30 min at 100°C. Once the process was completed, the tips were stored in a desiccator at room temperature until use.

Modification of the DeepTip™ probes with Halo ligand.

Both DeepTip™ SiN R11 and the JAC probes were modified with Halo ligand following the protocol used in the group of Doctor Jorge Alegre-Cebollada. Briefly, tips were incubated overnight, at room temperature, in a humid chamber and protected from light; in a 1 mM solution of HaloTag succinimidyl ester (O4) ligand (Promega P6751) in 50 mM borax buffer pH 8.5. Once the incubation time was over, the tips were washed five times

by immersion in mQ water and stored at 4°C in a humid environment and protected from light until use, without exceeding 7 days of storage.

Surface preparation with the NHalo-I27(8)-CCys protein.

Gold-coated glass cover slips were placed on the AFM holder and used as a support for the covalent binding of the NHalo-I27(8)-CCys protein. To do this, between 3-7 μ l of protein at a concentration of 0.2 mg/ml in HEPES buffer (0.1 M N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) pH 7.5) were deposited on the surface of the cover slip and allowed to incubate for around 1 min (incubation time was determined by observing the retraction of the edges of the droplet on the surface).

After the incubation time was over, the liquid cell of the atomic force microscope was closed, and the measurements began.

Acquisition of F-Z curves.

For the acquisition of F-Z curves, 3 JAC and 3 DeepTip™ probes (nominal value $k=0.01$ N/m for both probes) were used. The curves were acquired in liquid medium using HEPES buffer at room temperature. The spring constant of the cantilevers was calculated experimentally for each of them, before beginning the acquisition of curves using the thermal noise method. The optical sensitivity was calculated experimentally at the same time from the average value of the slope obtained from no less than 10 force-displacement curves. The operating parameters for the acquisition of F-Z curves were: contact force 1500 pN, Z amplitude 600 nm, approach and retraction speed 400 nm/s. Curve acquisition was carried out for at least 1 h.

Data analysis.

For all tips used during acquisition experiments, only the curves obtained during the first hour of the experiment were analysed with the objective of comparing a similar number of curves recorded with each probe. The curves were analysed with the IGOR PRO software using an analysis routine belonging to Jorge Alegre's group. The curves that presented the characteristic fingerprint of I27 were adjusted according to the wormlike chain (WLC) model to corroborate the unfolding process. Finally, a classification of the types of curves

obtained was carried out and the percentages of each type of curve were calculated for all the tips used in the tests.

4. RESULTS AND DISCUSSION.

4.1. Characterization of DeepTip™ AFM probes and MicroDeck™ substrates.

4.1.1. Functionality and temporal stability of the amino layer.

As mentioned in section 2.3.1, fluorescence microscopy was used to assess the presence of amino groups on the surface of DeepTip™ AFM probes and MicroDeck™ substrates.

DeepTip™ probes.

Fluorescence images of amino-functionalized AFM cantilevers of the models DeepTip™ SiN R67-150, DeepTip™ SiN R67-300, and DeepTip™ SiN R67-600 are shown in Figure 16.

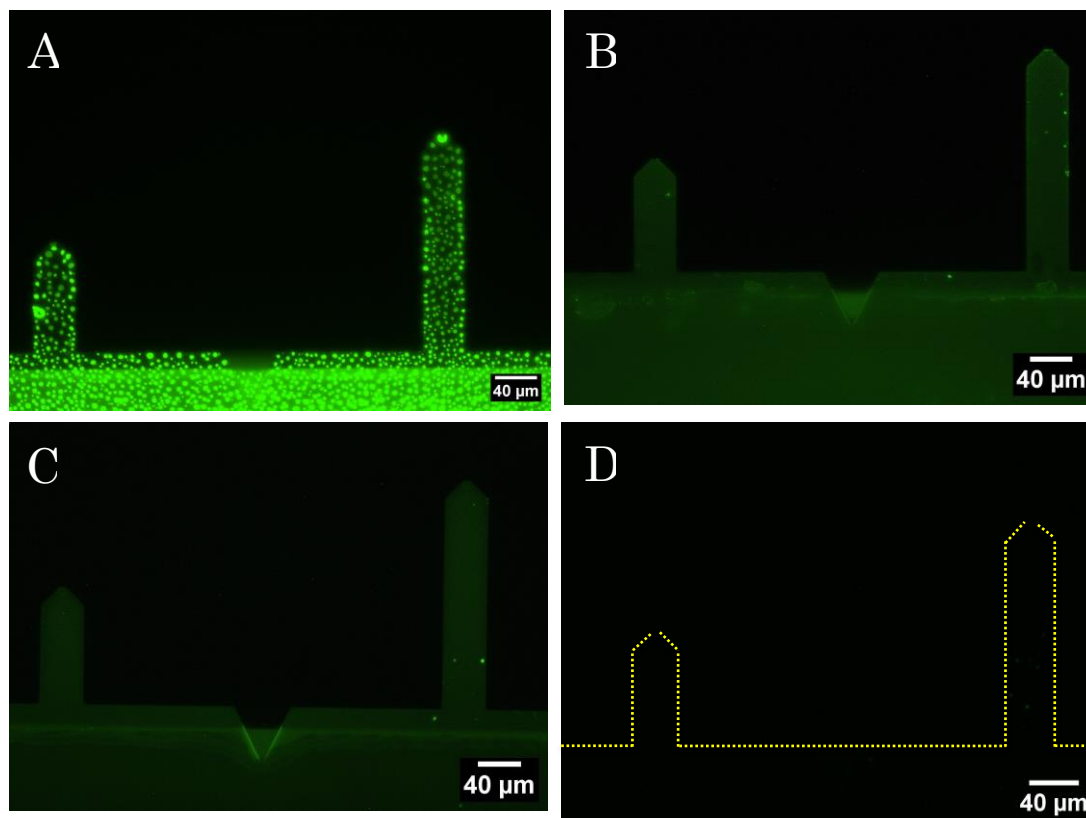


Figure 16. Fluorescence micrographs corresponding to various DeepTip™ probes. (A) DeepTip™ SiN R67-600, (B) DeepTip™ SiN R67-300, (C) DeepTip™ SiN R67-150 and (D) non-functionalized control sample.

In all cases, amino groups are found along the cantilevers, while the fluorescence intensity is significantly smaller in the nonfunctionalized control sample. There is a clear difference, however, between the three functionalized probes. As can be seen, DeepTip™ SiN R67-600 present inhomogeneous structures in contrast to the homogeneous surface observed in the DeepTip™ SiN R67-300 and DeepTip™ SiN R67-150 models

Fluorescence was analysed quantitatively in the two models with homogeneous layers to evaluate possible differences between both types. For this purpose, a statistical analysis was carried out by applying t-tests to the functionalized and control probes considering the following pairs: DeepTip™ SiN R67-150 vs DeepTip™ SiN R67-300, DeepTip™ SiN R67-150 vs control and DeepTip™ SiN R67-300 vs control. For all pairs, significant differences ($p < 0.001$) were obtained between the three probes as summarized in Figure 17. The comparison of both functionalized probes indicates that DeepTip™ SiN R67-300 presents a significantly higher fluorescence intensity. Since a high density of amine groups may translate to a high density of sensor molecules and represent a difficulty for recording single molecule interactions, the DeepTip™ SiN R67-150 model characterized by a homogeneous surface and a lower density of amine groups was the probe selected for most of the subsequent experiments.

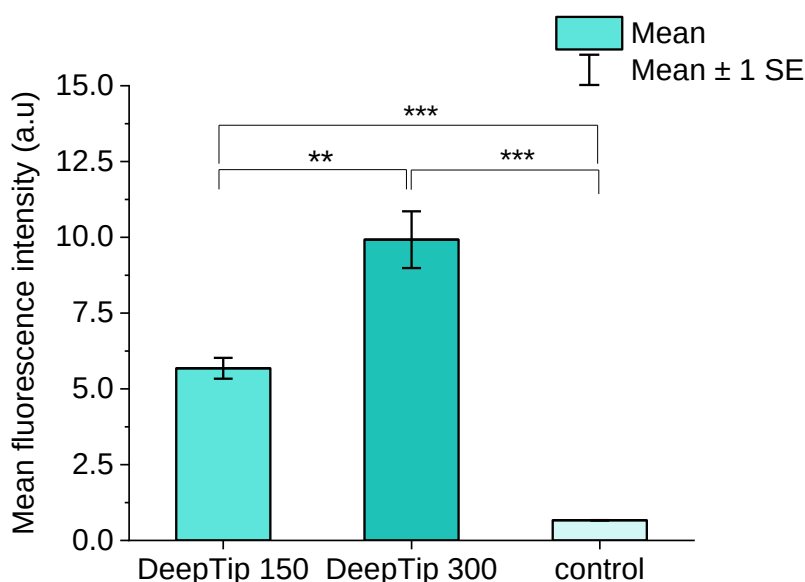


Figure 17. Bar chart comparing the average fluorescence intensity of DeepTip™ SiN R67-150, DeepTip™ SiN R67-300 and nonfunctionalized control probes.

Once it was decided that the standard tip to use in most experiments would be DeepTip™ SiN R67-150, it was considered relevant to characterize the temporal stability of its functional layer

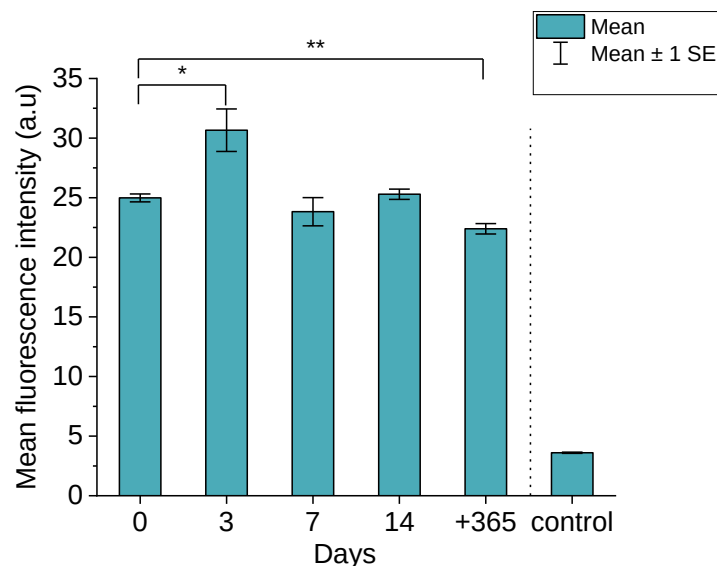


Figure 18. Bar chart comparing the mean fluorescence intensity of DeepTip™ SiN R67-150 at increasing times.

As can be seen in Figure 18, fluorescence intensity only shows a marginal decay with time even after more than one year. A slight significant increase in fluorescence intensity is observed at day 3 with respect to day 0. The main idea proposed to explain this increase is the lack of washing during the rinsing steps of the fluorescent reagent used during the incubation process. For samples older than one year, a significant variation is also observed with respect to day 0. In this case the decrease in fluorescence intensity may be due to the degradation of the amine group surface due to the time elapsed. For the samples at day 7 and 14 no significant variations are observed with respect to day 0. Finally, all samples show a significant difference ($p < 0.001$) respect to the control sample without amino groups at day 0.

Since no special storage conditions were implemented, simply consisting of depositing the samples in a p48 multiwell inside a cupboard at room temperature and protected from light, this result is a proof of the robustness of the functionalized probes.

In addition to the results obtained from the DeepTip™ SiN R67 series presented above, other DeepTip™ models were also characterized using fluorescence microscopy. The micrographs shown in figure 19 summarize the results retrieved from these probes.

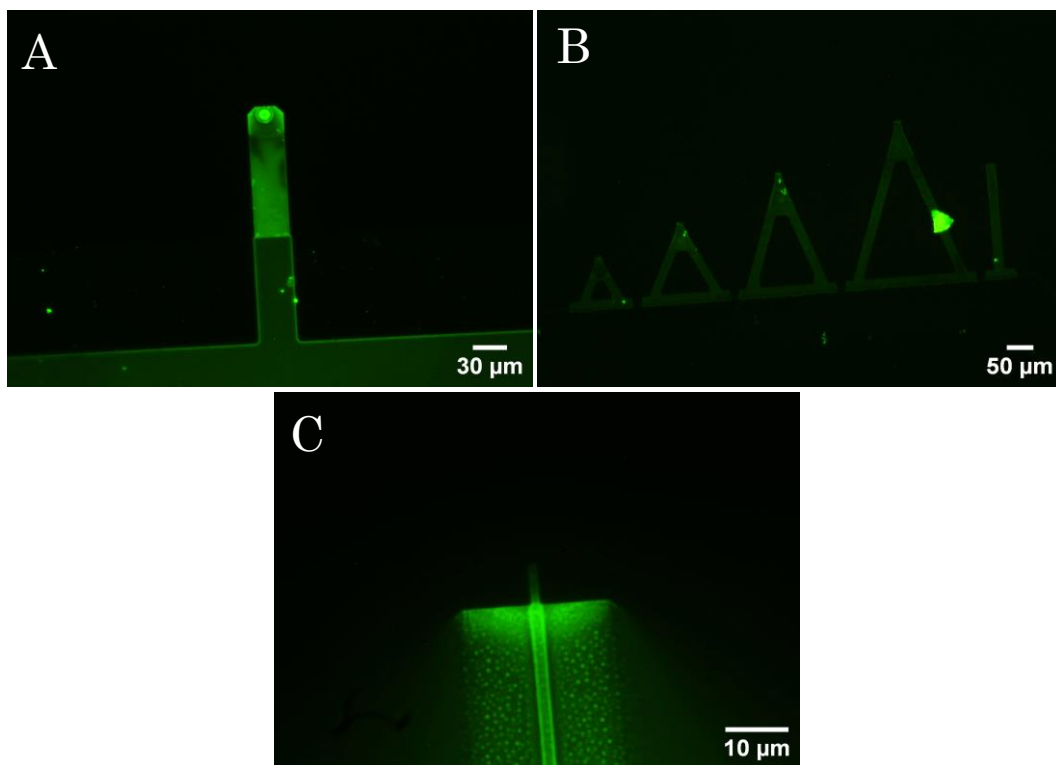


Figure 19. Fluorescence micrograph of the DeepTip™ SiN R11 (A), DeepTip™ SiN R15 T125 (B) and DeepTip™ SiO R1200 (C) models

MicroDeck™ substrates.

MicroDeck™ substrates were also checked using fluorescence microscopy, and the results obtained from this technique are summarized in Figure 20 for the MicroDeck-G-series.

Additionally, mica-based surfaces MicroDeck™-m-300 were also checked, as it is a widely used substrate in atomic force microscopy.

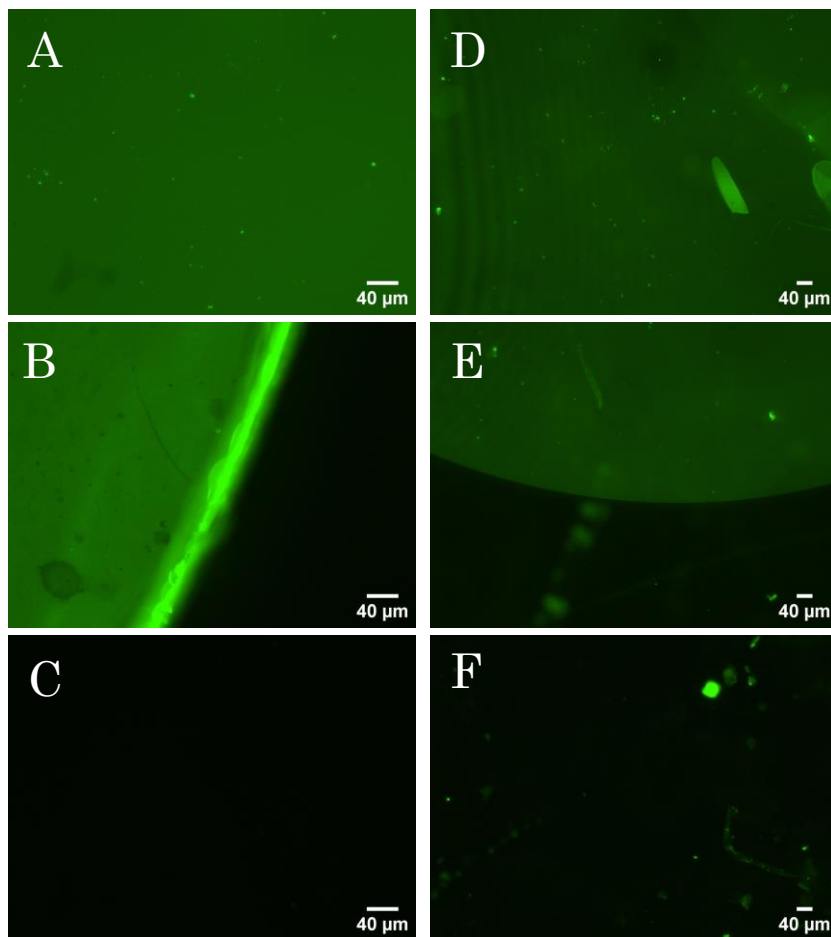


Figure 20. Fluorescence micrograph of MicroDeck-G-150 (A, B) and MicroDeck-m-300 (D, E) substrates. Control substrates, glass (C), mica (F).

4.1.2. Surface topography of functionalized samples

In this section, the result of the measurement of the surface topography obtained from AFM images of both DeepTip™ probes and MicroDeck™ substrates will be shown and discussed.

AFM probes

The analysis of the surface roughness of functionalized cantilevers has been studied from two approaches.

Firstly, comparing the variations in roughness between the two functionalized AFM probes as shown in Figure 21, and with the nonfunctionalized (control) AFM

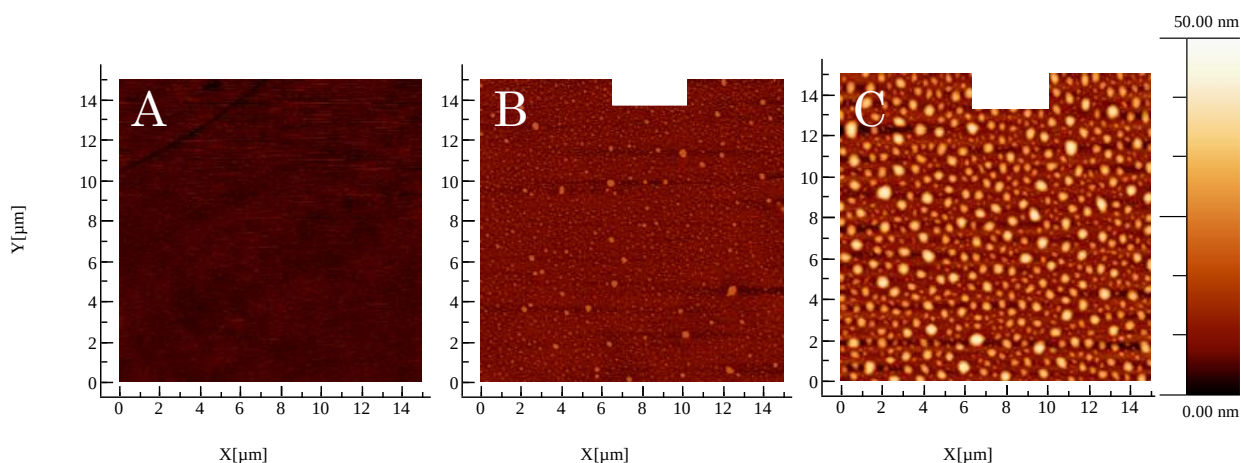


Figure 21. Representative AFM topography images obtained using. Control sample (A), DeepTip™ SiN R67-150 (B) and DeepTip™ SiN R67-300 (C)

probe. As can be seen, surfaces roughness is larger in the DeepTip™ SiN R67-300 probe than in DeepTip™ SiN R67-150 which may be related to the information provided by the manufacturer with respect to the thicker functionalized layer of the former. In addition, a few structures like bumps are identified in both functionalized probes. The analysis of the profile of these structures on the topography images yields a height of 35-40 nm for the DeepTip™ SiN R67-300 model and of 15-20 nm for the DeepTip™ SiN R67-150. The values of the root mean square (RMS) and of the average roughness (Ra) are indicated in Table 4. (Definition of Ra and RMS are found in annex B)

Table 4 RMS and Ra surface roughness values of different models of DeepTip™ and nonfunctionalized control probes

	DeepTip™ SiN R67-300	DeepTip™ SiN R67-150	Control
Ra (nm)	30 ± 10	7 ± 2	6 ± 3
RMS (nm)	9 ± 1	2.8 ± 0.7	1.7 ± 0.8

Additionally, the variation in surface roughness along the cantilever was also studied for both functionalized probes and presented in Figure 22.

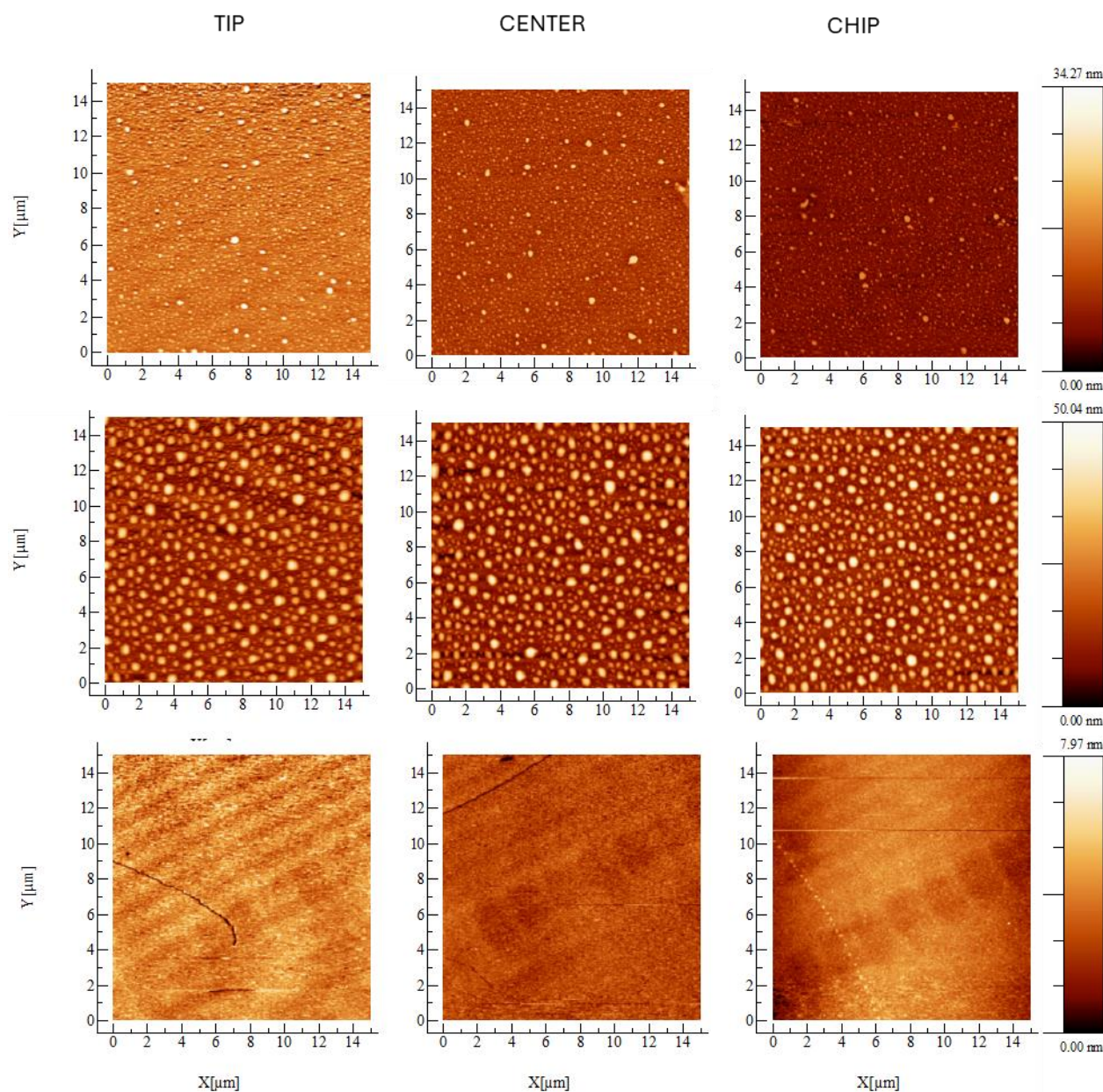


Figure 22. Representative AFM topography images at different positions along the cantilever in the DeepTip™ SiN R67-150 (top), DeepTip™ SiN R67-300 (middle) models and nonfunctionalized control sample (bottom).

Micrographs of the region near the tip of the cantilever (Tip), of its central area (Center) and, finally, of the region close to the chip (Chip) are shown. The comparison of the three images from both functionalized probes indicates that the functionalization leads to a

homogeneous surface along the entire cantilever, since no notable differences are observed between the different regions.

MicroDeck™ substrates

Representative micrographs of the MicroDeck-G-150 substrates are shown in Figure 23 and compared with nonfunctionalized control substrates. Surface roughness for both functionalized and control substrates was calculated for two different sizes: 2x2 μm and 0.5x0.5 μm , in three different functionalized samples and in a control substrate.

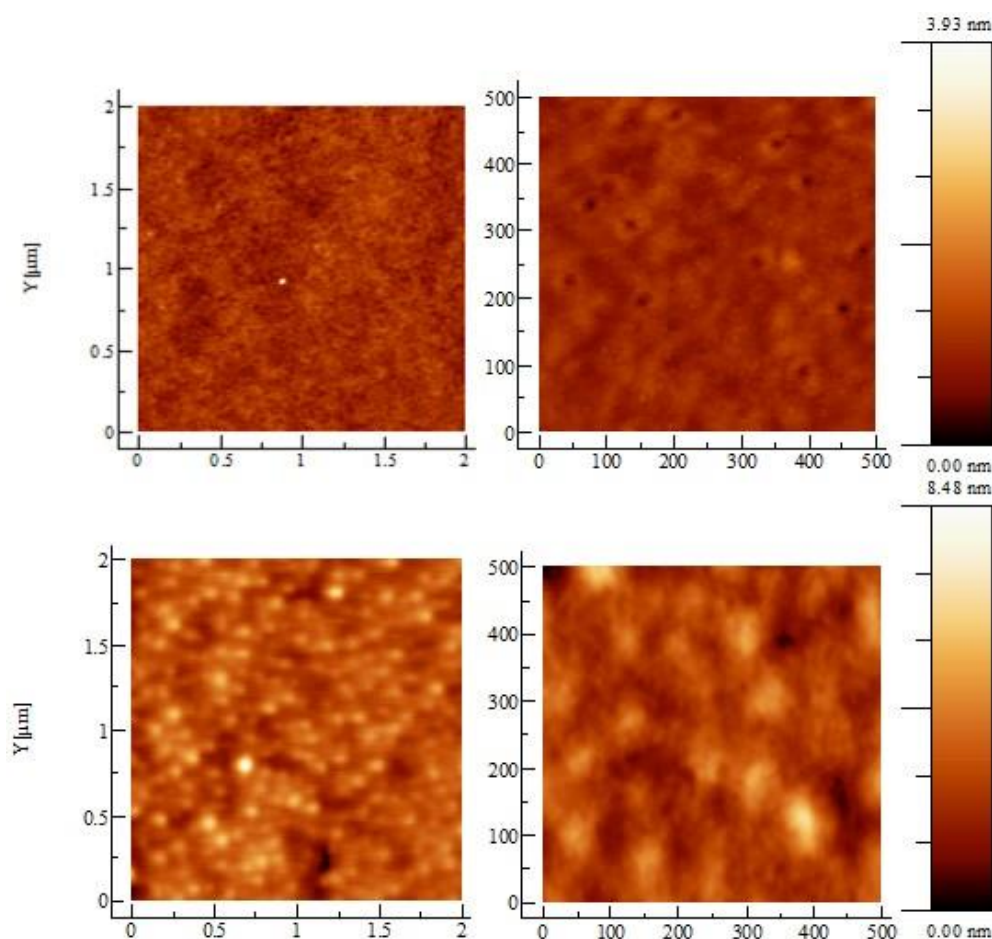


Figure 23. Representative AFM topography images at two different scan sizes of nonfunctionalized control surfaces (top) and MicroDeck™-G-150 surfaces (bottom)

Table 5 summarizes the values obtained for the surface roughness of both control and functionalized substrates. As can be seen, the surface roughness in one of the functionalized samples is significantly larger than the values obtained from the other two

samples. In particular, these latter values are only slightly larger than those measured on the nonfunctionalized control substrate.

The measured surface roughness was considered as adequate for using MicroDeck™ functionalized substrate for the rest of the experimental campaign.

Table 5. RMS roughness values of different samples belonging to MicroDeck-G150 surfaces.

SAMPLE	RMS at 2x2 μm	RMS at 0.5x0.5 μm
CONTROL	0.19 nm	0.14 nm
1°	0.74 nm	0.81 nm
2°	8.43 nm	3.38 nm
3°	0.66 nm	0.23 nm

4.1.3. SEM observation of functionalized cantilevers.

The DeepTip™ SiN R67-150 and DeepTip™ SiN R67-300 models were observed by scanning electron microscopy as detailed in section 2.3.3 since it was of interest to determine the geometry of the tips of these functionalized AFM probes in detail.

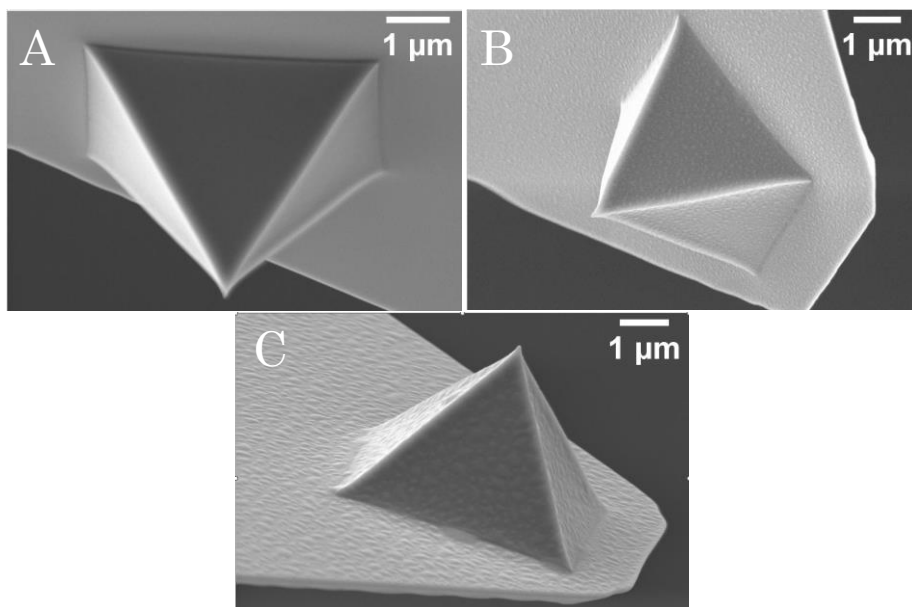


Figure 24. SEM micrographs of AFM tips (A) control sample, (B) DeepTip™ SiN R67-150, and (C) DeepTip™ SiN R67-300

As can be seen in the micrographs, the functionalized probes DeepTip™ SiN R67-150 and DeepTip™ SiN R67-300 present an island-like topography on the surface, in accordance with the topography images presented in the previous section. Furthermore, and considering the geometry of the tip, it is observed that it maintains the pyramidal geometry ending in a sharp apex. No modifications were observed in the images that give rise to double tips or a convolution of the tip. This means that the functional tips can be used reliably for subsequent experiments where these characteristics are necessary, such as obtaining images.

4.1.4. Characterization of resonance frequency and spring constant of DeepTip™ cantilevers.

On the part of the company Bioactive Surfaces SL, there was interest in establishing the possible influence of the functionalization process on the variation of the intrinsic parameters of resonance frequency and spring constant of the cantilevers. The results obtained from measuring the resonance frequency and spring constant of DeepTip™ SiN R67-150 and DeepTip™ SiN R67-300 cantilevers are shown in Table 6 and compared with the values of the control (non-functionalized) probes. Since both controls and

functionalized probes present two cantilevers, the measurements were undertaken on both.

Table 6. Average values of resonance frequency and spring constant of cantilevers belonging to different DeepTip™ models.

Dimension of cantilever	Resonance Frequency (kHz)	Spring constant (pN/nm)
100x40 μm (DeepTip™ 150)	45 ± 1	180 ± 50
200x40 μm (DeepTip™ 150)	12.6 ± 0.2	26 ± 4
100x40 μm (DeepTip™ 300)	$45.8 \pm 0,6$	200 ± 50
200x40 μm (DeepTip™ 300)	$12.7 \pm 0,1$	28 ± 8
100x40 μm (control)	46.7 ± 0.5	220 ± 50
200x40 μm (control)	12.73 ± 0.09	32 ± 9

As can be seen in Table 6, the average values of resonance frequency and spring constants are in the range of those presented by the control (non-functionalized) cantilevers. Since the variation of the controls results from the micromanufacturing process of the probes, so that different batches may exhibit different parameters, it may be concluded that functionalization does not lead to an increase in this intrinsic variation.

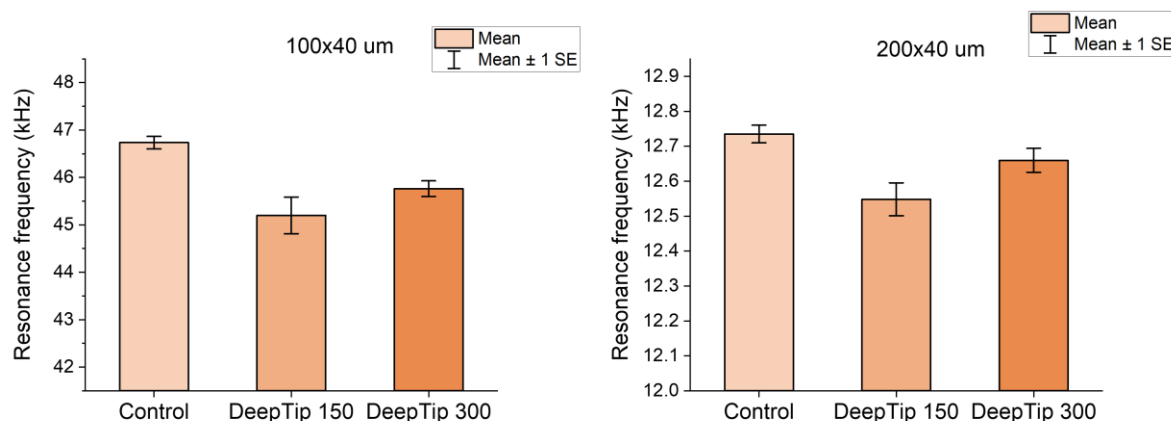


Figure 25. Bar chart showing the average values of resonance frequency for cantilevers of two different geometries belonging to the DeepTip™ SiN R67-150 and DeepTip™ SiN R67-300 probes.

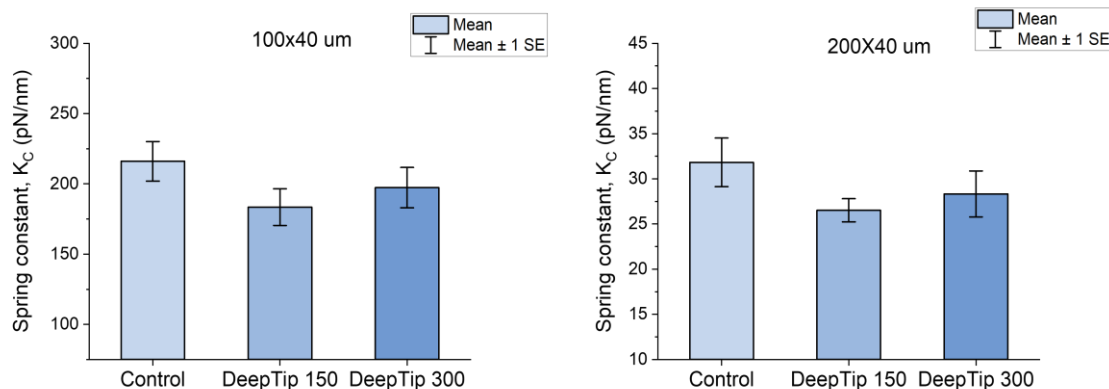


Figure 26. Bar chart showing the average values of spring constant for cantilevers of two different geometries belonging to the DeepTip™ SiN R67-150 and DeepTip™ SiN R67-300 probes.

4.2. Chemical modifications of DeepTip™ probes and MicroDeck™ substrates.

4.2.1. Chemical modification with thiols

DeepTip™ probes

DeepTip™ SiN R67-150 were modified with the NHS-sulfo-LC-SPDP compound to modify the surface with thiol groups according to the procedure discussed in section 3.4.1.

In Figure 27, it can see a representative fluorescence image obtained after this chemical modification. Additionally, images of the two controls used are shown, one of them with amino groups on the surface and the other with aldehyde groups (see section 3.4.1).

From a qualitative point of view, a clear difference can be seen between the image that corresponds to the surface modified with thiols and the controls, which is compatible with the presence of thiols on the surface after the modification process.

The fluorescence intensity was quantified to confirm that there was a statistically significant difference, as determined by the t-test (Figure 27 D). As can be seen, there is a statistically significant difference between the sample modified with thiols and the control that has amino groups ($p < 0.01$), and between the surface modified with thiols and that modified with aldehyde groups ($p < 0.001$). In contrast, the difference in the average fluorescence intensity between the controls is not statistically significant.

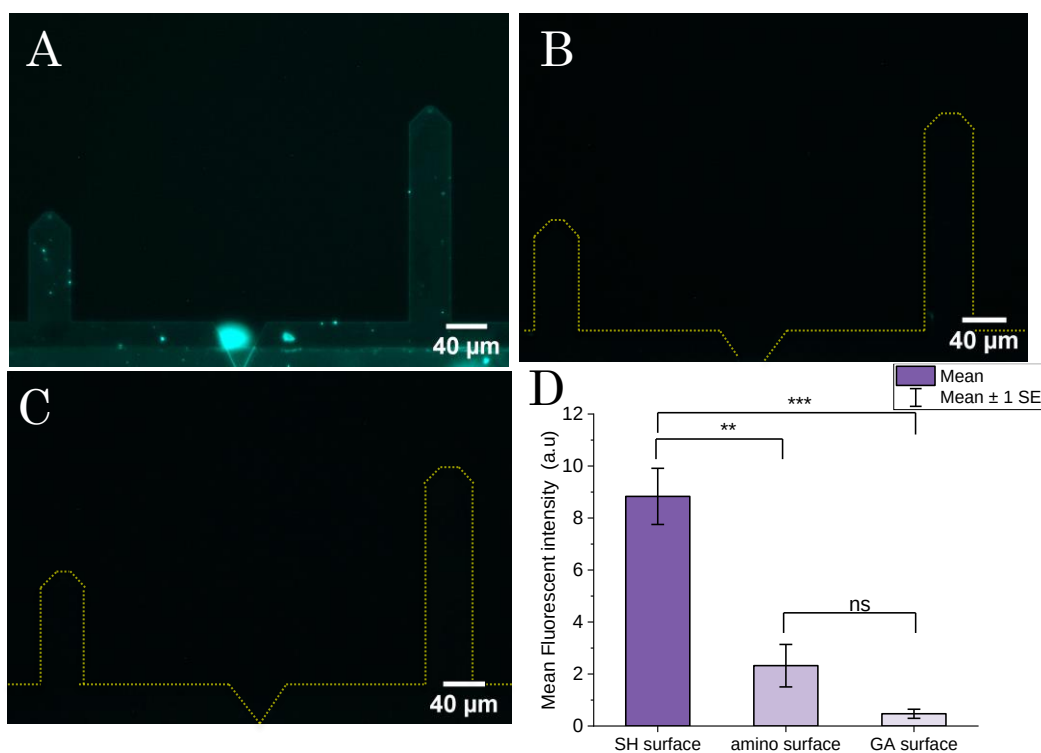


Figure 27. Representative fluorescence micrographs of samples modified with sulfo-LC-SPDP (A), control sample with amino groups (B) and sample modified with aldehyde groups (C). Bar chart (D) comparing the average fluorescence intensity between the different samples.

MicroDeck™ substrates

MicroDeck™-G-150 surfaces were incubated with either NHS-sulfo-LC-SPDP or, alternatively, with NHS-PEG5K-OPSS compounds to modify the surface with thiol groups according to the procedure discussed in section 2.4.1.

As was done with the DeepTip™ probes, the results obtained for the micro deck samples have been analysed both qualitatively (Figure 28) and quantitatively (Figure 29).

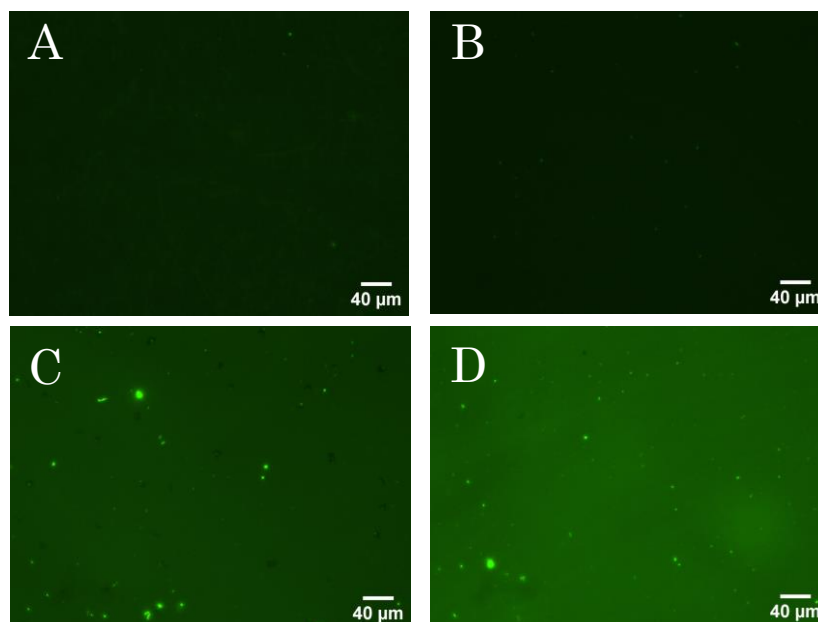


Figure 28. Representative fluorescence micrographs of MicroDeck™ surfaces with amino groups (A) and modified with aldehyde groups (B), OPSS (C) and sulfo-LC-SPDP (D).

In Figure 28 a clear difference is observed between the control samples and those modified with thiols. In addition, a slight difference in fluorescence intensity is also observed between the functionalized samples modified with thiol groups.

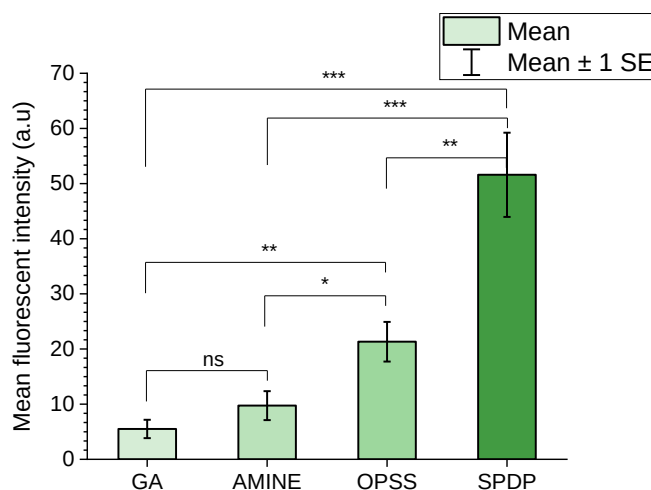


Figure 29. Bar chart comparing the average fluorescence intensity between the different probes modified with thiol groups.

t-tests were performed again in order to assess whether the observed differences were statistically significant. As shown in Figure 29, no significant difference is found between the control samples, while a significant increase in fluorescence is observed in both thiol-

modified probes. As mentioned from the comparison between the corresponding micrograph, a statistically significant difference is also observed between the substrate modified with OPSS and that modified with LC-SPDP.

Since both thiol-modified samples were incubated in solutions with the same concentration (w/v) of the corresponding molecule, while the molecular weight of OPSS is larger than that of LC-SPDP, it may be hypothesized that the difference in fluorescence stems from an incubation with a reduced number of crosslinkers for the former.

4.2.2. Chemical modification with azide and alkyne (click chemistry).

As mentioned in the introductory chapter, the azide/alkyne (click) chemistry has great relevance and special interest in the fields that aim to study biological systems due to its high efficiency and specificity. Hence, there has been interest in modifying the DeepTip™ SiN R67-150 samples with these compounds, since it can open the door to new strategies for immobilizing biomolecules in the field of affinity microscopy.

The results obtained after the application of the protocols described in section 3.4.2 are presented below and, as in the case of the modification with thiols, analysed both quantitatively and qualitatively.

4.2.2.1. Samples modified with NHS-PEG12-DBCO.

In this case, two controls were used: (i) DeepTip™ samples that were not incubated with DBCO, but incubated with a fluorophore, and (ii) non-functionalized samples incubated with DBCO. The aim of these double controls was to rule out non-specific cross reactions between the fluorophore and the amino groups on the surface, and additionally, to corroborate that the lack of amino groups on the surface prevents the binding of DBCO. Figure 30 shows representative images of each condition.

As can be seen, the highest fluorescence intensity is observed in the image that corresponds to the functionalized probes incubated with DBCO. This supports that the compound has modified the original surface (with amino groups) with DBCO groups.

The quantitative analysis of average fluorescence intensities shows that there is a statistically significant difference between the controls and the DBCO-modified probe.

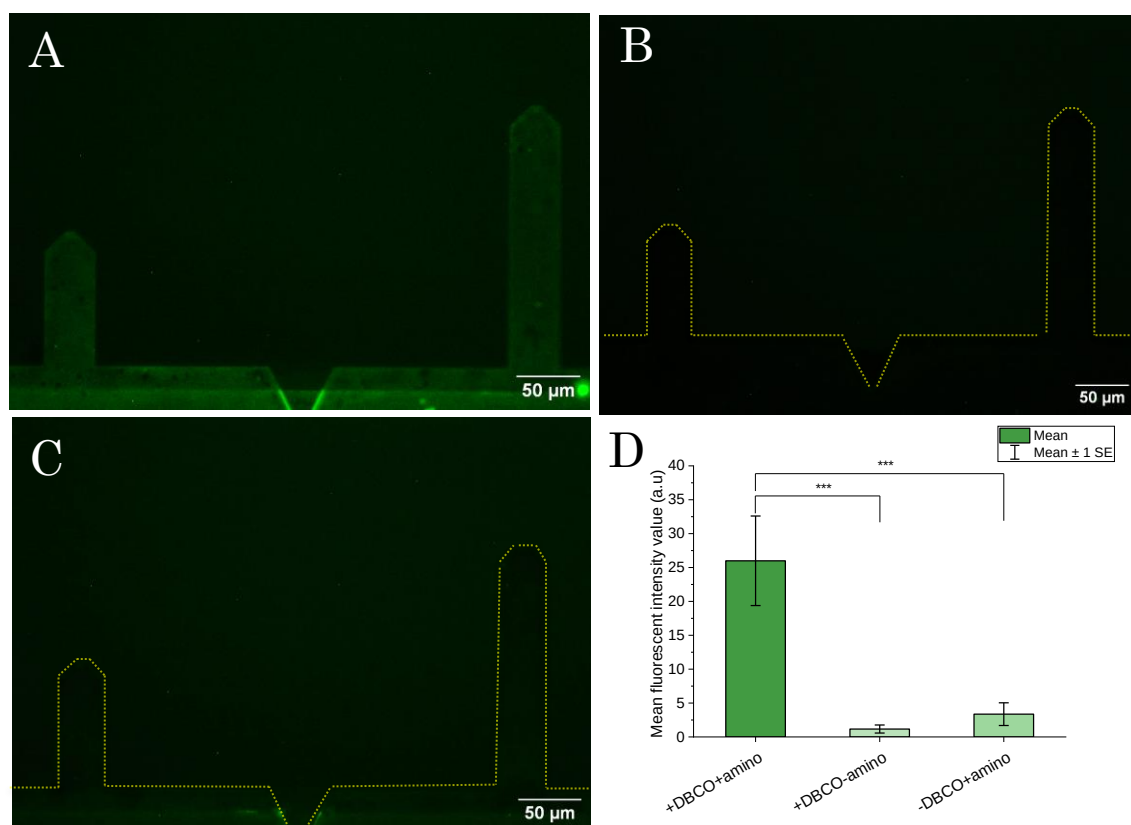


Figure 30. Representative fluorescence micrographs of probes modified with DBCO (A), nonfunctionalized probe incubated with DBCO (B), and functionalized probe not modified with DBCO (C). Bar chart comparing the average fluorescence intensity between the different samples (D).

4.2.2.2. Chemical modification with NHS-PEG4-azide.

The characterization of the azide-modified probes parallels that described in 4.2.2.1, in particular with respect to the controls (in this case, the nonfunctionalized probe was incubated with azide instead of DBCO).

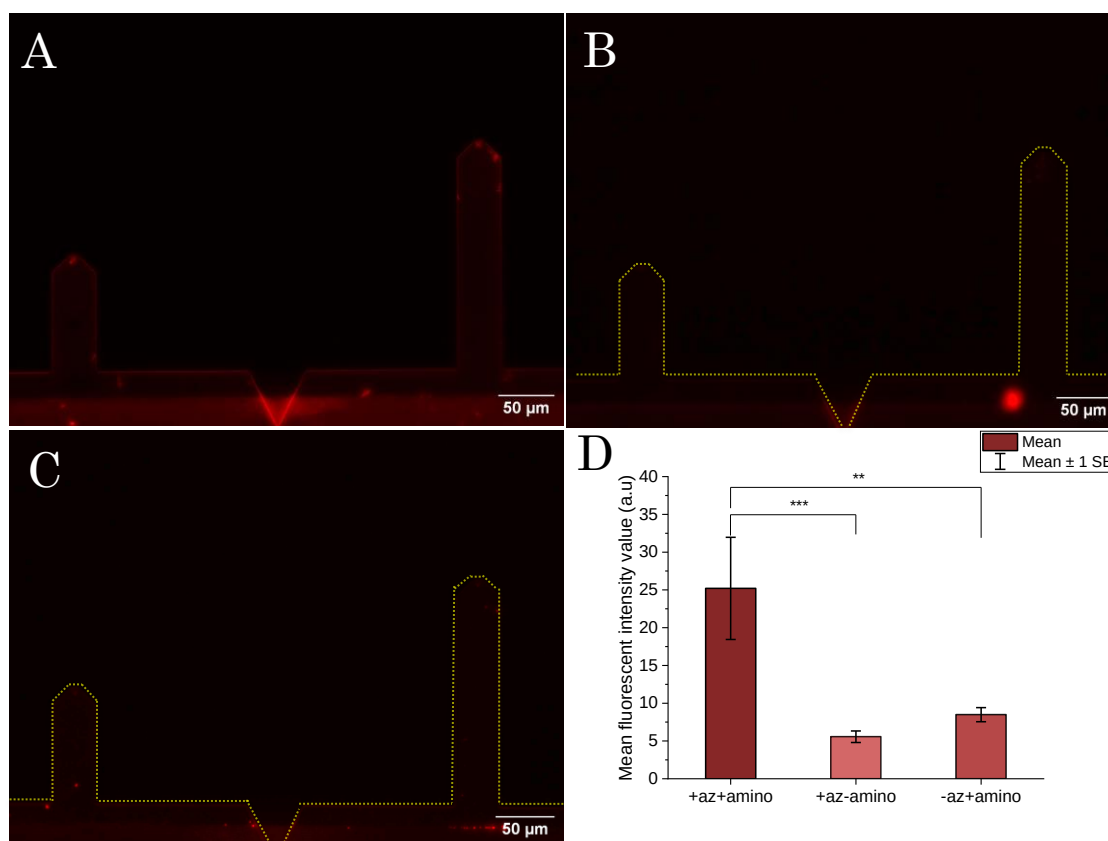


Figure 31. Representative fluorescence micrographs of probes modified with azide (A), nonfunctionalized probe incubated with azide (B) and functionalized probe not modified with azide (C). Bar chart (D) comparing the average fluorescence intensity between the different samples.

As can be seen in Figure 31, similar results to those obtained for DBCO modification are obtained, indicating that the modification with azide groups has also been successful.

4.3. Decoration of the modified surfaces with biomolecules.

4.3.1. Decoration with streptavidin-biotin

In this section the results on the decoration of the functionalized and chemically modified probes with biotin-PEG will be shown.

Figure 32 shows representative images of probes decorated with biotin (see section 3. 4.3), where streptavidin-FITC was used as a fluorophore, and indicates the efficiency of this decoration procedure qualitatively. It can be observed how the brightness is even greater in the region of the tip indicating that the density of biotin molecules must be larger in this area.

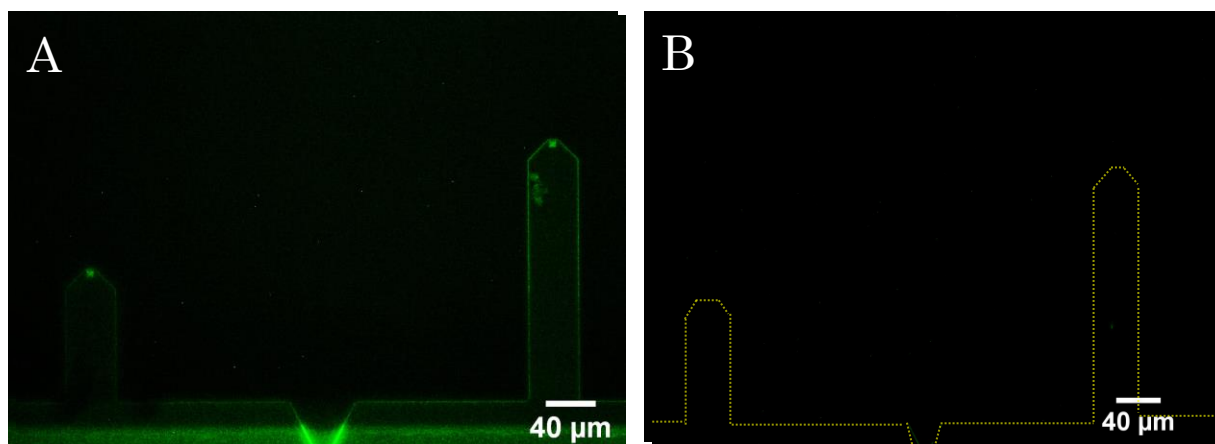


Figure 32. Representative fluorescence micrographs of probes decorated with biotin-PEG-NHS (A) and control probe (B).

4.3.2. Decoration with antibodies. Temporal stability of the samples.

In this section the results on the decoration of the functionalized and chemically modified probes with antibodies will be shown. As seen below, in the protocol used, decoration with antibodies takes the previous decoration with streptavidin as starting point.

Figure 33 shows fluorescence micrographs of a cantilever decorated with a biotinylated anti-LDH antibody and a control sample (the control sample consists of DeepTip™ SiN R67-150 probes decorated with biotin and streptavidin and that were not incubated in the anti-LDH antibody solution). A heat map filter has been applied to the images to, qualitatively, enhance the observation of the variations in the fluorescence intensity of the surfaces. As can be seen by the increased colour intensity in the region close to the AFM tip, it may be assumed that this area presents a greater density of antibodies. This fact is relevant for the usage of these probes in the affinity atomic force microscopy experiments that follow.

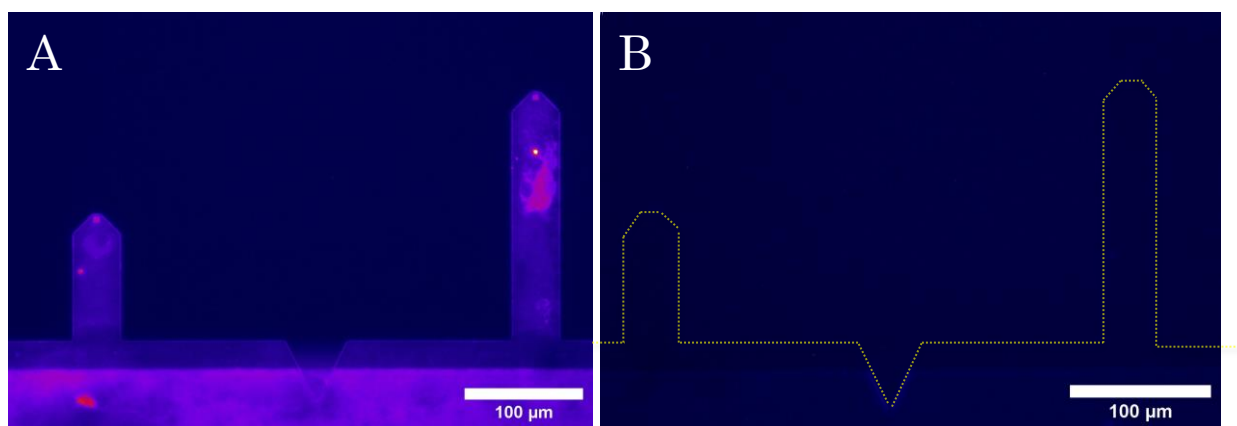


Figure 33. Representative fluorescence micrographs of probes decorated with an anti-LDH antibody (A) and control probe (B) obtained with a secondary fluorescent antibody.

The fluorescence intensity was analysed quantitatively, showing a significant statistical difference ($p < 0.001$) between the antibody-decorated probe and the control.

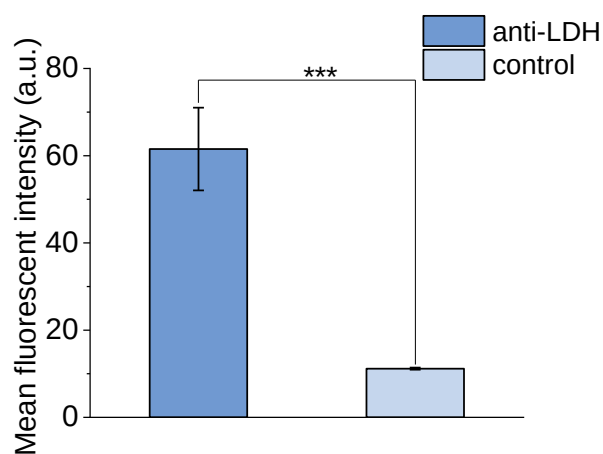


Figure 34. Bar chart comparing the average secondary antibody fluorescence intensity between the anti-LDH-decorated probe and the control.

Figure 35 shows the results of the temporal stability of the modified samples.

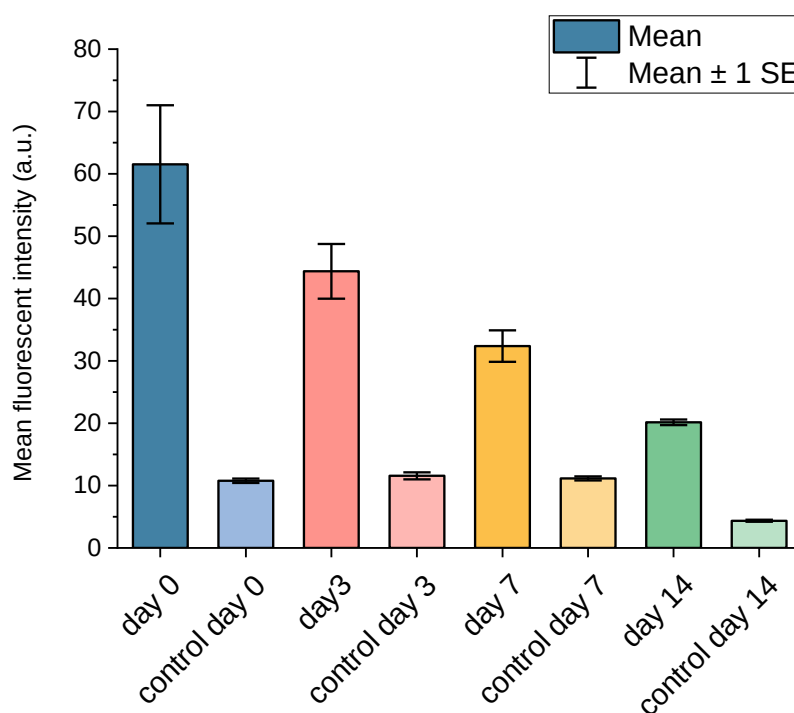


Figure 35. Bar chart comparing the mean fluorescence intensity of DeepTip™ SiN R67-150 decorated with an anti-LDH antibody over time.

As can be seen in the graph, the fluorescence intensity decreases with time. The most likely explanation for this decrease may be that, although the samples were preserved in a buffer (PBS pH 7.4) at 4°C, it is likely that antibodies denature progressively. The denaturation of the primary antibody implies that the secondary antibody used may not be adequately recognizing the anti-LDH antibody. Another possible explanation is that due to the time samples are maintained in the buffer, the biotinylated antibodies dissociate from the streptavidin molecules. In any case, and as shown in Table 7, there are still significant differences between the control samples and the samples decorated with antibodies up to 14 days after being modified, indicating that a significant number of residual functional molecules remain on the surface. However, it would not be advisable to use this type of modified tips after 7 days after decoration since the number of functional antibodies seems to be halved at this time.

Table 7. Significance p-values for samples incubated with antibody vs. control samples for different days after the decoration process.

Condition	p Value			
	Day 0	Day 3	Day 7	Day 14
Sample vs Control	<0.01	<0.01	<0.01	<0.01

4.3.3. Decoration of thiol-modified surfaces.

4.3.3.1. Decoration with a protein lacking cysteines

Streptavidin was selected as model system for decorating a surface with a protein without free thiol groups (without cysteine residues exposed on the surface). Streptavidin was previously modified with the sulfo-LC-SPDP crosslinker through the amine groups of the protein as explain in section 3.5.2.1.

To check whether the protein had been modified correctly, the absorbance at 280 nm (to detect the presence of protein and free NHS ester groups) and 343 nm (to detect the presence of pyridine-2-thione) of the modified protein solution was measured at different times of the modification process. Alternatively, another protein without free thiol groups, Prup3, was also used to establish the versatility of the procedure. Table 8 (Prup3) and Table 9 (streptavidin) show the results of the modification process.

Table 8. Absorbance values obtained during the modification process of the Prup3 protein.

Sample	Filtration	Redox state	ABS 280 nm	ABS 343 nm
Prup3-SPDP	No	Oxidized	1.003	0.022
Prup3-SPDP	No	Reduced	0.978	1.023
Prup3-SPDP	Yes	Oxidized	0.064	0.006
Prup3-SPDP	Yes	Reduced	0.066	0.047

Table 9. Absorbance values obtained during the modification process of the streptavidin protein.

Sample	Filtration	Redox state	ABS 280 nm	ABS 343 nm
streptavidin-SPDP	No	Oxidized	0.928	0.020
streptavidin-SPDP	No	Reduced	1.027	1.115
streptavidin-SPDP	Yes	Oxidized	0.145	0.005
streptavidin-SPDP	Yes	Reduced	0.174	0.115

As can be seen in the results, before filtering the protein solution (in order to isolate the modified protein fraction from the sulfo-LC-SPDP molecules that had not reacted), it is observed that the absorbance values at 343 nm in the reduced state of the sulfo-LC-SPDP are higher than the absorbance values in the oxidized state, because in the reduced state the pyridine-2-thione molecule has been released. This result is repeated for both proteins. When the protein is filtered and the modified protein fraction is obtained without free sulfo-LC-SPDP molecules, it is observed that the absorbance at 343 nm of the modified protein solution in the oxidized state is negligible while it increases considerably in the reduced state. This increase indicates that the pyridine-2-thione molecule has been released. The absorbance value at 343 nm in the reduced state, however, is considerably lower after filtration. This decrease in the absorbance value implies that after filtration, by reducing the modified protein, the pyridine-2-thione can only come from the sulfo-LC-SPDP molecules attached to the protein.

Regarding the values at 280 nm, no major variations are observed between the oxidized and reduced states, but considerable variations are observed after filtering. The explanation for this comes from the fact that not only the protein absorbs at 280 nm but also the NHS ester group of the sulfo-LC-SPDP molecule. When filtering, the free sulfo-LC-SPDP molecules are retained in the column. This implies that the resulting absorbance at 280 nm after filtration comes from the proteins, again indicating that filtration leads to the purification of the modified protein.

Once the modified streptavidin was obtained, it was used in the oxidized state to fix it to thiol-modified MicroDeck™-G-150 surfaces. To verify that the protein was fixed to the surfaces, a colorimetry test based on the horse radish peroxidase (HRP) enzyme was used as explained in section 3.5.2.2. In this case, biotin-HRP was employed due to the high affinity between biotin and streptavidin. In the HRP-based colorimetry assay, a solution containing the molecule 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide is used. When TMB is oxidized by hydrogen peroxide (a reaction catalysed by HRP) the solution turns to blue. The oxidation reaction is stopped by adding sulfuric acid, which causes the colour to change from blue to yellow and the absorbance can be measured at 450 nm.

Table 10 shows the absorbance results at 450 nm obtained from the colorimetry tests after fixing different amounts of streptavidin on the surfaces.

Table 10. Absorbance values obtained from surfaces decorated with streptavidin after colorimetric assays.

ABS (a.u)	Concentration of streptavidin-SPDP			
	0,5 $\mu\text{g/ml}$	1 $\mu\text{g/ml}$	2 $\mu\text{g/ml}$	4 $\mu\text{g/ml}$
	0.227	0.3	0.377	0.36
	0.196	0.341	--	0.478
	0.08	0.352	0.581	0.652
	0.079	0.309	0.571	0.504

It can be seen that the absorbance value obtained in the colorimetric assay increases as the concentration of the streptavidin solution used in the incubations increases. This indicates that after the decoration process, a measurable amount of protein remains attached to the surface. It can also be observed that solutions with a concentration of 2 $\mu\text{g/ml}$ or greater yield similar values of absorbance. This result suggests that the surface may be saturated with the protein under these conditions. The values obtained for the 0.5 $\mu\text{g/ml}$ solutions are quite variable probably due to the effect of small errors in the measurement of the volumes for these samples.

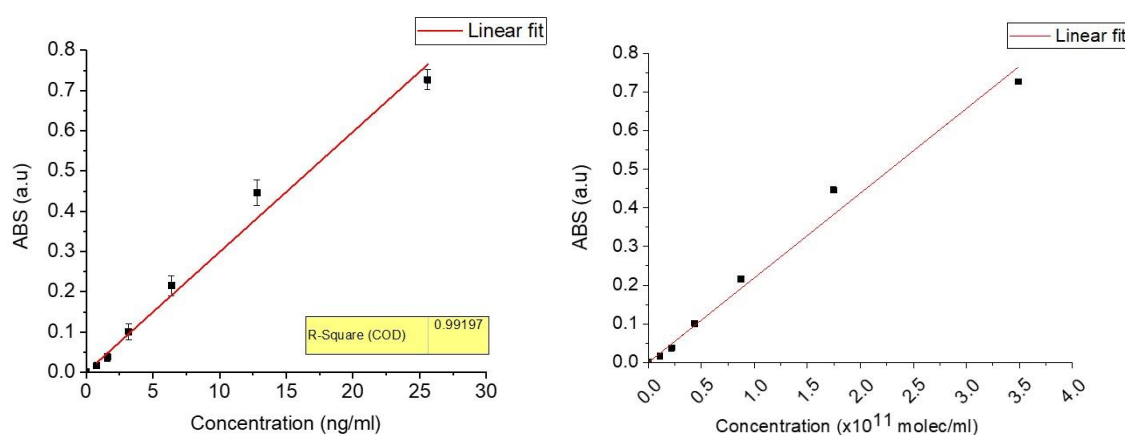


Figure 36. Biotin-HRP standard line for colorimetry assays.

Considering the absorbance results, the density of molecules on the surface has been estimated. To do this, calibration curves were carried out at different concentrations of biotin-HRP, and it was assumed that after the binding of streptavidin to the surface, only two biotin binding sites were available on streptavidin [28]. Taking this into account, after

decoration, it is estimated that there are around 7.5×10^{10} molecules/cm² of streptavidin (for the incubation with 1 µg/ml of streptavidin).

4.3.3.2. Decoration using a protein with free cysteines.

Considering that crosslinkers based on pyridyl disulfide can covalently bind proteins that have free thiol groups in their structures, the enzyme lactate dehydrogenase was used as a model protein of the decoration of a surface with a protein that contains free thiol groups.

Lactate dehydrogenase is an enzyme that catalyses the reduction of pyruvate to lactate through the oxidation of NADH to NAD⁺. Structurally, it is a tetramer whose subunit has a mass of around 36 kDa. The choice of this protein is not only due to the fact that it has free cysteines, which is an essential requirement for the process that was to be carried out, but also due to the possibility of being detected using UV-vis spectrophotometry techniques. In this regard, LDH can be detected through the optical activity of the NADH molecule at 340 nm. Consequently, the consumption of NADH during the catalysis of pyruvate to lactate resulting in the appearance of NAD⁺ is reflected in a significant decrease of absorbance.

Following this rationale, the protocol explained in section 3.5.2.3 was developed to covalently attach the enzyme LDH to MicroDeck™-G-150 substrates modified with either sulfo-LC-SPDP or OPSS-PEG-HNS crosslinkers.

The results obtained from the enzymatic assays at different concentrations of LDH carried out on the substrates are presented in Figure 37. Furthermore, a nonfunctionalized glass coverslip was used as a control substrate, to quantify the protein that might be bound to the surface by physical adsorption.

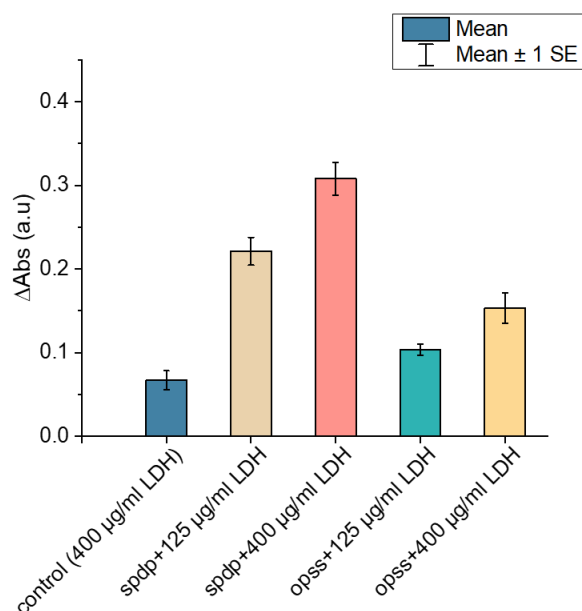


Figure 37. Bar chart showing the absorbance variation of samples decorated with different amounts of LDH through different thiol-based cross-linkers.

As can be seen, the absorbance variation corresponding to the control is lower than that observed in any of the decorated samples, as expected by the absence of a covalent bond between the protein and the surface. As expected also, in those samples that had been modified with cross-linkers, those that were incubated with a smaller amount of protein had lower absorbance variation values. Considering the samples with different crosslinker, those modified with sulfo-LC-SPDP yield a greater variation in absorbance, that corresponds to a larger amount of immobilized protein. This last result may be due to the larger density of sulfo-LC-SPDP molecules on the surface, consistently with the results found by fluorescence microscopy, and might be related with its lower molecular weight when compared with the OPSS crosslinker.

T-tests were performed in order to establish the statistical significance of the results presented in Figure 37 and shown in Table 11.

Table 11. Statistical significance of the differences obtained from the enzymatic test between substrates decorated with LDH under different conditions.

CONDITION	p VALUE
Physical adsorption vs spd+125 µg/ml LDH	<0.01
Physical adsorption vs spd+400 µg/ml LDH	<0.01
Physical adsorption vs OPSS+125 µg/ml LDH	<0.05
Physical adsorption vs OPSS+400 µg/ml LDH	<0.05
spd+125 µg/ml LDH vs spd+400 µg/ml LDH	<0.05
OPSS+125 µg/ml LDH vs OPSS+400 µg/ml LDH	NS
spd+125 µg/ml LDH vs OPSS+400 µg/ml LDH	<0.05

4.4. Affinity atomic force microscopy and force spectroscopy experiments.

4.4.1. Characterization of the biotin-streptavidin system

4.4.1.1. Affinity atomic force microscopy studies with DeepTip™ tips:

The results shown below were published in December 2022 in the journal *Molecules* with the title '*High-Yield Characterization of Single Molecule Interactions with DeepTip™ Atomic Force Microscopy Probes*' [111].

To evaluate the efficiency of the DeepTip™ probes combined with the chemical modification and decoration procedures presented above for the characterization of molecular interactions through affinity AFM, the biotin-streptavidin system was used as a model.

The first set of experiments in this system used MicroDeck™-Si-900 substrates decorated with streptavidin as described in section 3.6.1.1. Experiments proceeded by recording 250 force-displacement (F-Z) curves with a single tip on the substrate. The substrates were then incubated with a biotin-albumin solution to block the streptavidin binding sites, and 250 additional F-Z curves were recorded under this blocked condition using the same tip. In summary, each probe-substrate pair was used to measure a total of 500 curves. The experiment was duplicated with a new probe-substrate pair.

All the curves were analysed to determine both the adhesion force and the number of interaction peaks. These parameters were used to classify the recorded curves into six different groups, as illustrated in Figure 38.

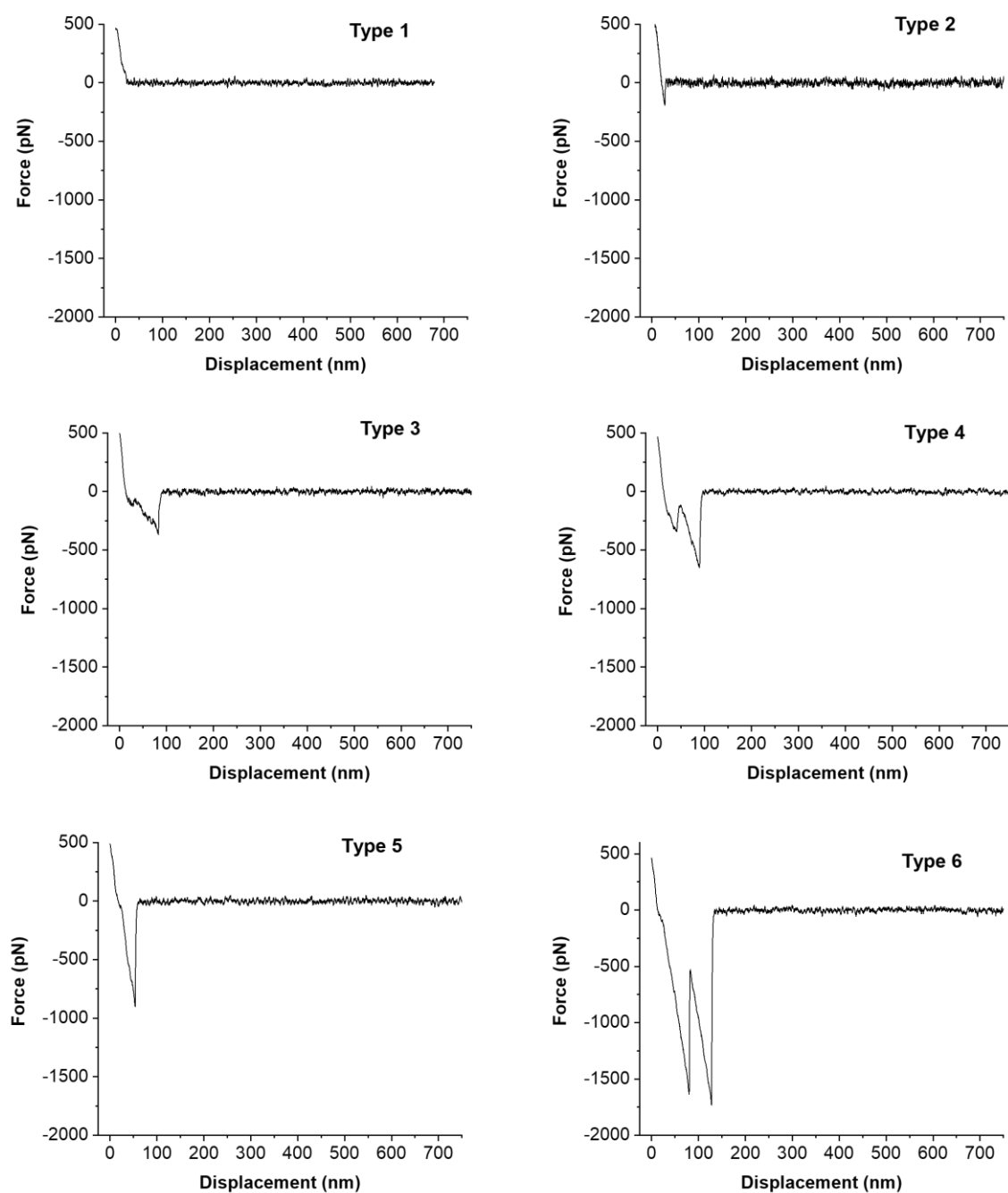


Figure 38. Representative curves of each of the six types in which the F-Z curves of the streptavidin-biotin system were classified. A seventh type (not shown) is comprised by the discarded curves.

The curves were classified based on two criteria: (i) the maximum adhesion force and (ii) the number of peaks present in the curve. Using these criteria, six types were defined:

1. No Interaction Curves: No adhesion events are observed.
2. Non-Specific Interaction Curves: Low adhesion, with an upper limit of the adhesion force at 200 pN.
3. Single Interaction Curves: High adhesion event, with an upper limit of the adhesion force at 650 pN.
4. Multiple Interactions–Independent detachment curves: Several consecutive high adhesion events, with an upper limit of the adhesion force at 650 pN.
5. Multiple Interactions–Simultaneous Detachment Curves: Very high adhesion event, with an upper limit of the adhesion force at 2000 pN.
6. Multiple Interactions–Combined Independent and Simultaneous Detachment Curves: A combination of multiple simultaneous and consecutive very high adhesion events, with an upper limit of the adhesion force at 2000 pN.

A seventh type was established with those curves discarded as experimental artefacts.

It is important to note that establishing a quantitative criterion for classifying the F-Z curves enables the automation of the procedure. This allows for the convenient classification of a much larger number of curves in future studies. However, for the current work, the classification of the curves according to this criterion was done manually.

Figure 39 shows the distribution of the number of adhesion events as a function of the maximum adhesion force for surfaces decorated with functional streptavidin and for surfaces with blocked streptavidin. As can be seen, blocking the streptavidin on the surface with biotin-BSA decreases the number of adhesion events at adhesion strengths above 400 pN, indicating that streptavidin is not available to interact with the biotin on the tip.

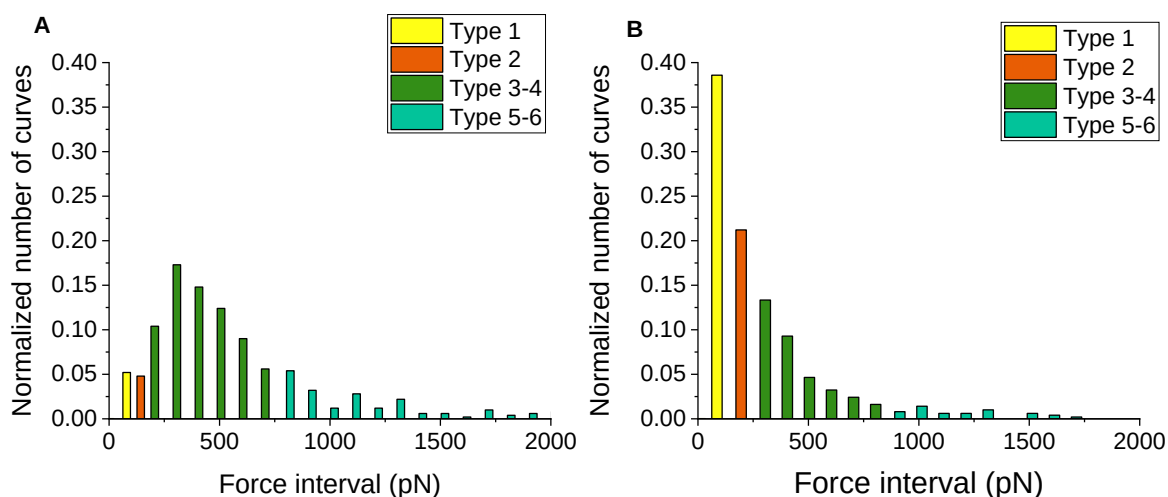


Figure 39. Histogram displaying the number of curves grouped by the maximum value of adhesion force value for (A) functional streptavidin and (B) streptavidin blocked with biotin-BSA. The type in which the curves are classified is indicated in the legend.

To assess the robustness of the procedure, particularly with respect to the repetitive interaction between the functionalized tip and the substrate, the distribution of curves into the seven types was analysed with respect to the order of each curve along the temporal series, as shown in Figure 40. This figure illustrates the difference in the percentage of a given type of curve over intervals of 50 consecutive curves along the temporal series, compared to the arithmetic mean calculated for that type across the entire set of curves for a given experiment, either in the pristine condition or after incubation with biotin-BSA. The absence of any defined trend in the temporal series with regard to the number of curves belonging to a given group suggests that there is no significant degradation of the tip due to its repetitive interaction with the sample and proves the robustness of the procedure .

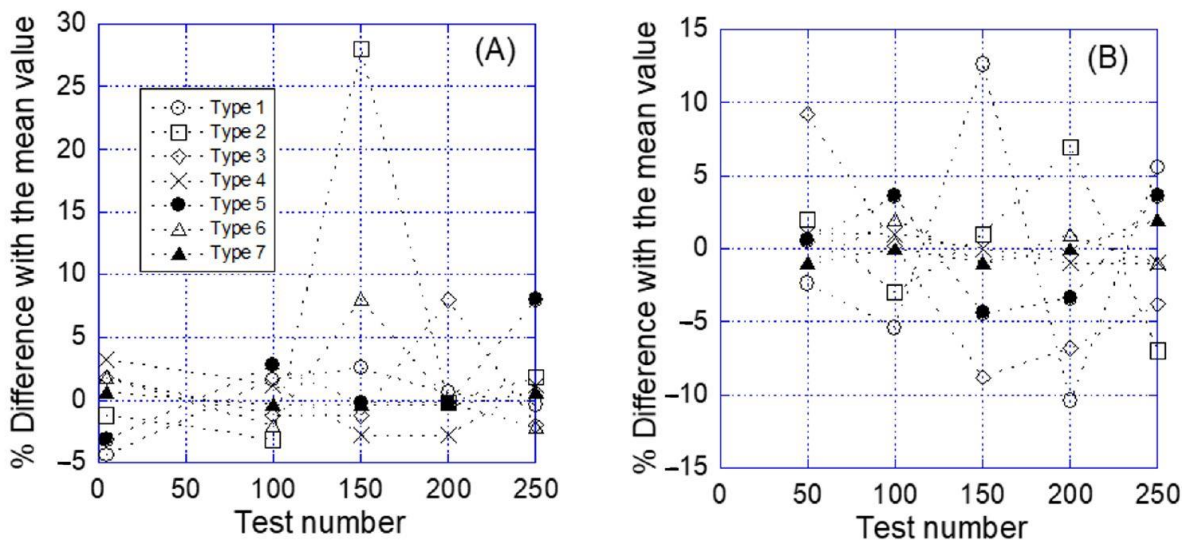


Figure 40. Difference in the percentage of curves corresponding to a given type as a function of the number of experiments for (A) pristine substrate decorated with streptavidin and (B) after incubation with biotin-BSA. The percentage difference is calculated by subtracting the percentage of curves of a given type in an interval of 50 curves (e.g., curves from 51 to 100) from the arithmetic mean obtained considering all the curves during the complete experiment

The primary aim of affinity AFM is to identify genuine sensor-target molecule interactions (especially single molecular interactions). To achieve this goal, the experimental system must meet three fundamental criteria: (a) absence of interaction in the absence of the target molecule, (b) sufficient resolution to discern genuine molecular interactions between sensor and target molecules, and (c) capability for repeated experiments across hundreds, if not thousands, of interaction events. As demonstrated above, DeepTip™ probes meet these three requirements when employed to characterize the interaction between biotin and streptavidin, although it is necessary to establish conditions for the distinction between genuine sensor-target molecule interactions and unspecific interactions. This distinction will be based on the main features that characterize an individual F-Z curve and on their statistical distribution.

In this context, an event will be defined as successful if a significant adhesion force is recorded when there is high confidence in the presence of the target molecule on the substrate. Specifically, in this study, a successful event is characterized by the identification of an interaction event involving at least one biotin and one streptavidin molecule. Conversely, an unsuccessful event occurs when no or very low adhesion force is recorded despite the expected presence of the ligand.

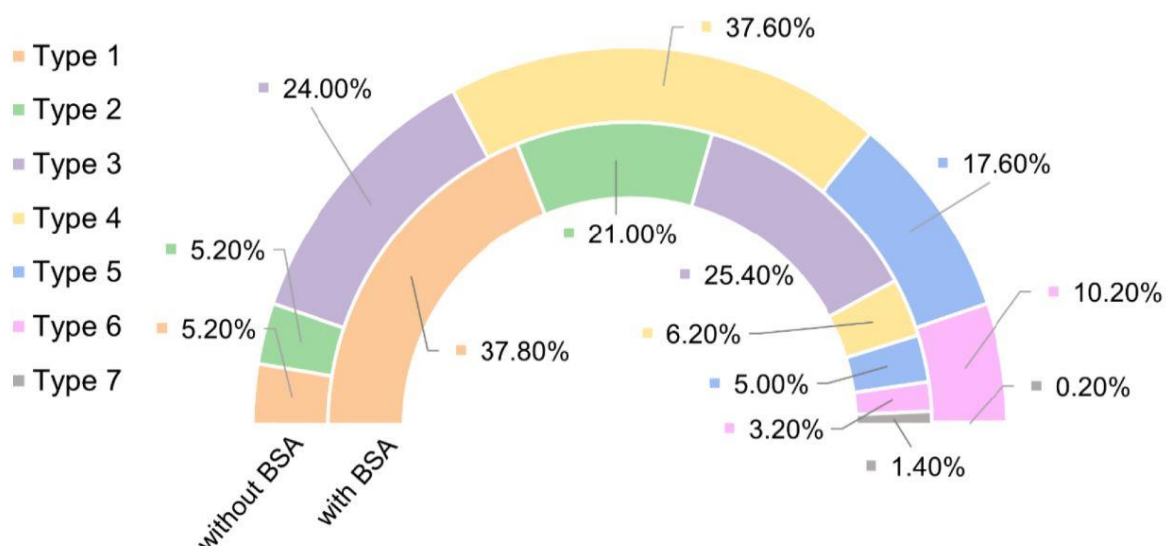


Figure 41. Chart showing the percentages of each group of curves under both experimental conditions: pristine streptavidin-decorated substrate (without BSA) and after incubation with BSA (blocked streptavidin). The groups are defined as follows: Type 1: no interaction; Type 2: nonspecific interaction; Type 3: single interaction; Type 4: multiple interactions with independent detachment; Type 5: multiple interactions with simultaneous detachment; Type 6: multiple interactions with combined independent and simultaneous detachment; Type 7: discarded F-Z curves.

Following this rationale, curves categorized as Type 7 are consistently regarded as unsuccessful events. Regarding the definition of successful events, a distinction can be made between experiments conducted on the pristine streptavidin-decorated surface and those performed after incubation with BSA. In the initial experiments, the surface is presumed to be fully coated with streptavidin, making any Type 1 event be considered unsuccessful. Conversely, blocking the streptavidin surface with BSA leads to a notable reduction in the availability of accessible streptavidin (ideally, no accessible streptavidin molecules should remain after BSA blocking, although this process may not have a 100% yield). Therefore, Type 1 curves in this second set of experiments do not necessarily signify an unsuccessful event.

While it could be argued that some valuable information could potentially be gathered from curves of Type 2, their analysis would necessitate a more exhaustive study, which falls outside the scope of this work. Therefore, these curves were not included among the count of successful events.

Regarding the first requirement mentioned above, the lack of observable interaction in 37% of the curves—after blocking the streptavidin on the substrate with biotin-BSA—clearly indicates that there is no significant nonspecific interaction between the functionalized tip and the substrate. Such nonspecific interactions would pose a serious issue for analysing streptavidin-biotin interactions, particularly when examining single interactions, as they would reduce the resolution of the procedure. In all other curves, except those excluded as experimental artefacts (approximately 1% of the data), the interaction between the probe and the substrate can be identified and quantified by measuring the adhesion force during the retraction phase of the force-displacement (F-Z) curve. Consequently, it is necessary to get a reliable interpretation on the information contained in types 3, 4, 5 and 6 that will proceed by considering the maximum adhesion force and the number adhesion events (peaks) in each curve, as well as their distribution in experiments with pristine streptavidin or after blocking with biotin-BSA.

The interpretation of each of these four types relies on both theoretical considerations and experimental features. To begin with, the adhesion force in the streptavidin-biotin system has been characterized in previous studies, reporting values ranging from approximately 200 pN to 1000 pN [95] with an average value of approx. 400 pN for the interaction between one biotin molecule and one streptavidin subunit. These values were derived from comparisons between experimental data and molecular dynamics simulations [110]. Genuine streptavidin-biotin interactions might show values around 100 pN that would result from specific pulling geometries of a biotin-streptavidin interaction [110] or from a reduced mechanical stability of the biotin binding to the tip [112]. In particular, it was found that adhesion forces of 200 pN or less are typically observed when biotin functionalization of the AFM is achieved through non-covalent interactions [96, 104], such as when cantilevers are coated with biotinylated bovine serum albumin (biotin-BSA) [103]. Additionally, the loading rate significantly influences the quantitative values of the adhesion forces obtained in experiments [57, 106, 110].

In contrast, several studies report adhesion forces in the range of 600 pN to 1000 pN [95, 113], and in some cases, up to 2000 pN [100]. These higher values align with simulations of the maximum force required to desorb a streptavidin molecule from a solid substrate in an aqueous environment, estimated to be around 500 pN [114]. This implies that the adhesion force between biotin and streptavidin should be at least of this magnitude.

Regarding the number of interaction events, some studies that report the highest adhesion force values also present force-displacement (F-Z) curves showing multiple biotin-

streptavidin interaction events, which detach independently from one another, a pattern observed in several works [95, 113]. This kind of multiple events is to be expected when there are sufficiently high densities of biotin molecules on the tip and streptavidin proteins on the substrate. Additionally, the independence of the detachment events indicates that the biotin (or streptavidin) molecules involved in these interactions are sufficiently spaced apart to ensure independent detachment. It might be argued that the highest value of $F = 2000$ pN [100] could result from two biotin–streptavidin interactions being sufficiently close to one another, allowing for the simultaneous detachment of both pairs of interacting molecules. These results could likely be refined further with the use of an elastic linker between the biotin and the AFM tip [115, 116], as will be used for the system LDH-anti-LDH antibody (see below). The results presented above align with the previous discussion. Thus, it can be argued that the single high adhesion events (Type 3) correspond to the interaction of one biotin molecule with one streptavidin molecule. Similarly, the curves classified as multiple high adhesion events with independent detachment (Type 4) correspond to interactions between two or more biotin molecules and two or more streptavidin molecules, separated by a sufficient distance so as to observe individual detachment events. Curves characterized by very high adhesion forces, exceeding 1000 pN (Type 5), may be assigned to interactions involving more than one biotin–streptavidin pair, when the molecules are so close that detachment occurs simultaneously. Lastly, Type 6 curves imply a combination of independent (Type 4) and simultaneous (Type 5) detachments of several biotin–streptavidin pairs within a single F - Z curve. This interpretation of the F - Z curves is also consistent with the frequency of appearance of each type, depending on the substrate used—either pristine streptavidin-decorated or streptavidin blocked with biotin–BSA. In the initial experiment on the pristine streptavidin, the most frequent type of curves corresponds to the multiple interaction with independent detachment (Type 4), consistently with the high density of immobilized molecules expected on both the DeepTip™ probe and the MicroDeck™ substrate. This type is followed in frequency by the single interaction (Type 3), and then by multiple interactions with simultaneous detachment (Type 5) or a combination of simultaneous and independent detachments (Type 6).

Notably, the curves that do not show any interaction event (Type 1) and those showing a nonspecific event (as defined in this work) with an adhesion force below 200 pN represent only 10% of the total curves. Consequently, the use of DeepTip™ probes results in a success rate of 90% concerning the total number of F - Z curves recorded. These proportions

are consistently modified after blocking the streptavidin molecules with biotinylated albumin. Under this condition, the majority of curves correspond to Type 1 (no interaction), and the combination of Type 1 and Type 2 (nonspecific interaction) accounts for more than 50% of the total curves. The next largest fraction of curves corresponds to the single molecular interaction (Type 3), which is expected due to some streptavidin molecules not being adequately blocked after incubation with biotinylated albumin.

In this case, the inversion in the proportion of single events (Type 3) and multiple events with independent detachment (Type 4) can be explained by the presence of isolated streptavidin molecules after blocking the substrate with albumin. A similar reduction in the proportion of curves corresponding to multiple events (Types 5 and 6) is observed after blocking the streptavidin molecules.

4.4.1.2. Dynamic Force Spectroscopy on biotin-streptavidin model with DeepTip™ probes.

After establishing the possibility of identifying target molecules (streptavidin) on a substrate with sensor molecules (biotin) decorating the AFM tip, the interaction between these two molecules was further studied using the DeepTip™ probes. For this purpose, dynamic force spectroscopy studies were undertaken with the aim of comparing the results obtained with the DeepTip™ probes with those previously published in the literature on the interaction between a single streptavidin molecule and a single biotin molecule. In this case, Deep Tip™ SiN R11 tips decorated with NHS-PEG-biotin and streptavidin decorated agarose beads as a source of streptavidin were used as indicated in section 3.6.1.2.

The study began by performing F-Z curves on the streptavidin-biotin system at four different retraction speeds. Once the curves were recorded, in this case only those that presented a single specific adhesion event were considered for the data analysis. To ensure that the adhesion event was specific, the part of the curve corresponding to the extension of the PEG spacer was fitted to the FJC model as had been done in previous studies [117]. Two parameters can be obtained for each curve using this analysis: (i) the loading rate and (ii) the maximum adhesion force or, in other words, the breaking force of the interaction. Representative curves belonging to each speed are shown in figure 42.

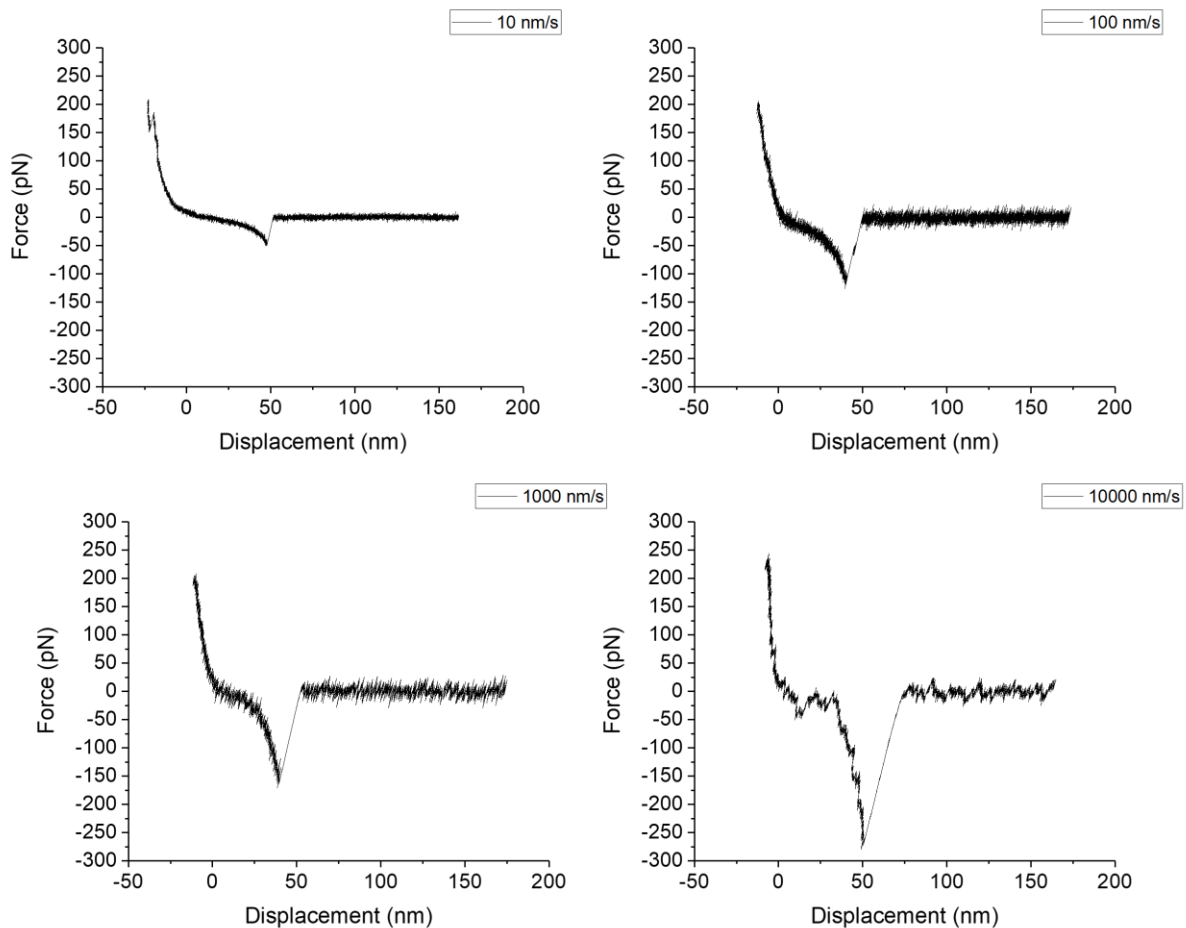


Figure 42 Representative F-Z curves of the streptavidin-biotin system for each retraction speed.

The average loading rate values obtained for each of the retraction speeds were the: 65.6 pN/s (10 nm/s), 742.7 pN/s (100 nm/s), 7615.6 pN/s (1000 nm/s), 77415.3 (10000 nm/s) pN/s.

The mean values of adhesion force obtained for each retraction speed are presented in Figure 43 (top). With all this, the average breaking force for each speed was represented against the natural logarithm of the average value of the loading rate obtained for each breaking force. The data were fitted to a straight line and the value of the slope and the intercept with the ordinary axis were obtained. These results can be seen in figure 43 (bottom).

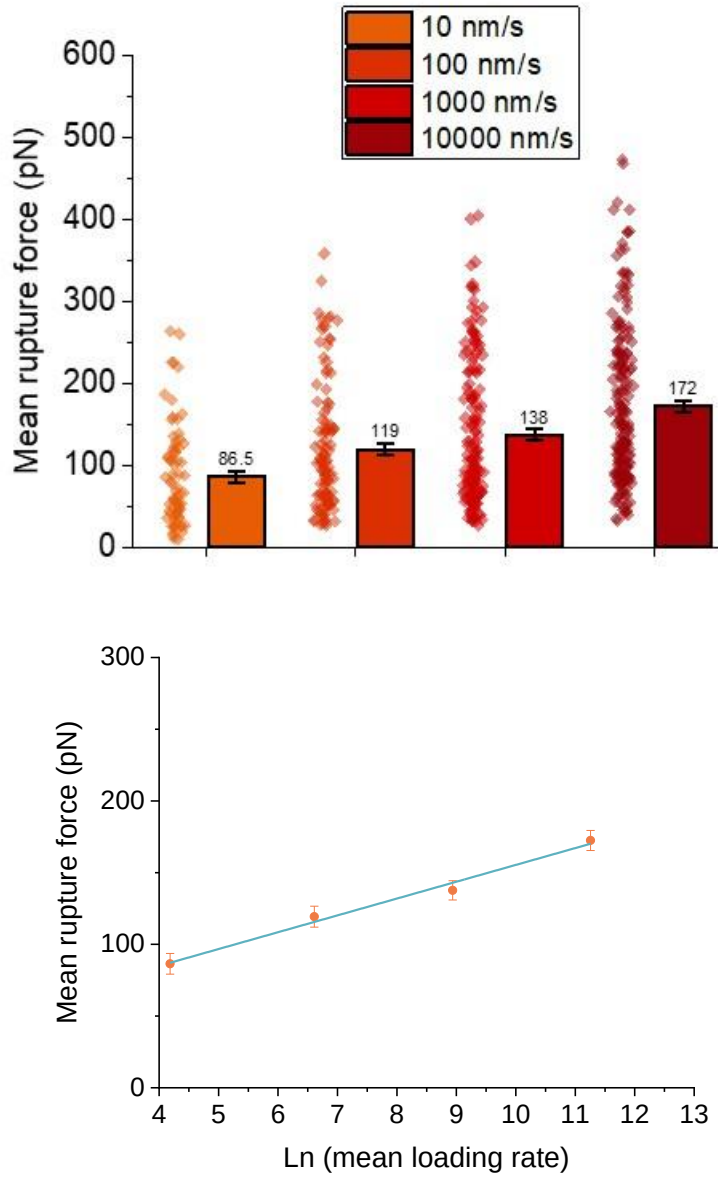


Figure 43. Top: Mean rupture force values obtained for each retraction speed used. Bottom: Representation of the values of mean rupture force vs Ln of the loading rate. The linear fit is shown in blue ($R^2=0.98$, Pearson's $r=0.99$).

As can be seen in the graph, there is a direct linear relationship between the average breaking force obtained and the natural logarithm of the loading rate (as indicated by the value of the Pearson correlation coefficient obtained from the linear fit $p = 0.99$). These results agree with what is expected in the Bell-Evans-Ritch model which establishes that the value of the breaking force increases with the loading rate.

From the linear fit of the data, the slope $m=11.73$ pN and $b=38.07$ pN have been obtained. By introducing these values in equation (12) and as indicated in [85], it has been possible

to obtain the value of parameter $x_p = 0.35$ nm as the distance along the reaction coordinate between the bound state and the transition state. These values are very close to those previously presented in other studies [103, 106, 107], which validates the results obtained here. The data obtained from the linear fit in this work have been superimposed with previously published results (Figure 44) and it is observed the consistency between both sets of data [106].

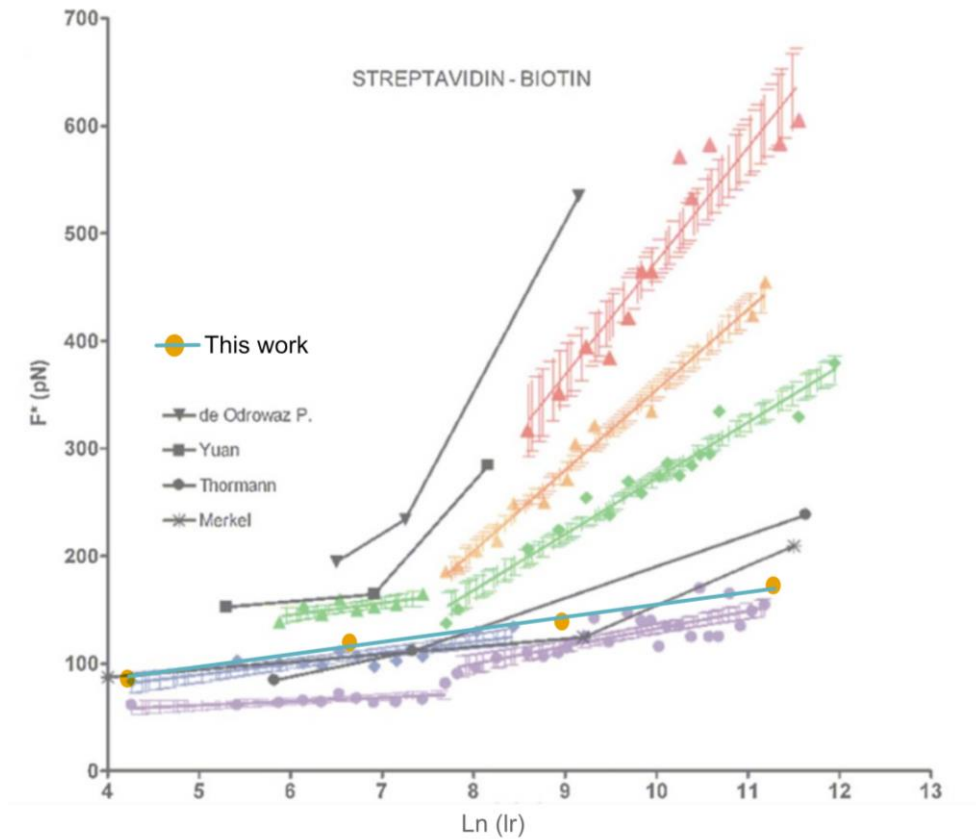


Figure 44. Comparison of the results obtained by applying the Bell-Evans model to the data presented in this Thesis with those found in previously published works [106].

Specifically, it is observed that the data from this work coincide with the data obtained by Teulon et al ($\text{Ln}(\text{lr}) < 8$) [106] and with those of Merkel et al [118]. According to what was stated in the work of Teulon et al, these data are attributed to unique interactions between the biotin molecule and one of the active sites of streptavidin, so that it can be inferred that the results obtained in this work also correspond to this situation.

4.4.1.3. Affinity microscopy in the biotin-streptavidin system: Comparison of various functionalized AFM probes.

Finally, the biotin-streptavidin system was used for comparing the results obtained using different functionalized AFM probes. With this aim, experiments were performed with either commercial DeepTip™ probes or with probes functionalized with amino groups by immersion silanization. Both functionalized probes were decorated with biotin following the protocol indicated in section 3.4.3. Agarose beads decorated with streptavidin were used as substrates for these experiments.

In order to carry out the analysis, the curves obtained with each tip were divided into four types: Curves without interaction, curves with specific interactions where only one adhesion event was observed, curves where multiple specific interaction events were observed and finally, curves that presented nonspecific interaction. Representative curves of each type are shown in Figure 45.

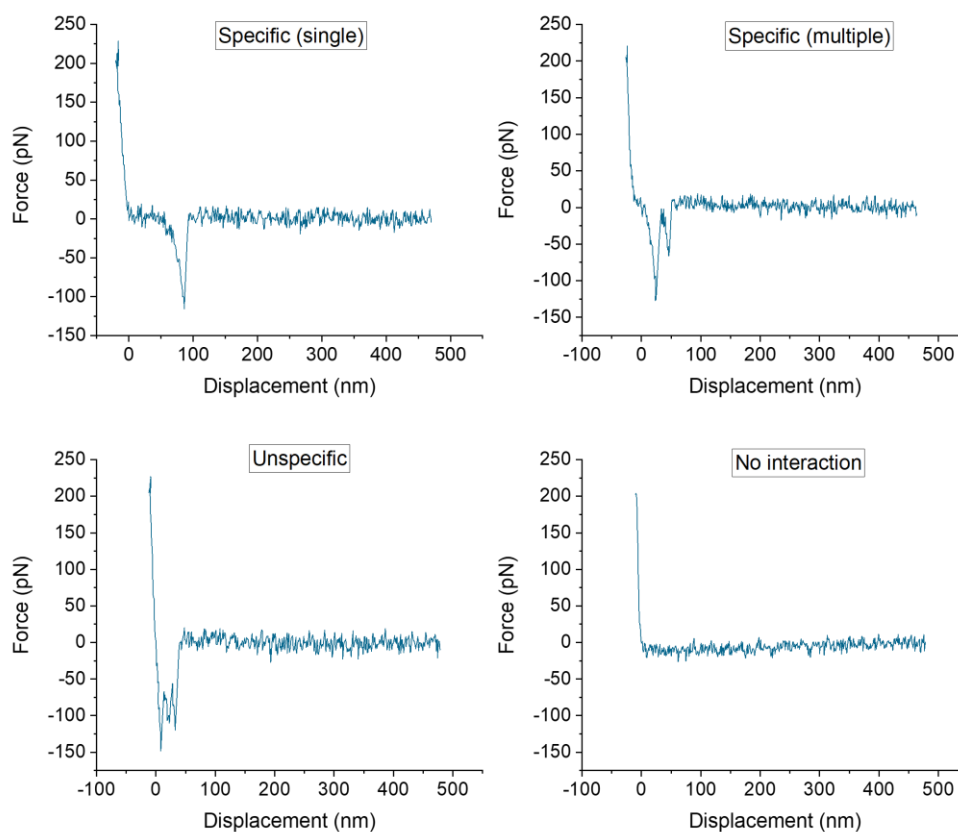


Figure 45. Representative curves of each of the groups in which the F-Z curves were classified.

Once the curves were classified, the percentages of each type acquired with either functionalized AFM probe were calculated as shown in Figure 46. F-Z curves were recorded with two probes of each type.

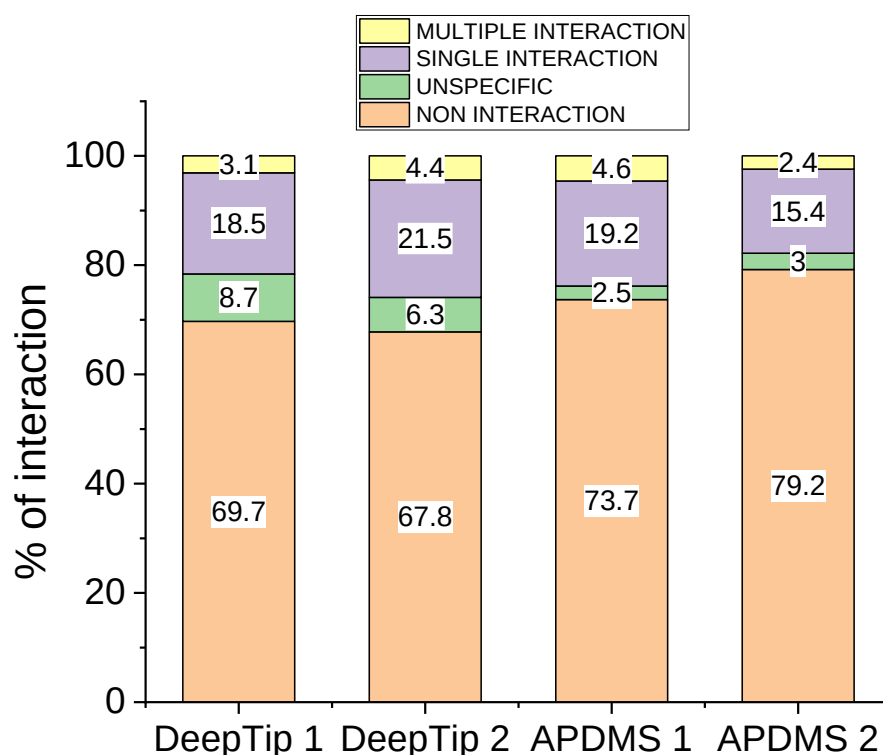


Figure 46. Percentages obtained for each type of curve according to the functionalized AFM probe used to perform the test.

Focusing on the type of curves that correspond to specific single interactions, which provide more specific information on the interaction object of study, a slightly higher proportion is obtained with the commercial DeepTip™ probes.

4.4.2. Characterization of the lactate dehydrogenase-anti-(lactate dehydrogenase) antibody system

The results of the affinity microscopy measurements carried out using the LDH/anti-LDH system presented below were published in *Identification of*

individual target molecules using DeepTipTM Atomic Strength Decorated with Antibodies Microscopy probes' [40].

The critical aspect in any affinity atomic-force microscopy experiment lies in correctly determining whether a given F–z curve arises from a genuine sensor molecule–target molecule interaction or from an unspecific event. Furthermore, the assignment must be self-consistent and not require supplementary validation from alternative experimental techniques. In this context, the interaction between LDH (target) and anti-LDH (sensor) was used as model system for performing affinity AFM with DeepTipTM probes. In contrast to the streptavidin-biotin system discussed above, the lower intensity of the interaction between antibody and antigen requires a more subtle analysis for the identification of genuine interactions between sensor and target as presented in detail below.

Following this rationale, the F-Z curves recorded from the interaction between LDH and anti-LDH molecules were classified using three features:

1. The number of peaks
2. The value of the adhesion force (determined by the minimum of the F–z curve)
3. The presence or absence of the elastomeric trait in the region prior to the peak(s)

Following these three criteria, the F–z curves obtained from the affinity AFM tests were classified into seven types:

1. Type 1: No interaction
2. Type 2: Elastomeric, one peak
3. Type 3: Non-elastomeric, one peak
4. Type 4: Double peak, elastomeric
5. Type 5: Double peak combined elastomeric/non-elastomeric.
6. Type 6: Threefold or fourfold peaks
7. Type 7: Discarded curves

A representative curve for each of the first six types is presented in Figure 47.

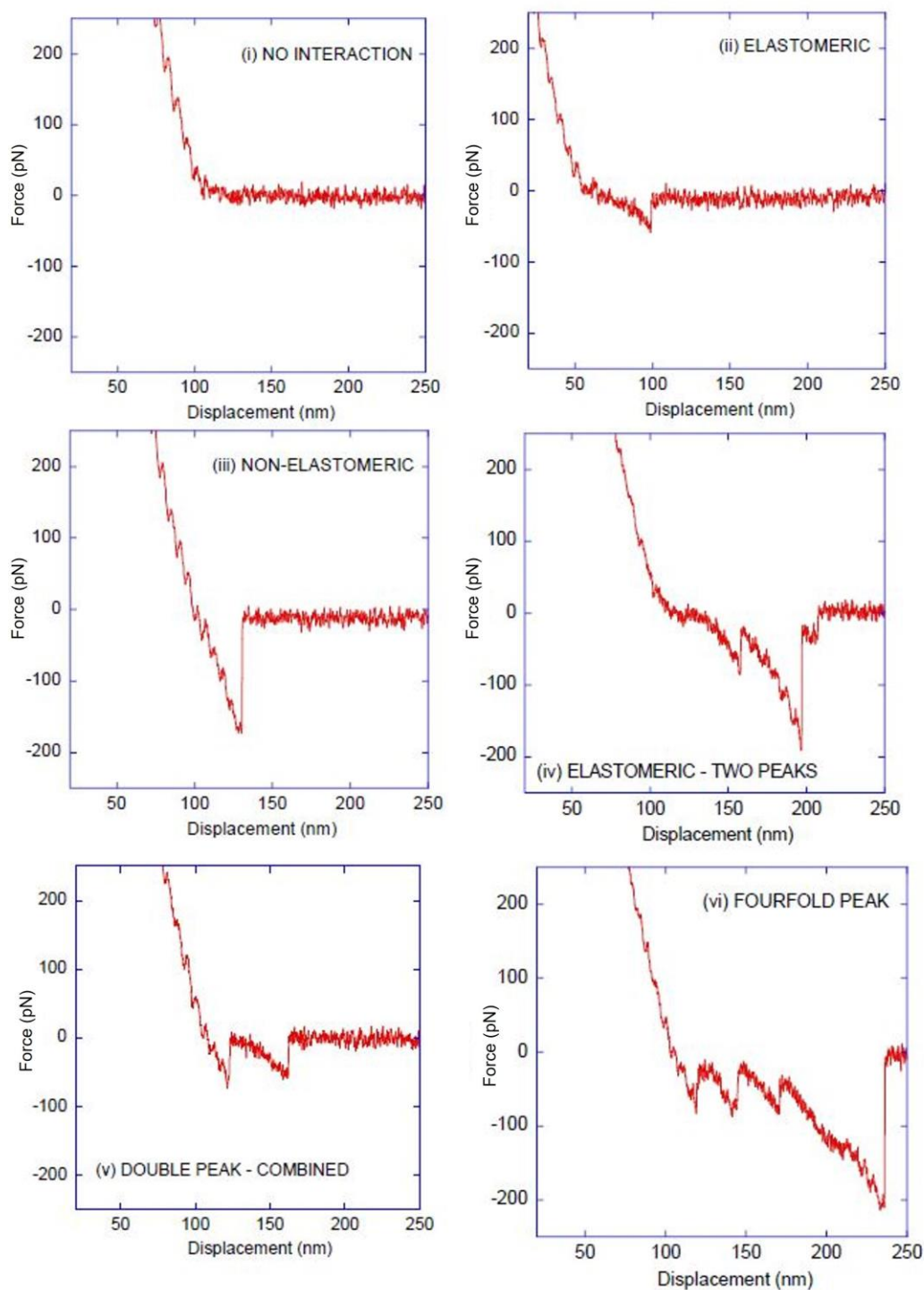


Figure 47. Representative force–displacement (F – z) curves obtained from the interaction between LDH and anti-LDH antibody for the types: (i) no-interaction; (ii) elastomeric; (iii) non-elastomeric; (iv) elastomeric with two peaks; (v) double-peak combined; and (vi) three- or four-peaks. Compression forces are positive, and positive displacement corresponds to the retraction of the AFM probe from the substrate.

Type 7 includes curves with abnormal shapes, which were discarded from the analysis and are not depicted in Figure 47.

Based on this classification, the number of curves that belong to each group was determined as shown in Figure 48. Notably, 47% corresponded to Type 1 (no interaction), while 7% corresponded to Type 7 (discarded curves). Consequently, slightly less than 50% of the curves potentially contain information on the interaction between a target molecule and a sensor molecule (antibody).

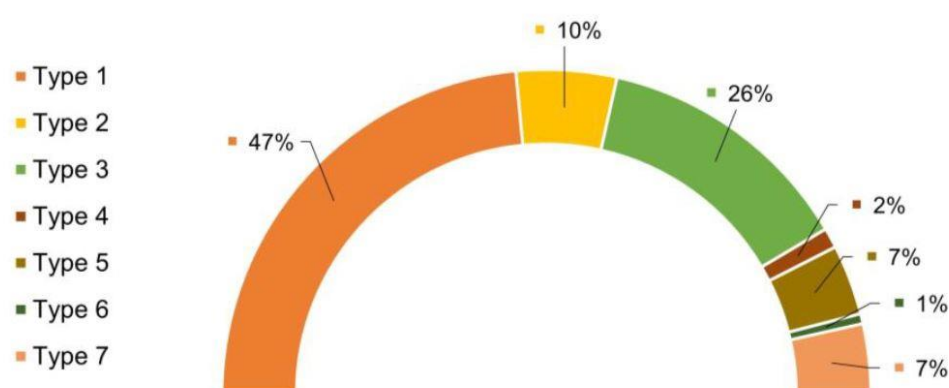


Figure 48. Percentages of each type of F-Z curves: Type 1: 47%—no-interaction; Type 2: 10%—elastomeric curves; Type 3: 26%—non-elastomeric; Type 4: 2%—elastomeric two-peaks; Type 5: 7%—combined elastomeric/non-elastomeric; Type 6: 1%—threefold or fourfold elastomeric peaks; Type 7: 7%—discarded curves.

At this moment, it becomes crucial to identify those F-z curves that contain information on genuine LDH–anti-LDH interactions. Curves with multiple peaks and the elastomeric trait, (Types 4, 5, and 6), hold particular significance. The elastomeric trait is characterized by an increasing absolute value of the F–z slope preceding the peak at increasing z values. This trait is attributed to the stretching of the polyethylene glycol (PEG) spacer connecting the antibody to the AFM probe and corresponds to the mechanical behaviour of polymer chains with substantial conformational freedom (elastomeric behaviour) [119].

In association with the elastomeric trait, the fluctuation in the force value following post-peak unloading serves as another criterion for accurately identifying a genuine interaction. This variation corresponds to the magnitude of force lost when one or more bonds break. The curves depicted in Figure 47 illustrate that these values are

approximately 50 pN (Figures 47 ii, iv–vi) and multiples thereof (e.g., 150 pN; Figures 47 iv, vi). It's challenging to attribute the presence of two peaks in the same curve, each with a maximum force of approximately 100 pN, and exhibiting the expected elastomeric trait from the unfolding of the PEG spacer to two unrelated nonspecific interaction events between the probe and the substrate. In a previous work [120], this combination of features was used as a benchmark for the identification of genuine sensor–target interactions in a given F–z curve. In accordance with this approach, the one-peak elastomeric curves with adhesion forces within this range are also regarded as corresponding to genuine sensor–target molecule interactions. Consequently, the proportion of F–z curves reflecting genuine interactions amounts, to at least, 18% (Type 2 + Type 4 + Type 6) of the total number of curves.

Once the curve classification criteria were established, those that presented a single adhesion peak in combination with the characteristic shape of the elastomeric section of the PEG spacer extension (type 2) were taken and used to calculate the distribution of adhesion values obtained from the F-Z curves (Figure 49).

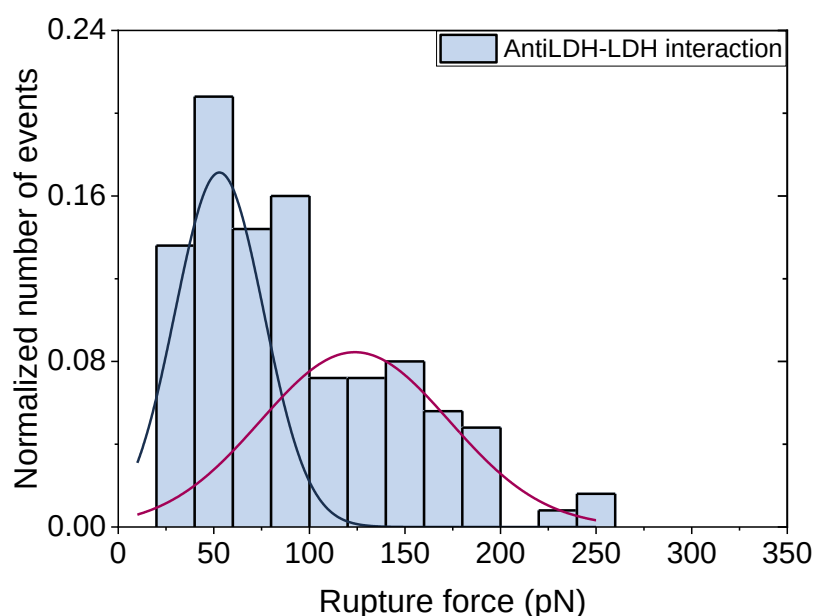


Figure 49. Distribution of adhesion forces of the LDH/anti-LDH system obtained from type 2 curves.

The average value of the adhesion force obtained, for all the peaks considered, was 120 ± 90 pN. Additionally, all values were plotted in a histogram to see the shape of the distribution. As seen in Figure 49, the distribution is fairly wide and may be considered as a bimodal distribution with one maximum at approx. 55 pN and another maximum at approx. 140 pN. These values are similar to adhesion values found in the literature for an antibody-antigen interaction, such as those obtained by Berquand et al for the interaction between Antilysozyme Fv Fragments and lysozyme [121].

To try to interpret the shape of the distribution obtained for the antibody used, it may be relevant mentioning that this anti-LDH antibody is polyclonal. Polyclonal antibodies are produced by different clones of B lymphocytes, making them structurally different from each other and, although all polyclonal antibodies for a given antigen recognize this antigen (in this case LDH), recognition may proceed with different intensity. Consequently, it is possible that the wide distribution of interaction forces is due to the polyclonal nature of the antibody.

Before beginning with the analysis of non-elastomeric peaks, it is crucial to consider some additional aspects of force–displacement curves in more depth. Firstly, the significance of recording a sufficient number of no-interaction curves should be emphasized. No-interaction curves serve as a self-consistency test for the entire experimental procedure, since their observation throughout the whole experiment eliminates the possibility of a possible bias of the data due to, for example, the contamination or the degradation of the tip.

It is also worth highlighting the recording of peaks with multiple peaks exhibiting the elastomeric trait in this system where antibodies serve as sensor molecules. The observation of these curves can be correlated with the structure of an antibody [122] and, in particular, with the existence of two Fab regions capable of independently binding the corresponding antigen. In this context, single-peak curves can be interpreted as the binding of one Fab region of the anti-LDH antibody to the LDH protein, while double, threefold, and fourfold peaks would correspond to the binding of two, three, or four Fabs to the corresponding antigens, respectively. This interpretation aligns with the distribution of curves for each elastomeric type: one peak—Type 2 (10%) > two peaks—Type 4 (7%) > three and four peaks—Type 6 (1%), reflecting the decreasing probability of more than one Fab binding to the antigen. It is worth noting that the interaction of both

Fabs from two independent antibodies, which may be bound to different sites of the same streptavidin molecule, could contribute significantly to the four-peak/Type 6 curves.

It is also worth mentioning that the proposed experimental procedure on the LDH-anti-LDH model system aims to test the robustness of the entire scheme under favourable, although not optimal, conditions with respect to the interactions between sensor and target molecules. Consequently, no effort was made, for instance, to control the orientation of LDH during immobilization, meaning that some proteins may not present accessible epitopes recognizable by the antibody.

Additionally, the conditions for decorating the substrates with LDH were chosen to achieve significant surface coverage, rather than depositing a protein monolayer on the material. Furthermore, an alternative procedure using individual Fabs instead of complete antibodies as sensor molecules could be considered. While this approach might reduce the variability of adhesion peaks, it could also decrease the number of curves with double or multiple peaks, which are crucial for distinguishing genuine from nonspecific interactions.

Lastly, and as indicated above, it is important to acknowledge that using a polyclonal anti-LDH antibody represents a limitation with respect to the reproducibility of measured adhesion forces, since the scattering of these values is likely to be greater than when using a monoclonal antibody. However, and despite these non-fully optimized procedures, the possibility of obtaining reliable results regarding the number of genuine sensors–target interactions recorded underpins the robustness of the entire experimental approach. Even when considering only the curves that exhibit at least one elastomeric peak, the ratio of successful interaction events reaches a value of 13%. This is significantly higher than the usual percentages of successful interaction events reported in other works, which typically fall within the range of 0.1–1% [104, 110]. The successful ratio increases even further to 20% when considering Type 5 curves (with one elastomeric/one non-elastomeric peak).

To interpret the significance of non-elastomeric curves and ploughing on prior research [120], figure 50 displays the distribution of peaks exhibiting either the elastomeric or non-elastomeric trait within the force range of 50 pN to 200 pN. While it is possible that peaks with lower adhesion force values could correspond to specific interactions, potentially reflecting different pulling geometries [110], it is also plausible that these low-intensity

peaks might arise from events other than genuine LDH–anti-LDH interactions. Due to this uncertainty, curves with low-intensity peaks were excluded from the subsequent statistical analysis. On the other hand, it could be argued that peaks with higher adhesion forces do indeed correspond to this specific interaction, whether as single or multiple events. However, justifying these high values of adhesion forces would necessitate additional analysis in future experimental campaigns. Furthermore, high adhesion forces are scattered from the chosen upper limit at 200 pN up to values exceeding 1000 pN, severely limiting the reliable application of goodness-of-fit tests if this range is included.

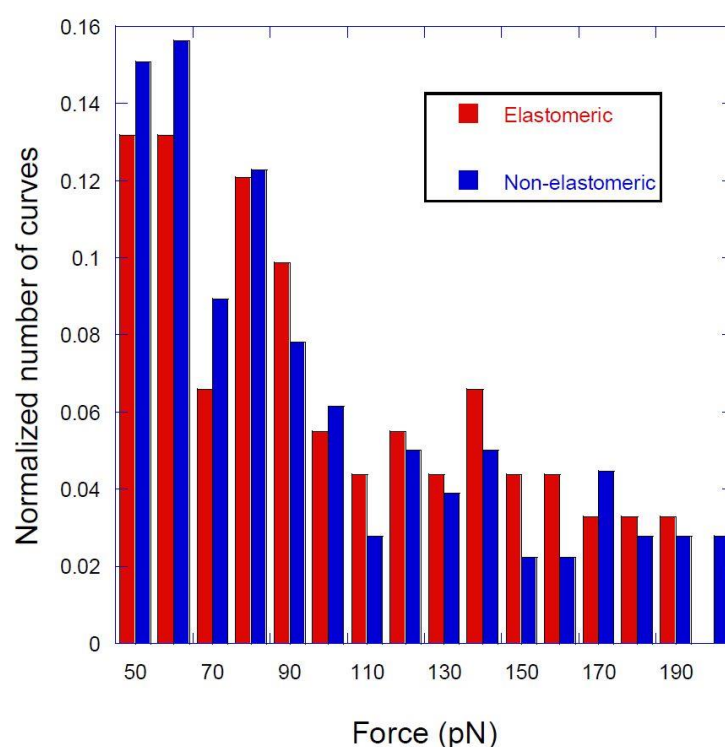


Figure 50. Statistical distribution of the force–displacement curves corresponding to elastomeric and non-elastomeric peaks as a function of the measured adhesion force.

In this context, the interpretation of non-elastomeric peaks (Type 3 and Type 5 curves) can be established by comparing the distribution of both types of peaks normalized to the total number of curves of each type. The concurrence of both distributions supports the genuine character of the non-elastomeric peaks as resulting from the LDH–anti-LDH antibody interaction. This is because it would be unlikely for two unrelated events (specific target–sensor molecule interactions and other unspecific interactions) to yield a common statistical distribution.

As described elsewhere [120], the distribution of non-elastomeric curves may be taken as a reference and compared with that of the elastomeric curves. The choice of the distribution of non-elastomeric curves as a reference distribution is suggested by the larger number of this type of curves (by over three times) with respect to the elastomeric ones. The comparison of both distributions follows the χ^2 test of goodness of fit and involves dividing the range of adhesion forces between 50 pN and 200 pN into 10 pN intervals.

The value of the χ^2 parameter for the distribution of elastomeric curves using the distribution of non-elastomeric curves as a reference is $\chi^2 = 9.9$. This is compared with the value of χ^2 ($f = 15$) = 25.0 for the 15 degrees of freedom considered (the number of intervals in which the range of forces is divided minus one). Since χ^2 ($f = 15$) = 25.0 > χ^2 (=9.9), it is concluded that both sets of events correspond to the same statistical distribution.

This concurrence of statistical distributions supports the assignment of non-elastomeric peaks to genuine LDH–anti-LDH antibody interactions. Consequently, there is a percentage of almost 50% of successful events in which the genuine LDH-anti-LDH antibody interaction is observed when applying the experimental procedure presented in this work.

4.4.3. SMFS of titin. Yield comparison of functionalisation methods.

Although results of this type of experiment were already presented in section 4.4.1.3, there was interest in performing this comparison with a system in the context of SMFS for protein unfolding, instead of the interaction between sensor and target biomolecules. For this reason, the Halo-I27(8) protein was used as a model because it has a very characteristic unfolding fingerprint that would facilitate carrying out the study.

To evaluate the detection performance of specific events, 3 DeepTip™ SiN R11 tips and three qp-scont tips decorated with Halo ligand were used as detailed in section 3.6.3.

Initially the curves obtained were classified based on whether they presented the characteristic fingerprint of the unfolding of the protein (completely or partially), whether the curves presented non-specific adhesion events, or if no event was identified in the F-Z curve. Following this classification, 7 types of curves can be obtained:

1. 8i27: Curves with eight peaks corresponding to the unfolding of protein.
2. ≤ 7 i27: Curves that show seven or less peaks. Unfolding of one or several of the motifs is not complete, but the pattern is recognizable, indicating a specific adhesion event between the tip and the substrate.

3. Curves with non-specific adhesion. There is adhesion, but nothing resembling the fingerprint is observed.
4. Curves with an initial part of non-specific adhesion, followed by a fingerprint of ≤ 7 peaks.
5. Non-adhesion: Curves where no interaction is observed.
6. Multimers: curves that show more than 8 peaks in the fingerprint.
7. No detachment: curves where the adhesion is so strong that the tip does not detach from the surface.

A representative curve of each type is shown in Annex A.

Once the curves were classified, the percentage of each type was calculated for each tip used. The percentages obtained are shown in table 12.

Table 12 Percentages obtained from each type of curve depending on the type of tip used to acquire them.

Tip	Percentage of curves according to type						
	1	2	3	4	5	6	7
DT 1	1 %	4.5 %	20.5 %	6.7 %	52.2 %	1.0 %	14.0 %
DT 2	2.1 %	3.1 %	25.5 %	8.9 %	44.6 %	1.2 %	14.6 %
DT 3	0.5 %	2.1 %	15.5 %	2.9 %	72.1 %	0.2 %	6.7 %
JAC 1	0.4 %	2.5 %	18.7 %	4.9 %	60.5 %	0.0 %	13.1 %
JAC 2	1.7 %	2.2 %	24.0 %	8.3 %	35.2 %	0.8 %	27.8 %
JAC 3	2.1 %	3.7 %	53.9 %	4.8 %	27.9 %	0.6 %	7 %

From all the percentages obtained the data of types 1 and 2 will be discussed first. Although it is not the objective of this work or of this study in particular, this type of curves are the ones that can provide the most information regarding to the study of the protein unfolding process, therefore they are the ones that would be most interesting in this type of test. As can be seen, in four of the six tips (DT 1, DT 2, JAC 2 and JAC 3) the percentages obtained for type 1 are between 1%-2% while for type 2 they are in the range of 2.2%-4.5%. In the case of the DT 3 and JAC 1 tips the values reach just 0.5% for type 1 curves. To explain this result, since they are similar between tips that have been functionalized with amino groups in different ways, two ideas arise. The first is that the halo ligand decoration process had some procedural flaw, causing the tips to be incubated with a lower number

of molecules that results in a lower number of specific adhesion events or, on the other hand, that the amount of protein in these two cases was lower, giving rise to the same consequence. For type 2 of the curves, a similar trend is obtained, since at the points DT 1, DT 2, JAC 2 and JAC 3 somewhat higher values are obtained than for the points DT 3 and JAC 1. As for type 3 of curves that represent non-specific adhesions is very similar for all tips except for the JAC 3 tip, which presents around twice as many non-specific adhesions as the rest. This may be due to the surface being previously contaminated, but without more data it is not possible to clarify the cause. For the rest of the types of curves, the percentages obtained are similar for all the tips used.

Now, returning to type 1 of curves, the percentage obtained in general, regardless of the type of tip, is similar between tips, but very low. This low value is probably a consequence of the low density of I27 molecules on the surface. Considering that this type of assay in the context of SMFS seeks to measure unfolding of individual protein molecules, the easiest way to isolate the interaction is to greatly reduce the number of available molecules with which the tip can interact. Therefore, the performance is probably so low, not because of how the tip is functionalized but because of the sample preparation characteristics of this type of test.

5. CONCLUSIONS.

From this work it is possible to extract the following relevant conclusions:

In relation to the characterization of commercial DeepTip™ probes and MicroDeck™ substrates.

1. Both probes and substrates present thin films with reactive amino groups that have high stability over time, even under not particularly strict storage conditions
2. The functionalization process does not modify the intrinsic values of spring constant and resonance frequency of the probes.
3. The tips of the DeepTip™ probes maintain their geometry. No convolution phenomena or double tips are observed after the manufacturing process.
4. The surfaces of both the DeepTip™ probes and the MicroDeck™ substrates show a moderate increase in roughness compared with the control (non-functionalized) samples.

In relation to the chemical modification of the surfaces:

1. It is possible to modify the surfaces of both DeepTip™ probes and MicroDeck™ substrates with heterobifunctional cross-linkers based on NHS-ester. In this way, surfaces chemically modified with (1) pyridyl disulfide groups, (2) thiols, (3) DBCO and (4) azide can be obtained. Alternatively, the surfaces coated with biotin can be obtained.
2. The modified surfaces can be used to immobilized proteins, such as enzymes or antibodies, among others, while maintaining their function after covalent binding. The functionality of the biomolecules may be maintained for up to 14 days, as seen in the case of the anti-LDH antibody.

In relation to the performance of affinity microscopy and SMFS tests:

1. It is possible to perform affinity microscopy measurements using DeepTip™ probes and MicroDeck™ substrates. It is shown that the probes are stable throughout the F-Z experiments, with no degradation observed as the recording of F-Z curves progresses. DeepTip™ and MicroDeck™ substrates can also be used to perform DFS measurements, achieving results similar to those obtained in previous studies with other functionalized probes. In particular, it is possible to achieve molecular resolution with these probes and substrates, as long as the detection of the interaction between two molecules is concerned.
2. The performances obtained with DeepTip™ tips when detecting interaction events or performing unfolding measurements are similar to those obtained with other functionalized tips functionalized when applying the usual protocols. However, specifically developed protocols allow the recording of genuine interaction events between sensor and target molecule in a proportion close to 50 % that exceeds the usual yield obtained in this type of experiments.
3. A methodology has been developed based on the intensity of the interaction and the shape of the F-Z curve obtained that allows extracting both qualitative and quantitative information from the sample of interest.

With all this, it is concluded that the initial objective of this work is achieved

6. FUTURE WORK.

Although the proposed objectives have been achieved with the development of this work, it is always possible to go further. That's what this is all about, advancing in the search for knowledge and new technologies and processes. Affinity microscopy and SMFS are very versatile techniques that allow the study of biological systems in conditions very similar to biological environments. Starting from this basis, looking to the not-too-distant future, it can be a great help for the studies of more complex cases such as the molecular mechanisms behind autoimmune or neurodegenerative diseases, allergic processes, the study of infection processes of different pathogens or the study of cell adhesion processes on a molecular scale.

The work carried out has advantages over more traditional methods found in the literature, such as the use of already functionalized commercial tips, which means an optimization of sample preparation time, which translates into more time to carry out experiments of interest. . Furthermore, the protocols developed based on commercial products are simple and do not require extensive resources to carry them out. However, the door is open to many improvements and new ideas.

As future work to this already developed, the following ideas are proposed:

- Carry out studies using this type of commercial tips within the framework of affinity microscopy and SMFS on cell models, both eukaryotic and prokaryotic.
- Expand the use of crosslinkers within the framework of click chemistry, due to the specificity and versatility it presents. This can open up a wide range of new possibilities for surface modification. In addition, other very interesting options should be incorporated into the studies, such as the use of photoactivatable cross-linkers.

- Modify the molecules of interest, either through protein engineering techniques or through the use of cross-linkers, to develop new processes that allow their covalent immobilization and ideally with a specific orientation on the surface.
- Develop an open-source program that allows the processing of SMFS data (especially F-Z curves) regardless of the brand or model of microscope used in the same way that ImageJ/FIJI exists for image processing. Furthermore, it would be very useful for advances within the research community in this field to have a database accessible to everyone with the data and experimental conditions used and the biophysical parameters obtained from the studies carried out.

The study options are unlimited as long as the resources and interest in knowing exist.

REFERENCES

- [1] G. Binnig, C.F. Quate and C. Gerber, "Atomic force microscope," *Phys.Rev.Lett.*, vol. 56, no. 9, pp. 930.
- [2] X. Deng, F. Xiong, X. Li, B. Xiang, Z. Li, X. Wu, C. Guo, X. Li, Y. Li, G. Li, W. Xiong and Z. Zeng, "Application of atomic force microscopy in cancer research," *J Nanobiotechnol*, vol. 16, no. 1, -12, pp. 102-102.
- [3] H.- BUTT, P. SIEDLE, K. SEIFERT, K. FENDLER, T. SEEGER, E. BAMBERG, A.L. WEISENHORN, K. GOLDIE and A. ENGEL, "Scan speed limit in atomic force microscopy," *Journal of Microscopy*, vol. 169, no. 1, -01, pp. 75-84.
- [4] J.E. Sader, I. Larson, P. Mulvaney and L.R. White, "Method for the calibration of atomic force microscope cantilevers," *Rev.Sci.Instrum.*, vol. 66, no. 7, pp. 3789-3798.
- [5] A. Khan, J. Philip and P. Hess, "Young's modulus of silicon nitride used in scanning force microscope cantilevers," *J.Appl.Phys.*, vol. 95, no. 4, pp. 1667-1672.
- [6] J.P. Cleveland, S. Manne, D. Bocek and P.K. Hansma, "A nondestructive method for determining the spring constant of cantilevers for scanning force microscopy," *Rev.Sci.Instrum.*, vol. 64, no. 2, pp. 403-405.
- [7] J.E. Sader, J.W.M. Chon and P. Mulvaney, "Calibration of rectangular atomic force microscope cantilevers," *Review of scientific instruments*, vol. 70, no. 10, Oct, pp. 3967-3969.
- [8] I. Horcas, R. Fernández, J.M. Gómez-Rodríguez, J. Colchero, J. Gómez-Herrero and A.M. Baro, "WSXM: A software for scanning probe microscopy and a tool for nanotechnology," *Review of scientific instruments*, vol. 78, no. 1, Jan, pp. 013705.
- [9] J.L. Hutter and J. Bechhoefer, "Calibration of atomic-force microscope tips," *Rev.Sci.Instrum.*, vol. 64, no. 7, pp. 1868-1873.
- [10] F. Sumbul, N. Hassanpour, J. Rodriguez-Ramos and F. Rico, "One-step calibration of AFM in liquid," *Frontiers in Physics*, vol. 8, pp. 301.
- [11] J. Rodriguez-Ramos and F. Rico, "Determination of calibration parameters of cantilevers of arbitrary shape by finite element analysis," *Rev.Sci.Instrum.*, vol. 92, no. 4.
- [12] J. Salguero, J.M. Vazquez, M. Batista and I. Del Sol, "Recent Advances in Surface Functionalisation," *Coatings*, vol. 13, no. 3, -02-28.
- [13] H. Wang, R. Bash, J.G. Yodh, G.L. Hager, D. Lohr and S.M. Lindsay, "Glutaraldehyde Modified Mica: A New Surface for Atomic Force Microscopy of Chromatin," *Biophysical journal*, vol. 83, no. 6, pp. 3619-3625.

- [14] C.K. Riener, C.M. Stroh, A. Ebner, C. Klampfl, A.A. Gall, C. Romanin, Y.L. Lyubchenko, P. Hinterdorfer and H.J. Gruber, "Simple test system for single molecule recognition force microscopy," *Analytica chimica acta*, vol. 479, no. 1, pp. 59-75.
- [15] I. Neundlinger, A. Poturnayova, I. Karpisova, C. Rankl, P. Hinterdorfer, M. Snejdarkova, T. Hianik and A. Ebner, "Characterization of Enhanced Monovalent and Bivalent Thrombin DNA Aptamer Binding Using Single Molecule Force Spectroscopy," *Biophysical journal*, vol. 101, no. 7, Oct 05, pp. 1781-1787.
- [16] T. Mori, M. Asakura and Y. Okahata, "Single-Molecule Force Spectroscopy for Studying Kinetics of Enzymatic Dextran Elongations," *Journal of the American Chemical Society*, vol. 133, no. 15, Apr 20, pp. 5701-5703.
- [17] A. Ebner, P. Hinterdorfer and H.J. Gruber, "Comparison of different aminofunctionalization strategies for attachment of single antibodies to AFM cantilevers," *Ultramicroscopy*, vol. 107, no. 10, pp. 922-927.
- [18] R. Daza, L. Colchero, D. Corregidor, M. Elices, G.V. Guinea, F.J. Rojo and J. Pérez-Rigueiro, "Functionalization of atomic force microscopy cantilevers and tips by activated vapour silanization," *Appl.Surf.Sci.*, vol. 484, pp. 1141-1148.
- [19] P. Rezvanian, R. Daza, P.A. López, M. Ramos, D. González-Nieto, M. Elices, G.V. Guinea and J. Pérez-Rigueiro, "Enhanced biological response of AVS-functionalized Ti-6Al-4V alloy through covalent immobilization of collagen," *Scientific reports*, vol. 8, no. 1, pp. 3337.
- [20] A. Álvarez-López, L. Colchero, M. Elices, G.V. Guinea, J. Pérez-Rigueiro and D. González-Nieto, "Improved cell adhesion to activated vapor silanization-biofunctionalized Ti-6Al-4V surfaces with ECM-derived oligopeptides," *Biomaterials Advances*, vol. 133, pp. 112614.
- [21] A. Álvarez López, R. Tabraue Rubio, L. Colchero, G.V. Guinea, J. Pérez Rigueiro and D. González Nieto, "Versatility of Activated Vapor Silanization (AVS) functionalization for biomedical applications,".
- [22] J. Carlsson, H. Drevin and R. Axén, "Protein thiolation and reversible protein-protein conjugation. N-Succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent," *Biochem.J.*, vol. 173, no. 3, pp. 723-737.
- [23] S. Karumuthil-Melethil, N. Perez, R. Li, B.S. Prabhakar, M.J. Holterman and C. Vasu, "Dendritic Cell-Directed CTLA-4 Engagement during Pancreatic β Cell Antigen Presentation Delays Type 1 Diabetes," *The Journal of Immunology*, vol. 184, no. 12, -06-15, pp. 6695-6708.
- [24] H. You, R. Iino, R. Watanabe and H. Noji, "Winding single-molecule double-stranded DNA on a nanometer-sized reel," *Nucleic Acids Research*, vol. 40, no. 19, -07-05, pp. e151.
- [25] H. Zhou, J.W. Marks, W.N. Hittelman, H. Yagita, L.H. Cheung, M.G. Rosenblum and J.A. Winkles, "Development and Characterization of a Potent Immunoconjugate

Targeting the Fn14 Receptor on Solid Tumor Cells," *Molecular Cancer Therapeutics*, vol. 10, no. 7, -07-01, pp. 1276.

[26] A. Tobi, A.A. Willmore, K. Kilk, V. Sidorenko, G.B. Braun, U. Soomets, K.N. Sugahara, E. Ruoslahti and T. Teesalu, "Silver nanocarriers targeted with a CendR peptide potentiate the cytotoxic activity of an anticancer drug," vol. 4, no. 1, pp. 2000097.

[27] M. Wen, F. Zhou, C. Cui, Y. Zhao and X. Yuan, "Performance of TMC-g-PEG-VAPG/miRNA-145 complexes in electrospun membranes for target-regulating vascular SMCs," vol. 182, pp. 110369.

[28] C. Marcuello, R. de Miguel and A. Lostao, "Molecular recognition of proteins through quantitative force maps at single molecule level," vol. 12, no. 4, pp. 594.

[29] D. Zhao, D. Alizadeh, L. Zhang, W. Liu, O. Farrukh, E. Manuel, D.J. Diamond and B. Badie, "Carbon nanotubes enhance CpG uptake and potentiate antiglioma immunity," vol. 17, no. 4, pp. 771-782.

[30] H.C. Kolb, M.G. Finn and K.B. Sharpless, "Click chemistry: diverse chemical function from a few good reactions," vol. 40, no. 11, pp. 2004-2021.

[31] J. Lahann, "Click Chemistry for Biotechnology and Materials Science," ed. 1. Aufl., 2009.

[32] P. Thirumurugan, D. Matosiuk and K. Jozwiak, "Click Chemistry for Drug Development and Diverse Chemical–Biology Applications," vol. 113, no. 7, Jul 10, pp. 4905-4979.

[33] W. Xi, T.F. Scott, C.J. Kloxin and C.N. Bowman, "Click Chemistry in Materials Science," vol. 24, no. 18, May 01, pp. 2572-2590.

[34] N.J. Agard, J.A. Prescher and C.R. Bertozzi, "A strain-promoted [3 2] azide–alkyne cycloaddition for covalent modification of biomolecules in living systems," *J.Am.Chem.Soc.*, vol. 126, no. 46, pp. 15046-15047.

[35] P.M. Gramlich, C.T. Wirges, A. Manetto and T. Carell, "Postsynthetic DNA Modification through the Copper-Catalyzed Azide–Alkyne Cycloaddition Reaction," vol. 47, no. 44, pp. 8350-8358.

[36] Z. Chen, H. Meng, G. Xing, C. Chen, Y. Zhao, G. Jia, T. Wang, H. Yuan, C. Ye and F. Zhao, "Acute toxicological effects of copper nanoparticles in vivo," *Toxicol.Lett.*, vol. 163, no. 2, pp. 109-120.

[37] A.A. Alanizi, A.M. Sorlin, M.F. Parker, M. López-Álvarez, H. Qin, S.H. Lee, J. Blecha, O.S. Rosenberg, J. Engel and M.A. Ohliger, "Bioorthogonal Radiolabeling of Azide-Modified Bacteria Using [18F] FB-sulfo-DBCO," *Bioconjug.Chem.*, vol. 35, no. 4, pp. 517-527.

[38] D. Velázquez-Carreras, M. Gavilan-Herrera, I. Martinez-Martin, C. Suay-Corredera, A.C. Dumitru, E.H. Galán and J. Alegre-Cebollada, "Towards a new modular

polyprotein system compatible with single-molecule force spectroscopy by atomic force microscopy and magnetic tweezers," *Biophys.J.*, vol. 122, no. 3, pp. 304a.

[39] A. Noy, D.V. Vezenov and C.M. Lieber, "CHEMICAL FORCE MICROSCOPY,".

[40] D. Corregidor-Ortiz, R. Daza, L. Colchero, R. Tabraue-Rubio, J.M. Atienza, M. Elices, G.V. Guinea and J. Pérez-Rigueiro, "Identification of Individual Target Molecules Using Antibody-Decorated DeepTip™ Atomic-Force Microscopy Probes," vol. 9, no. 4, pp. 192.

[41] T. Hugel and M. Seitz, "The Study of Molecular Interactions by AFM Force Spectroscopy," *Macromol. Rapid Commun.*, vol. 22, no. 13, -09-01, pp. 989.

[42] M.L. Hughes and L. Dougan, "The physics of pulling polyproteins: a review of single molecule force spectroscopy using the AFM to study protein unfolding," *Rep. Prog. Phys.*, vol. 79, no. 7, -06-16.

[43] J.C. Gil-redondo, A. Weber and J.L. Toca-herrera, "Measuring biological materials mechanics with atomic force microscopy - Mechanical unfolding of biopolymers," *Microscopy Res & Technique*, vol. 85, no. 8, -05-02, pp. 3025.

[44] M. Chinnamani V., P. Bhadra, V.T. Fidal, S. Gayathri, A. Chadha and E. Bhattacharya, "Ultrasensitive detection of antigen-antibody interaction and triglycerides in liquid ambient using polysilicon cantilevers," *J Micromech Microengineering*, vol. 30, no. 12, DEC, pp. 125002.

[45] C. Lo Giudice, A.C. Dumitru and D. Alsteens, "Probing ligand-receptor bonds in physiologically relevant conditions using AFM," vol. 411, pp. 6549-6559.

[46] M. Grandbois, W. Dettmann, M. Benoit and H.E. Gaub, "Affinity imaging of red blood cells using an atomic force microscope," vol. 48, no. 5, pp. 719-724.

[47] I.H. Kim, M.N. Lee, S.H. Ryu and J.W. Park, "Nanoscale Mapping and Affinity Constant Measurement of Signal-Transducing Proteins by Atomic Force Microscopy," vol. 83, no. 5, Mar 1, pp. 1500-1503.

[48] R. McKendry, M. Theoclitou, T. Rayment and C. Abell, "Chiral discrimination by chemical force microscopy," vol. 391, no. 6667, pp. 566-568.

[49] A. Noy, "Chemical force microscopy of chemical and biological interactions," vol. 38, no. 11, pp. 1429-1441.

[50] D.V. Vezenov, A. Noy and P. Ashby, "Chemical force microscopy: probing chemical origin of interfacial forces and adhesion," *J.Adhes.Sci.Technol.*, vol. 19, no. 3-5, pp. 313-364.

[51] M. Grandbois, W. Dettmann, M. Benoit and H.E. Gaub, "Affinity Imaging of Red Blood Cells Using an Atomic Force Microscope," vol. 48, no. 5, May 01, pp. 719-724.

[52] J.W. Chung, D. Shin, J.M. Kwak and J. Seog, "Direct force measurement of single DNA-peptide interactions using atomic force microscopy," vol. 26, no. 6, pp. 268-275.

-
- [53] I. Neundlinger, A. Poturnayova, I. Karpisova, C. Rankl, P. Hinterdorfer, M. Snejdarkova, T. Hianik and A. Ebner, "Characterization of enhanced monovalent and bivalent thrombin DNA aptamer binding using single molecule force spectroscopy," *Biophys.J.*, vol. 101, no. 7, pp. 1781-1787.
- [54] D. Alsteens, V. Dupres, S. Yunus, J. Latgé, J.J. Heinisch and Y.F. Dufréne, "High-Resolution Imaging of Chemical and Biological Sites on Living Cells Using Peak Force Tapping Atomic Force Microscopy," vol. 28, no. 49, Dec 11, pp. 16738-16744.
- [55] A.C. Dumitru, E.T. Herruzo, E. Rausell, V. Ceña and R. Garcia, "Unbinding forces and energies between a siRNA molecule and a dendrimer measured by force spectroscopy," vol. 7, no. 47, pp. 20267-20276.
- [56] T.D. Becke, S. Ness, S. Sudhop, H.E. Gaub, M. Hilleringmann, A.F. Schilling and H. Clausen-Schaumann, "Covalent immobilization of proteins for the single molecule force spectroscopy," no. 138, pp. e58167.
- [57] F. Rico, A. Russek, L. González, H. Grubmüller and S. Scheuring, "Heterogeneous and rate-dependent streptavidin–biotin unbinding revealed by high-speed force spectroscopy and atomistic simulations," vol. 116, no. 14, Apr 2, pp. 6594-6601.
- [58] M. Koehler, C. Lo Giudice, P. Vogl, A. Ebner, P. Hinterdorfer, H.J. Gruber and D. Alsteens, "Control of Ligand-Binding Specificity Using Photocleavable Linkers in AFM Force Spectroscopy," *Nano Lett.*, vol. 20, no. 5, /05/13, pp. 4038-4042.
- [59] W. Ott, M.A. Jobst, C. Schoeler, H.E. Gaub and M.A. Nash, "Single-molecule force spectroscopy on polyproteins and receptor–ligand complexes: The current toolbox," vol. 197, no. 1, Jan, pp. 3-12.
- [60] G.V. Los, L.P. Encell, M.G. McDougall, D.D. Hartzell, N. Karassina, C. Zimprich, M.G. Wood, R. Learish, R.F. Ohana, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J. Zhu, A. Darzins, D.H. Klaubert, R.F. Bulleit and K.V. Wood, "HaloTag: A Novel Protein Labeling Technology for Cell Imaging and Protein Analysis," *ACS Chem.Biol.*, vol. 3, no. 6, /06/01, pp. 373-382.
- [61] I. Popa, R. Berkovich, J. Alegre-Cebollada, C.L. Badilla, J. Rivas-Pardo, Y. Taniguchi, M. Kawakami and J.M. Fernandez, "Nanomechanics of HaloTag Tethers," *J.Am.Chem.Soc.*, vol. 135, no. 34, /08/28, pp. 12762-12771.
- [62] B. Zakeri, J.O. Fierer, E. Celik, E.C. Chittock, U. Schwarz-Linek, V.T. Moy and M. Howarth, "Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin," vol. 109, no. 12, -09-21, pp. E690-E697.
- [63] J. Yin, P.D. Straight, S.M. McLoughlin, Z. Zhou, A.J. Lin, D.E. Golan, N.L. Kelleher, R. Kolter and C.T. Walsh, "Genetically encoded short peptide tag for versatile protein labeling by Sfp phosphopantetheinyl transferase," vol. 102, no. 44, -09-02, pp. 15815-15820.
- [64] D.A. Pippig, F. Baumann, M. Strackharn, D. Aschenbrenner and H.E. Gaub, "Protein–DNA Chimeras for Nano Assembly," vol. 8, no. 7, /07/22, pp. 6551-6555.

- [65] L.F. Milles, K. Schulten, H.E. Gaub and R.C. Bernardi, "Molecular mechanism of extreme mechanostability in a pathogen adhesin," vol. 359, no. 6383, Mar 30, pp. 1527-1533.
- [66] Y.F. Dufrêne, D. Martínez-Martín, I. Medalsy, D. Alsteens and D.J. Müller, "Multiparametric imaging of biological systems by force-distance curve-based AFM," vol. 10, no. 9, Sep, pp. 847-854.
- [67] K.C. Johnson and W.E. Thomas, "How Do We Know when Single-Molecule Force Spectroscopy Really Tests Single Bonds?" *Biophysical Journal*, vol. 114, no. 9, -05, pp. 2032-2039.
- [68] T. Hoffmann and L. Dougan, "Single molecule force spectroscopy using polyproteins," *Chem.Soc.Rev.*, vol. 41, no. 14, pp. 4781-4796.
- [69] A.M. Vera and M. Carrión-Vázquez, "Direct Identification of Protein-Protein Interactions by Single-Molecule Force Spectroscopy," *Angewandte Chemie*, vol. 128, no. 45, -10-13, pp. 14176-14179.
- [70] F. Rico, L. Gonzalez, I. Casuso, M. Puig-Vidal and S. Scheuring, "High-Speed Force Spectroscopy Unfolds Titin at the Velocity of Molecular Dynamics Simulations," *Science*, vol. 342, no. 6159, -11-13, pp. 741-743.
- [71] Z.T. Yew, M. Schlierf, M. Rief and E. Paci, "Direct evidence of the multidimensionality of the free-energy landscapes of proteins revealed by mechanical probes," vol. 81, no. 3 Pt 1, Mar 1, pp. 031923.
- [72] D.P. Sadler, E. Petrik, Y. Taniguchi, J.R. Pullen, M. Kawakami, S.E. Radford and D.J. Brockwell, "Identification of a Mechanical Rheostat in the Hydrophobic Core of Protein L," vol. 393, no. 1, Oct 16, pp. 237-248.
- [73] A. Raspadori, V. Vignali, A. Murello, G. Giachin, B. Samorì, M. Tanaka, C. Bustamante, G. Zuccheri and G. Legname, "Evidence of Orientation-Dependent Early States of Prion Protein Misfolded Structures from Single Molecule Force Spectroscopy," vol. 11, no. 9, Sep 16, pp. 1358.
- [74] H. Butt, B. Cappella and M. Kappl, "Force measurements with the atomic force microscope: Technique, interpretation and applications," vol. 59, no. 1, Oct 1, pp. 1-152.
- [75] J.N. Milstein and J. Meiners, "Worm-Like Chain (WLC) Model," pp. 2757-2760.
- [76] C. Bouchiat, M.D. Wang, J.-. Allemand, T. Strick, S.M. Block and V. Croquette, "Estimating the Persistence Length of a Worm-Like Chain Molecule from Force-Extension Measurements," vol. 76, no. 1, pp. 409-413.
- [77] S.B. Smith, Y. Cui and C. Bustamante, "Overstretching B-DNA: The Elastic Response of Individual Double-Stranded and Single-Stranded DNA Molecules," vol. 271, no. 5250, Feb 9, pp. 795-799.
- [78] M.D. Wang, H. Yin, R. Landick, J. Gelles and S.M. Block, "Stretching DNA with optical tweezers," vol. 72, no. 3, Mar, pp. 1335-1346.

-
- [79] N. Galvanetto, A. Perissinotto, A. Pedroni and V. Torre, "Fodis: Software for Protein Unfolding Analysis," vol. 114, no. 6, Mar 27, pp. 1264-1266.
- [80] P.R. Heenan and T.T. Perkins, "FEATHER: Automated Analysis of Force Spectroscopy Unbinding and Unfolding Data via a Bayesian Algorithm," vol. 115, no. 5, Sep 4, pp. 757-762.
- [81] G.I. Bell, "Models for the specific adhesion of cells to cells," vol. 200, no. 4342, May 12, pp. 618-627.
- [82] E. Evans and K. Ritchie, "Dynamic strength of molecular adhesion bonds," vol. 72, no. 4, Apr 1, pp. 1541-1555.
- [83] A. Lostao, K. Lim, M.C. Pallarés, A. Ptak and C. Marcuello, "Recent advances in sensing the inter-biomolecular interactions at the nanoscale – A comprehensive review of AFM-based force spectroscopy," vol. 238, May 31, pp. 124089.
- [84] H. Flyvbjerg, F. Jülicher, P. Ormos and F. David, "DYNAMIC FORCE SPECTROSCOPY," vol. 75, pp. 145-204.
- [85] M.J. Akhunzada, H.J. Yoon, I. Deb, A. Braka and S. Wu, "Bell-Evans model and steered molecular dynamics in uncovering the dissociation kinetics of ligands targeting G-protein-coupled receptors," vol. 12, no. 1, Sep 24, pp. 15972.
- [86] C.M. Dundas, D. Demonte and S. Park, "Streptavidin–biotin technology: improvements and innovations in chemical and biological applications," *Appl Microbiol Biotechnol*, vol. 97, no. 21, Nov 1, pp. 9343-9353.
- [87] J.H.T. Luong and S.K. Vashist, "Chemistry of Biotin–Streptavidin and the Growing Concern of an Emerging Biotin Interference in Clinical Immunoassays," vol. 5, no. 1, Jan 14, pp. 10-18.
- [88] G.L. Waldrop, H.M. Holden and M.S. Maurice, "The enzymes of biotin dependent CO₂ metabolism: What structures reveal about their reaction mechanisms," vol. 21, no. 11, /11/01, pp. 1597-1619.
- [89] J.H.T. Luong and S.K. Vashist, "Chemistry of Biotin–Streptavidin and the Growing Concern of an Emerging Biotin Interference in Clinical Immunoassays," vol. 5, no. 1, Jan 14, pp. 10-18.
- [90] Shuichi Miyamoto and Peter A. Kollman, "Absolute and relative binding free energy calculations of the interaction of biotin and its analogs with streptavidin using molecular dynamics/free energy perturbation approaches," vol. 16, no. 3, Jul, pp. 226-245.
- [91] P.C. Weber, J.J. Wendoloski, M.W. Pantoliano and F.R. Salemme, "Crystallographic and thermodynamic comparison of natural and synthetic ligands bound to streptavidin," vol. 114, no. 9, Apr 1, pp. 3197-3200.
- [92] P.S. Stayton, S. Freitag, L.A. Klumb, A. Chilkoti, V. Chu, J.E. Penzotti, R. To, D. Hyre, I. Le Trong, T.P. Lybrand and R.E. Stenkamp, "Streptavidin–biotin binding energetics," vol. 16, no. 1, Dec 31, pp. 39-44.

- [93] G.U. Lee, D.A. Kidwell and R.J. Colton, "Sensing Discrete Streptavidin-Biotin Interactions with Atomic Force Microscopy," vol. 10, no. 2, Feb 1, pp. 354-357.
- [94] E.L. Florin, V.T. Moy and H.E. Gaub, "Adhesion forces between individual ligand-receptor pairs," vol. 264, no. 5157, Apr 15, pp. 415-417.
- [95] J. Wong, A. Chilkoti and V.T. Moy, "Direct force measurements of the streptavidin-biotin interaction," vol. 16, no. 1, pp. 45-55.
- [96] C. Yuan, A. Chen, P. Kolb and V.T. Moy, "Energy Landscape of Streptavidin-Biotin Complexes Measured by Atomic Force Microscopy," vol. 39, no. 33, Aug 22, pp. 10219-10223.
- [97] E. Galligan, C.J. Roberts, M.C. Davies, S.J.B. Tendler and P.M. Williams, "Simulating the dynamic strength of molecular interactions," vol. 114, no. 7, Feb 15, pp. 3208-3214.
- [98] Y. Lo, Y. Zhu and T.P. Beebe, "Loading-Rate Dependence of Individual Ligand-Receptor Bond-Rupture Forces Studied by Atomic Force Microscopy," vol. 17, no. 12, Jun 12, pp. 3741-3748.
- [99] X. Zhang and V.T. Moy, "Cooperative adhesion of ligand-receptor bonds," vol. 104, no. 1, May 1, pp. 271-278.
- [100] M. de Odrowaz Piramowicz, P. Czuba, M. Targosz, K. Burda and M. Szymoński, "Dynamic force measurements of avidin-biotin and streptavidin-biotin interactions using AFM," vol. 53, no. 1, Jan, pp. 93-100.
- [101] J. Zhou, L. Zhang, Y. Leng, H. Tsao, Y. Sheng and S. Jiang, "Unbinding of the streptavidin-biotin complex by atomic force microscopy: A hybrid simulation study," vol. 125, no. 10, Sep 14, pp. 104905-7.
- [102] E. Thormann, P.L. Hansen, A.C. Simonsen and O.G. Mouritsen, "Dynamic force spectroscopy on soft molecular systems: Improved analysis of unbinding spectra with varying linker compliance," vol. 53, no. 2, Dec 1, pp. 149-156.
- [103] F. Rico and V.T. Moy, "Energy landscape roughness of the streptavidin-biotin interaction," vol. 20, no. 6, Nov, pp. 495-501.
- [104] S. Guo, C. Ray, A. Kirkpatrick, N. Lad and B.B. Akhremitchev, "Effects of Multiple-Bond Ruptures on Kinetic Parameters Extracted from Force Spectroscopy Measurements: Revisiting Biotin-Streptavidin Interactions," vol. 95, no. 8, Oct 15, pp. 3964-3976.
- [105] E.B. Walton, S. Lee and K.J. Van Vliet, "Extending Bell's Model: How Force Transducer Stiffness Alters Measured Unbinding Forces and Kinetics of Molecular Complexes," vol. 94, no. 7, Apr 1, pp. 2621-2630.
- [106] J. Teulon, Y. Delcuze, M. Odorico, S.W. Chen, P. Parot and J. Pellequer, "Single and multiple bonds in (strept)avidin-biotin interactions," vol. 24, no. 3, May, pp. 490-502.

-
- [107] S.M. Sedlak, M.S. Bauer, C. Kluger, L.C. Schendel, L.F. Milles, D.A. Pippig and H.E. Gaub, "Monodisperse measurement of the biotin-streptavidin interaction strength in a well-defined pulling geometry," vol. 12, no. 12, pp. e0188722.
- [108] P.M. Williams, "Analytical descriptions of dynamic force spectroscopy: behaviour of multiple connections," *Anal.Chim.Acta*, vol. 479, no. 1, pp. 107-115.
- [109] S.M. Sedlak, L.C. Schendel, M.C.R. Melo, D.A. Pippig, Z. Luthey-Schulten, H.E. Gaub and R.C. Bernardi, "Direction Matters: Monovalent Streptavidin/Biotin Complex under Load," vol. 19, no. 6, Jun 12, pp. 3415-3421.
- [110] S.M. Sedlak, L.C. Schendel, H.E. Gaub and R.C. Bernardi, "Streptavidin/biotin: Tethering geometry defines unbinding mechanics," vol. 6, no. 13, Mar, pp. eaay5999.
- [111] D. Corregidor, R. Tabraue, L. Colchero, R. Daza, M. Elices, G.V. Guinea and J. Pérez-Rigueiro, "High-Yield Characterization of Single Molecule Interactions with DeepTip™ Atomic Force Microscopy Probes," vol. 28, no. 1, Dec 27, pp. 226.
- [112] S. Senapati, S. Biswas, S. Manna, R. Ros, S. Lindsay and P. Zhang, "A Y-Shaped Three-Arm Structure for Probing Bivalent Interactions between Protein Receptor–Ligand Using AFM and SPR," vol. 34, no. 23, Jun 12, pp. 6930-6940.
- [113] M. Ludwig, W. Dettmann and H.E. Gaub, "Atomic force microscope imaging contrast based on molecular recognition," *Biophys.J.*, vol. 72, no. 1, pp. 445-448.
- [114] S. Lecot, Y. Chevolot, M. Phaner-Goutorbe and C. Yeromonahos, "Impact of Silane Monolayers on the Adsorption of Streptavidin on Silica and Its Subsequent Interactions with Biotin: Molecular Dynamics and Steered Molecular Dynamics Simulations," vol. 124, no. 31, Aug 06, pp. 6786-6796.
- [115] T. Puntheeranurak, I. Neundlinger, R.K.H. Kinne and P. Hinterdorfer, "Single-molecule recognition force spectroscopy of transmembrane transporters on living cells," vol. 6, no. 9, Sep 1, pp. 1443-1452.
- [116] B. Yang, Z. Liu, H. Liu and M.A. Nash, "Next Generation Methods for Single-Molecule Force Spectroscopy on Polyproteins and Receptor-Ligand Complexes," vol. 7, May 19, pp. 85.
- [117] M.R. Uhlig, C.A. Amo and R. Garcia, "Dynamics of breaking intermolecular bonds in high-speed force spectroscopy," vol. 10, no. 36, Sep 20, pp. 17112-17116.
- [118] R. Merkel, P. Nassoy, A. Leung, K. Ritchie and E. Evans, "Energy landscapes of receptor-ligand bonds explored with dynamic force spectroscopy," vol. 397, no. 6714, Jan 7, pp. 50-53.
- [119] J. Pérez Rigueiro, "BIOLOGICAL MATERIALS AND BIOMATERIALS ," ed. 1, 2023.
- [120] R. Daza, M. Garrido-Arandia, D. Corregidor-Ortiz, C.I. Pérez, L. Colchero, R. Tabraue-Rubio, M. Elices, G.V. Guinea, A. Diaz-Perales and J. Pérez-Rigueiro, "Statistical Study of Low-Intensity Single-Molecule Recognition Events Using

DeepTipTM Probes: Application to the Pr u p 3-Phytosphingosine System," vol. 8, no. 8, Dec 8, pp. 595.

[121] A. Berquand, N. Xia, D.G. Castner, B.H. Clare, N.L. Abbott, V. Dupres, Y. Adriaensen and Y.F. Dufrêne, "Antigen Binding Forces of Single Antilysozyme Fv Fragments Explored by Atomic Force Microscopy," vol. 21, no. 12, pp. 5517-5523.

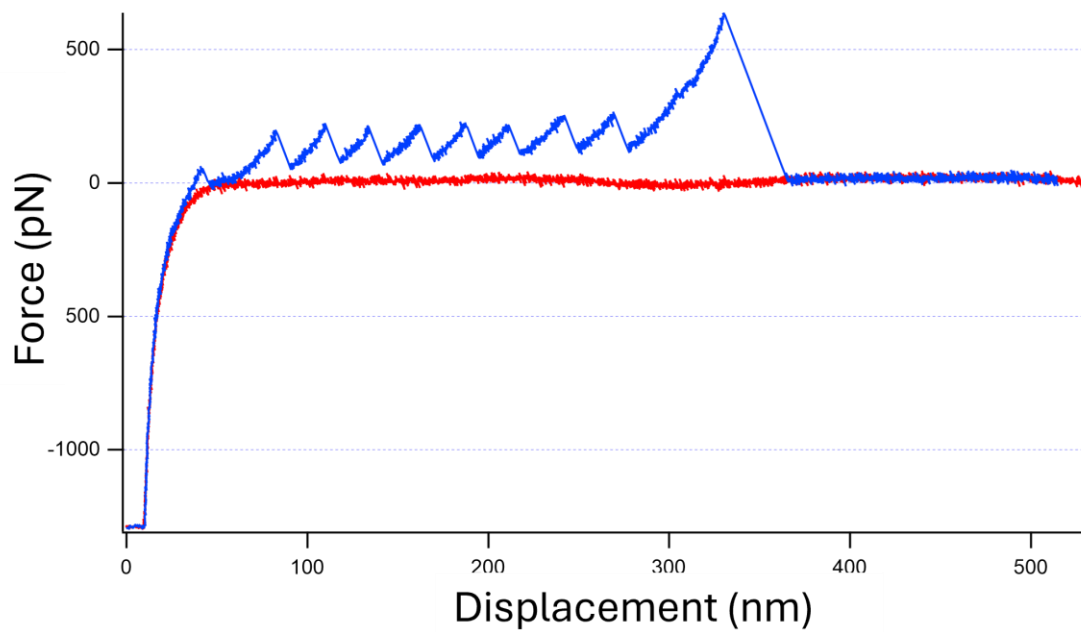
[122] A.K. Abbas, A.H. Lichtman and S. Pillai, "Cellular and molecular immunology," ed. Ninth edition, 2018.

[123] M. Duman, A. Ebner, C. Rankl, J. Tang, L.A. Chtcheglova, L. Wildling, H.J. Gruber and P. Hinterdorfer, "Atomic Force Microscopy (AFM) for Topography and Recognition Imaging at Single Molecule Level," pp. 102-112.

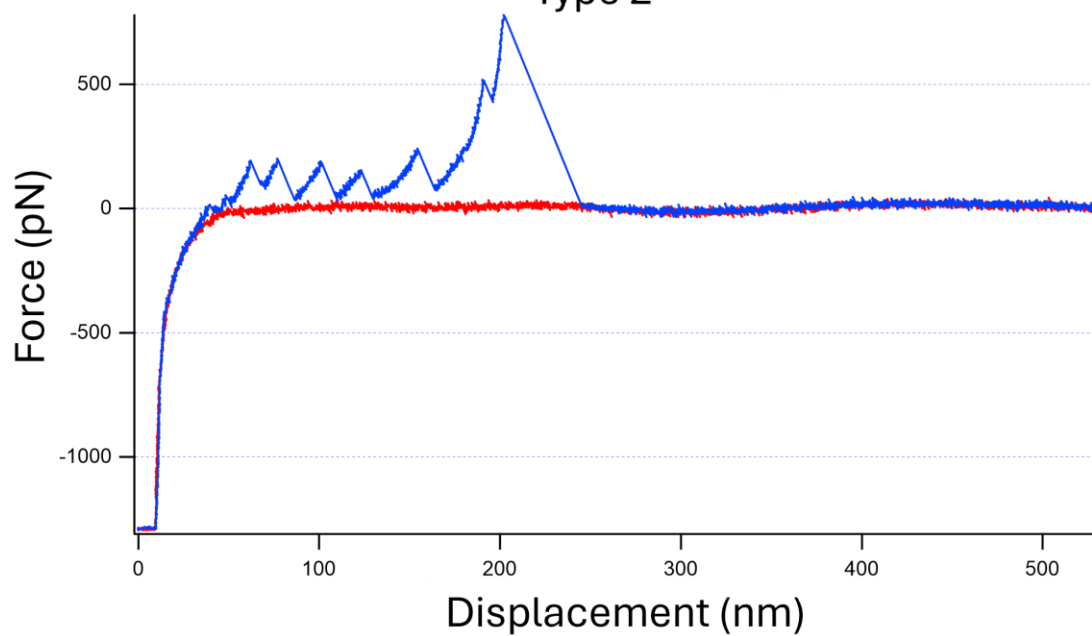
ANNEXES.

Annex A: Types of curves obtained from the SMFS experiment of titin unfolding

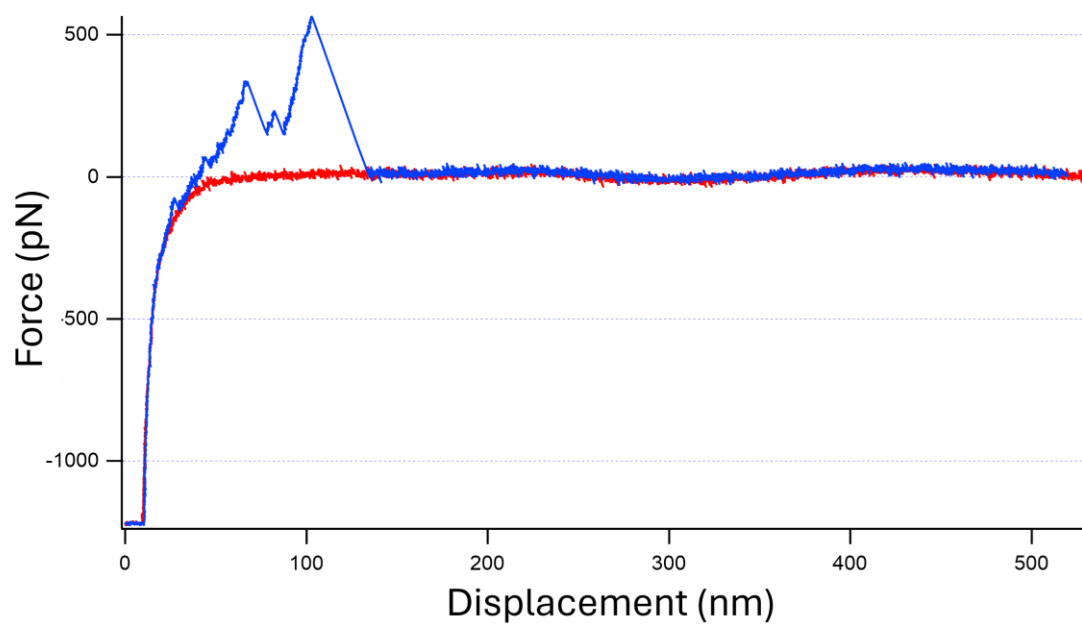
Type 1



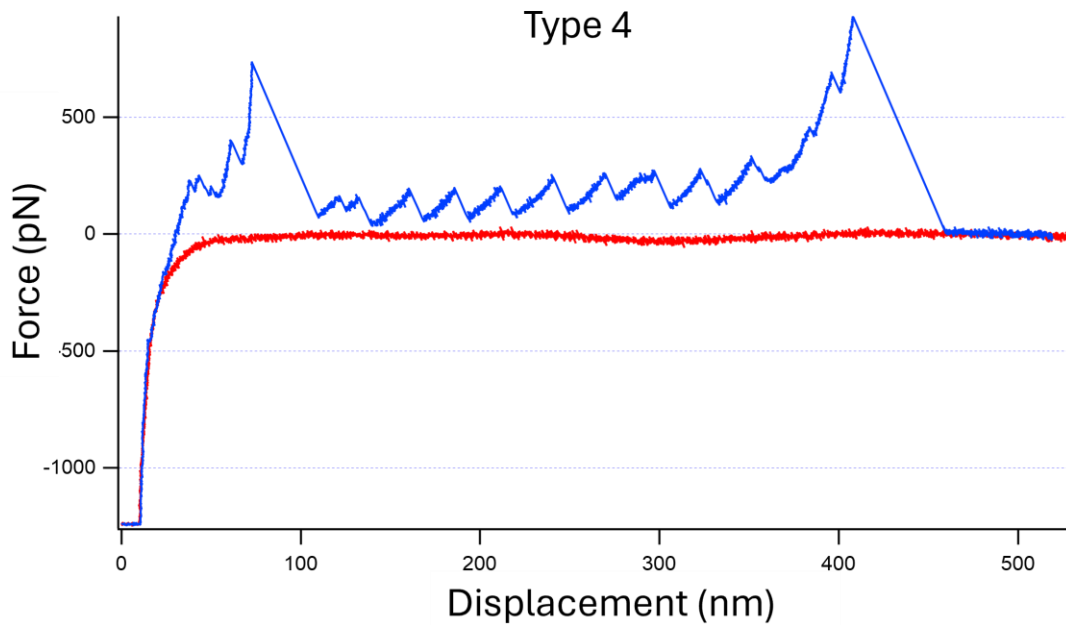
Type 2

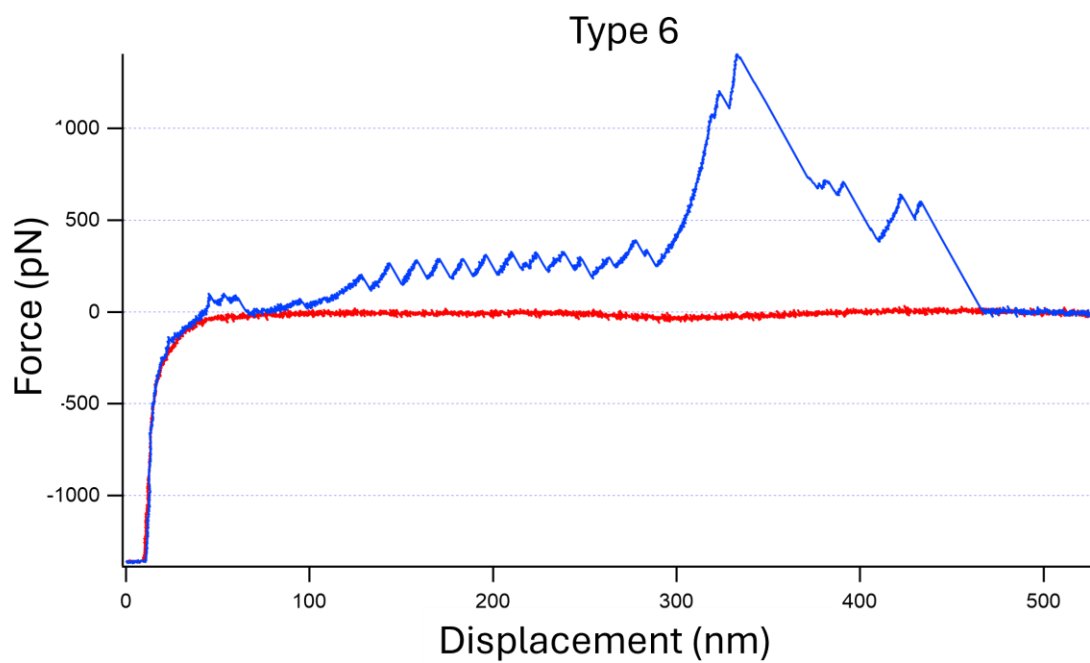
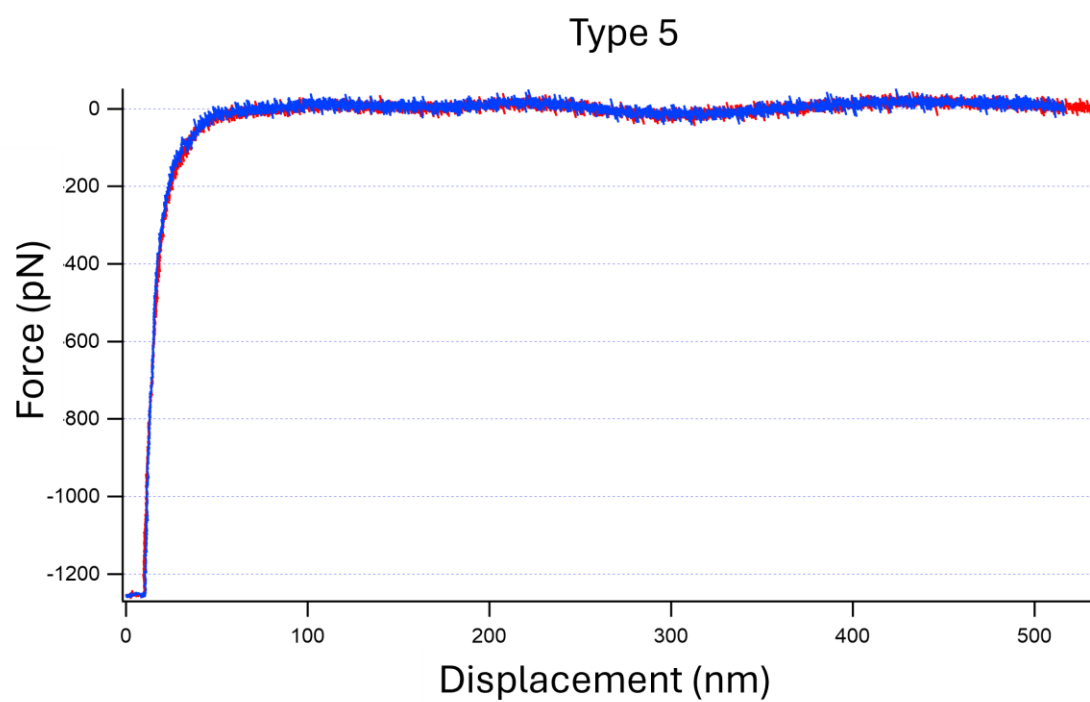


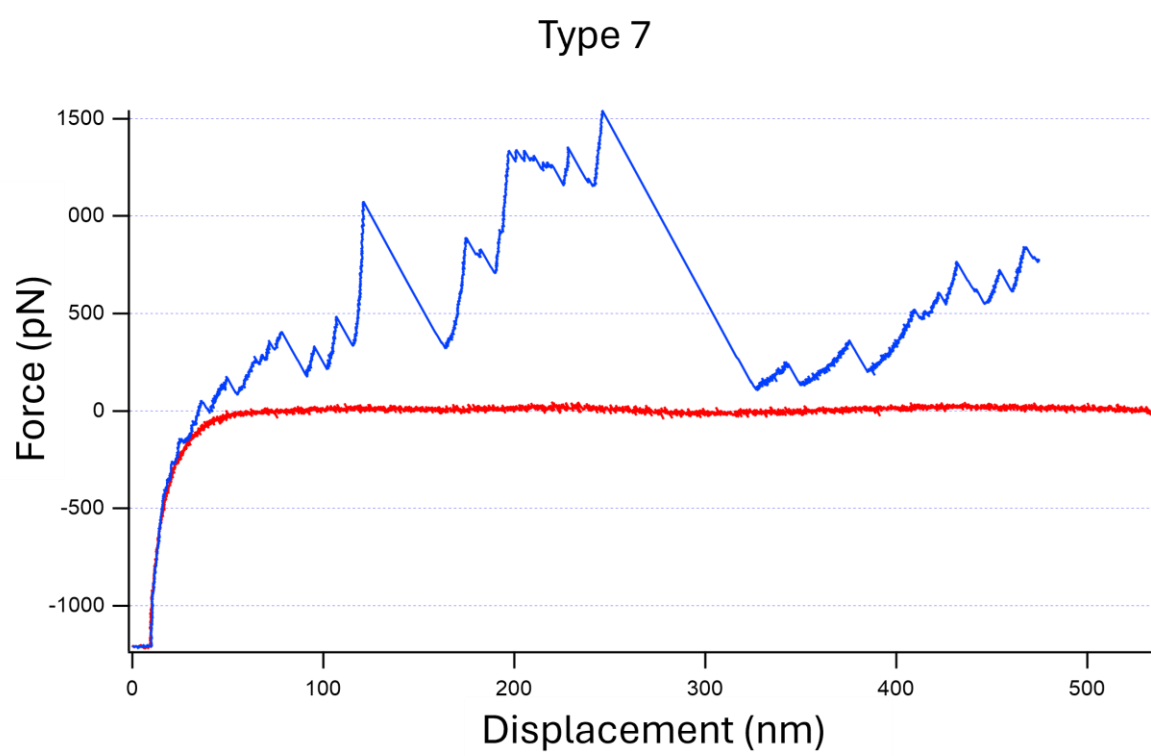
Type 3



Type 4







Annex B: Definitions of Ra and RMS values to characterise surface roughness.

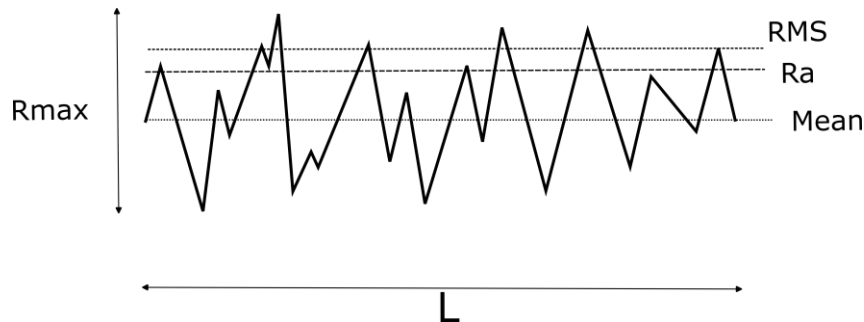
Ra and RMS both represent surface roughness, but they are calculated differently. Ra, or Roughness Average, is determined by averaging the microscopic peaks and valleys of a surface. On the other hand, RMS, or Root Mean Square, is calculated by taking the square root of the average of the squares of these microscopic peaks and valleys.

The formulas to calculate Ra and RMS values are presented below:

$$Ra = \left(\frac{1}{L}\right) \int_0^L |Z(x)| dx$$

$$RMS = \left(\left(\frac{1}{L}\right) \int_0^L Z(x)^2 dx\right)^{\frac{1}{2}}$$

Where L is the evaluation length and Z(x) is the profile height function.



The picture shows a generic representation of a profile of length L in which the values of Ra and RMS are plotted.