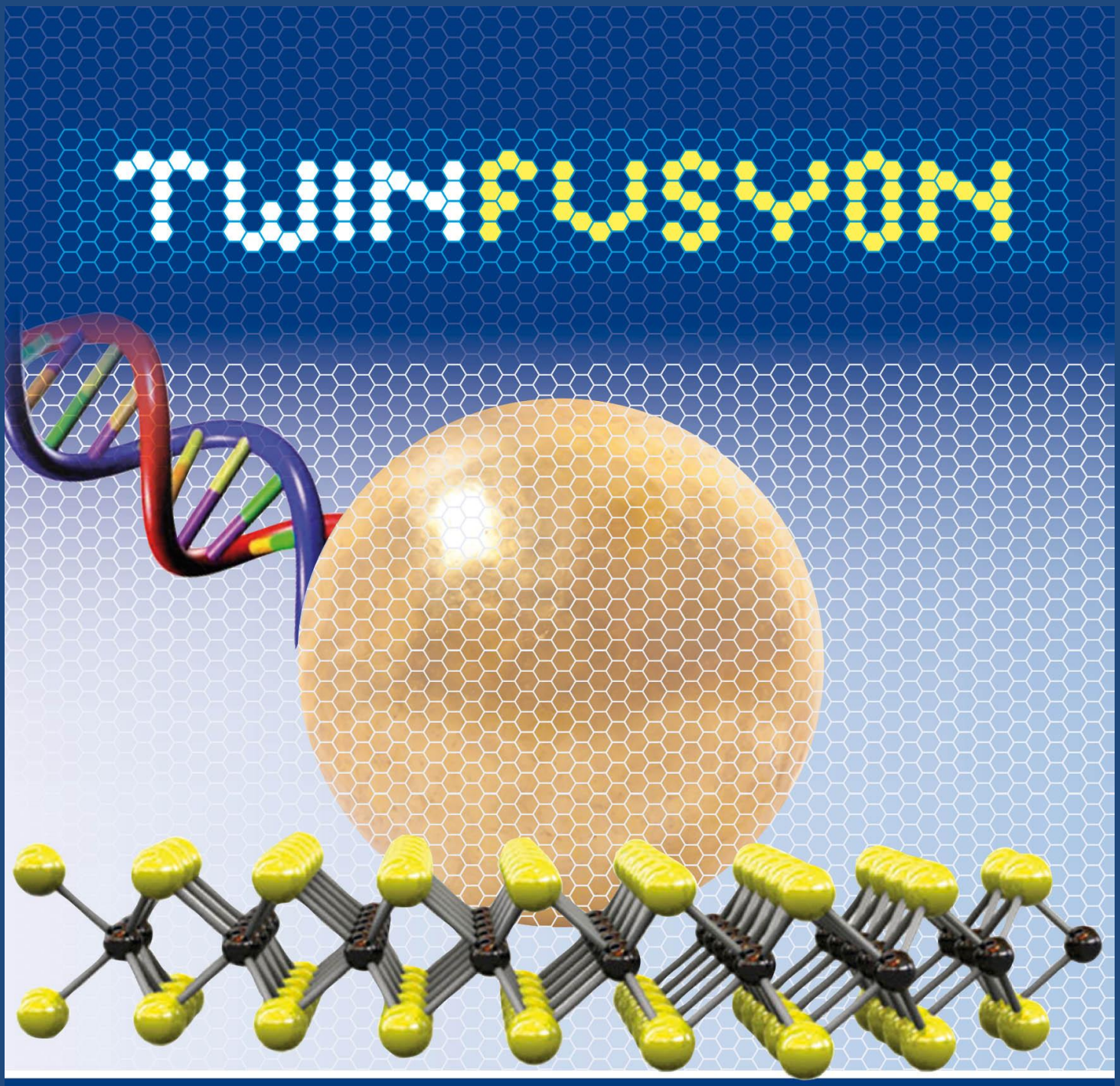
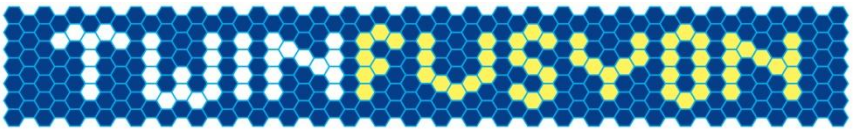


BEST-PRACTICES FOR BIOFUNCTIONALISATION OF INNOVATIVE MATERIALS

This project has received funding from the *European Union's Horizon 2020 research and innovation programme* under grant agreement No 692034. This booklet reflects only the author's view and the Research Executive Agency is not responsible for any use that may be made of the information it contains.

Eds: Maria Losurdo, Milan Orlita, Kurt Hingerl, Josef Humlicek

ISBN 978-3-200-05312-0 (eBook)



Twinning for Improving Capacity of Research in Multifunctional Nanosystems for Optronic Biosensing

H2020-TWINN-2015-692034

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ISBN 978-3-200-05312-0 (eBook)

"When nature finishes to produce its own species, man begins using natural things in harmony with this very nature to create an infinity of species"
(Leonardo da Vinci)

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1 Abbreviations

2D = bidimensional

Ab = antibody

CVD = chemical vapour deposition

DMF = dimethilformamide

EG = epitaxial graphene

EPL = expressed protein ligation

FET = field effect transistor

GO = graphene oxide

GBMs = graphene based materials

IgG = immunoglobulin-G

IPA =

LSPR = localized surface plasmon resonance

NP = nanoparticle

ODCB= ortodichlorobenzene solvent

PEG = Polyethylene glycol

RGO = reduced graphene oxide

TEM = transmission electron microscopy

THF = tetrahydrofuran

TMDs= transition metal dichalcogenides

2 SUMMARY

The major challenge in developing biosensors is to provide a high specificity to target biomolecules. The detection and study of specific biomolecules, proteins, drugs, DNA, antibodies, enzymes, virus, etc. require biofunctionalised probes of materials and nanoparticles that have a strong affinity for the target biomolecule while suppressing non-specific interactions.

Among the plethora of materials and nanostructures that are currently applied or investigated for bioapplications, functionalised graphene, graphene-based nanocomposites, novel 2D-materials as well as plasmonic nanoparticles are gaining increasing attention in biomedical applications because of their unique and highly enriched physical and chemical properties. Following graphene [1], an atlas of other 2D materials have emerged recently [2]. Among the novel 2D-materials, semiconducting 2D TMDs transition metal dicalchogenides (MoS_2, WS_2, $MoSe_2$, and WSe_2) are gaining attention from scientific community.

In addition, inorganic nanoparticles, such as magnetic and plasmonic nanoparticles, can be grown on the surface of graphene and related 2D materials, enabling further expansion of functional 2D-based nanocomposites with interesting optical and magnetic properties that can be useful for multimodal technologies for biosensing, imaging and imaging-guided cancer therapeutic applications
Among the various nanoparticles, of particular interest are plasmonic gold nanoparticles (Au NPs) that offer a suitable platform for multifunctionalisation with a wide range of organic or biological ligands for the selective binding and detection of small molecules and biological targets [3].

Therefore, herein we describe aspects related to the biofunctionalisation of the three presently most investigated and interesting materials of:

- Graphene,
- MoS_2,
- Gold nanoparticles.

Despite the explosion of interest in the development of 2D- and nanoparticle-based systems for bio-applications, the descriptions of procedures for biofunctionalisation of those materials vary from article to article. In this labyrinth of biofunctionalisation, various methods, practices, and procedures are given with the emphasis of papers being on the final biosensor performance.

Indeed, an important contributing factor toward that goal is making it clear just how biofunctionalisation of materials, transducers, or devices is carried out, highlighting the fundamental importance of ensuring that research is reliable, robust, and reproducible. Providing reliable information on the biofunctionalisation permits other researchers to both verify the validity of the research and repeat experiments to ensure that the results obtained are reproducible. Therefore, best-practices and protocols about how bioconjugates are made are highly needed. In order to grasp the complexity and variety of the practices in biofunctionalising surfaces, over published 2000 protocols [4] only one paper was found as „protocol“ summarising the procedures used to make nano-graphene bioconjugates for various biomedical applications [5].

The major scope of this report is to share an overview of the methodological know-how in biofunctionalisation of graphene-, 2D, and Au-NPs based materials to guide researchers in this field toward fabricating bioconjugates with high reproducibility.

3 GRAPHENE-BASED MATERIALS BIOFUNCTIONALISATION

There are various kinds of graphene-based materials (GBMs) and their properties and behaviour are closely related to the method of production.

Exfoliated graphene (G) [6]	
Chemical vapor deposition (CVD) graphene [7]	Epitaxial Graphene (EG) [8]
Reduced graphene oxide (RGO) [9]	Graphene oxide (GO) [9]

To endow graphene-based materials with sensing capabilities, it is necessary to functionalise them with recognition molecules that bring the detection targets onto the GBMs surface through specific interactions and possibly also assist in signal transduction.

Experimentally CVD and epitaxial graphene unavoidably contains edges, basal plane fluctuations, vacancies, grain boundaries and other chemical impurities. Similarly flakes of exfoliated graphene has edges. By altering the electronic structure, these structural ‘‘imperfections’’ can alter the chemical properties and reactions of graphene, and therefore their response during biofunctionalisation. The defects of graphene, which are potential site for anchoring of biomolecules, are shown in Figure 1.

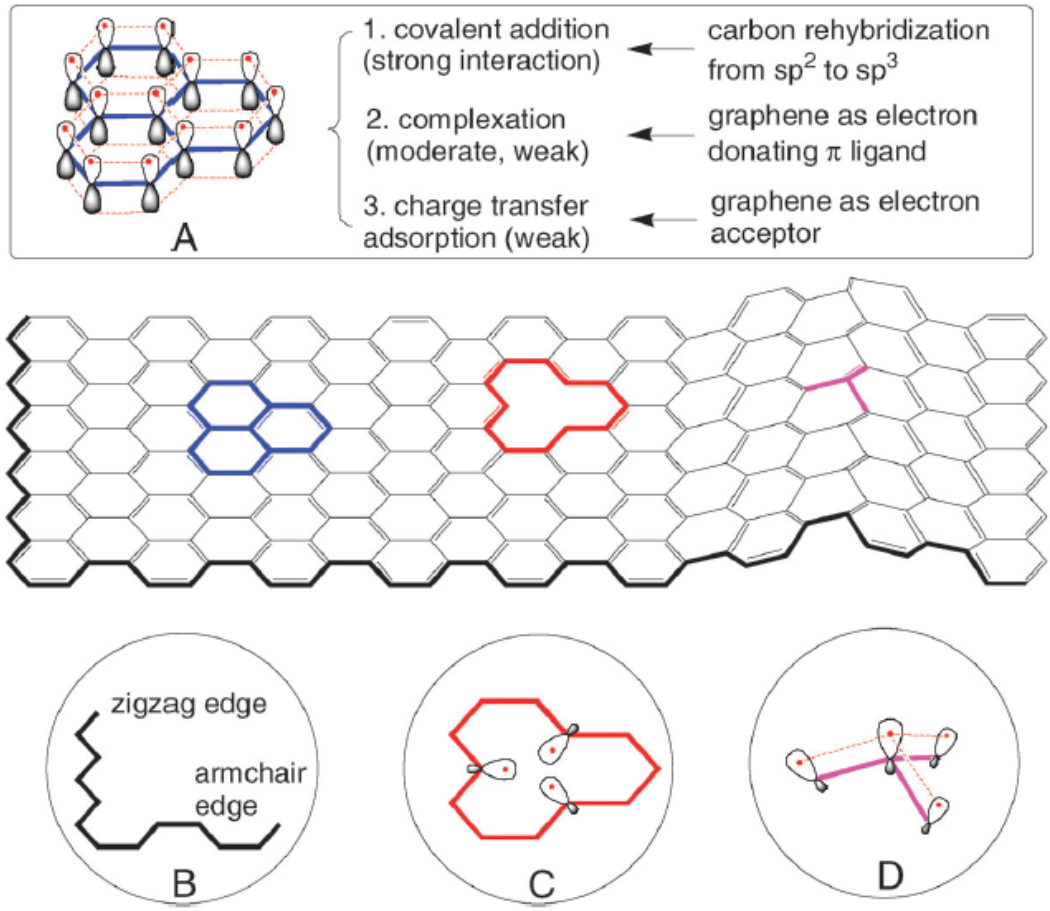


Figure 1: Structure and main defects characterising CVD, epitaxial-, and exfoliated graphene [readapted from ref. 10]

The main difference between graphene and GO/RGO is the presence of oxygen functionalities in GO as shown in Figure 2. As a result, graphene is hydrophobic whereas GO is hydrophilic and easy to disperse in organic solvents, water, and different matrixes. With respect to electrical conductivity, GO functions as an electrical insulator, because of the disturbance of its sp^2 bonding networks. On the other hand, GO contains both aromatic (sp^2) and aliphatic (sp^3) domains, which further expands the types of interactions that can occur with biomolecules interacting with the surface. In fact, the main

advantage of GO is that the oxygen functional groups, shown in Figure 2, can serve as active sites for interaction with molecules and covalent biofunctionalisation. For example, carboxylic (-COOH) groups can react with the -NH_2 group of proteins and aminoacids through amide linkages. As an example, horseradish peroxidase (HRP) and lysozyme can be spontaneously immobilized on GO because the individual GO sheet is enriched with oxygen-containing groups, which makes it possible to immobilize enzymes without any further surface modifications or coupling reagents, simply through strong electrostatic and hydrogen-bond interactions between oxygen groups of the GO and nitrogen groups of the enzymes (see Figure 2) [11]

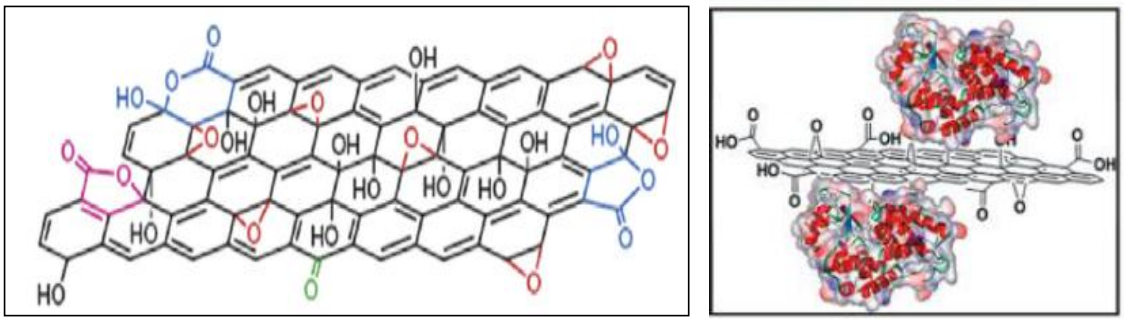


Figure 2: Structure and oxygen-based functional groups of graphene oxide, GO (left) and of enzyme GO-bound HRP (right) using hydrogen bonds.

From the comparison of graphene and GO it can be inferred that the biofunctionalisation of graphene-based materials depends not only on the analyte but also on the graphene category that should always be specified in relation to the specific biofunctionalisation strategy, and consequently a different source of graphene can require different biofunctionalisation steps.

Furthermore, numerous graphene-based biosensing platforms are being investigated and developed based on different transduction mechanisms [12], such as electronic, electrochemical, optoelectronic and nano-electromechanical sensing, as summarised in the Table 1. There have been a number of demonstrations of graphene-based sensors; however, high sensitivity and selectivity needs to be engineered by attaching various functional groups to graphene, either covalently or non-covalently, requiring different biofunctionalisation strategies thereof.

In all those types of sensors graphene-based materials have been interfaced with a large variety of biomolecules and cells as summarised in Figure 3.

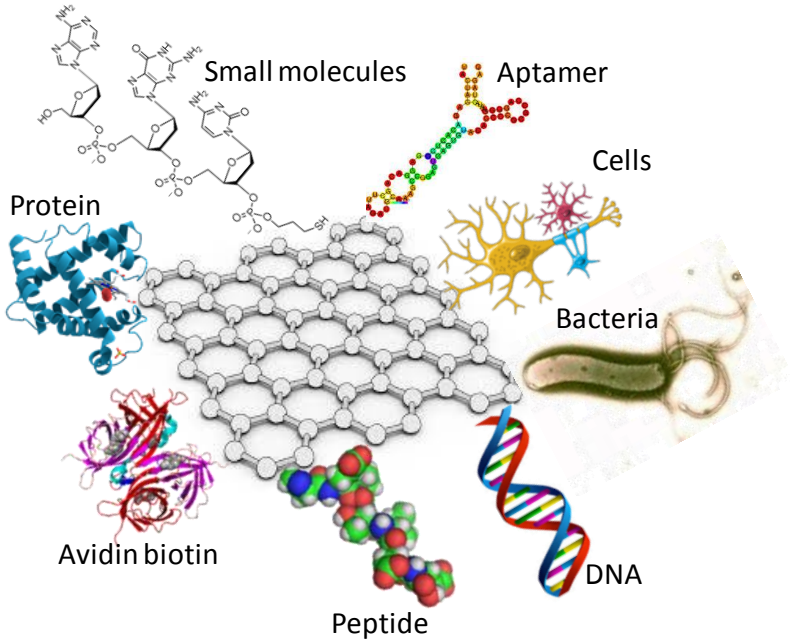
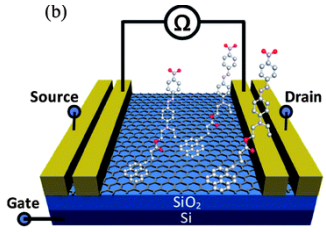
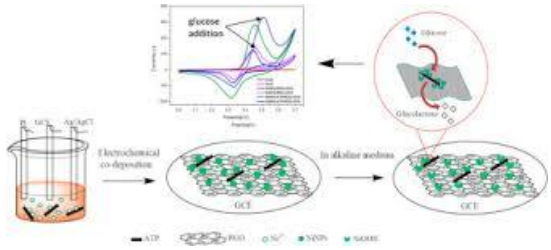
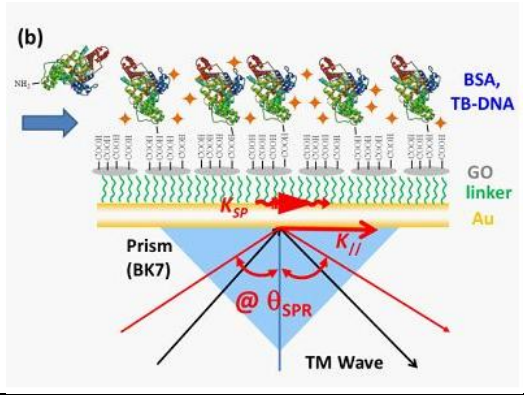
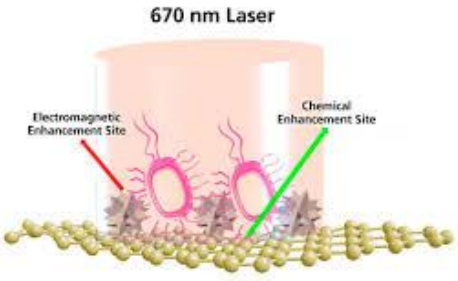
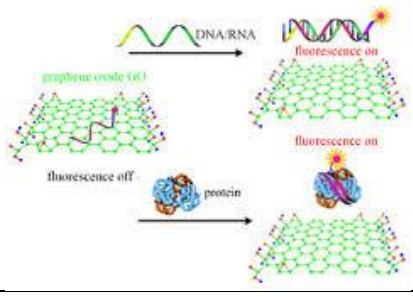


Figure 3: Graphene functionalised with avidin–biotin, peptides, NAs, proteins, aptamers, small molecules, bacteria, and cells through physical adsorption or chemical conjugation.

Table 1. Main types of graphene-based sensors depending on transduction mechanism

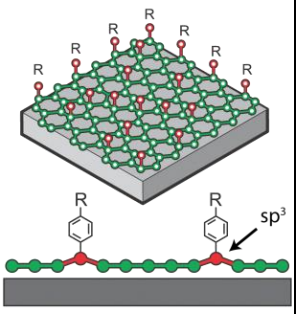
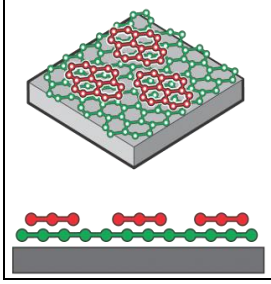
Type of Sensor	Type of Analyte
GRAPHENE IN BIO-FIELD-EFFECT TRANSISTORS (GFET) 	• DNA hybridization [13] • Immunoglobulin G (IgG) [14] • Aptamers • Proteins • Enzymes • Bacteria [15]
GRAPHENE IN ELECTROCHEMICAL BIOSENSORS (Amperometric, potenziometric, capacitive, impedimetric) 	• DNA hybridization and polymorphism [16] • Glucose [17] • Hemoglobin [18] • Dopamine [19] • DNA [20]
OPTICAL RESONATORS and LSPR-SENSORS 	Bacteria [21] Cells [22] Antibodies [23] Proteins [24]
SERS (Surface Enhanced Raman Spectroscopy) 	• Rhodamine 6G [25] • Cellular uptake mechanisms [26] • Imaging cancer cells [27]
Fluorescence Immonosensing 	• Aptamer-based detection of thrombin [28] • Virus detection [29]

Consequently, a myriad of different strategies for biofunctionalisation of graphene-based materials are being applied and investigated, making therefore difficult to compare and or establish standard biofunctionalisation protocols.

This report is not meant to be complete and exhaustive but to give some indications about how to get oriented in this labyrinth.

The functionalisation strategies can be grouped into two general categories: ***covalent*** and ***non-covalent*** [30], whose characteristics are summarised in the Table below:

Table 2. Two general categories of functionalisation strategies

Category	Characteristics	Interactions	Examples
Covalent functionalisation 	• Compromises the sp^2 structure of graphene lattice, resulting in defects and loss of the electronic properties. • Stable and specific	Covalent links can be formed by • nucleophilic substitution, • electrophilic addition, • condensation, • addition • amide coupling reactions, • cross-linking, • click chemistry. Two general routes are used based on reactions between (a) free radicals and the C=C bonds on the graphene planes (b) organic functional groups and the oxygen-containing groups of GO or those on the edges of graphene.	• Diazonium salt [31] •Phthalocyanines [32] • Porphyrins [33] • Dopamines, [33] • Aminoacids [33]
Non-covalent functionalisation 	• The extended π system is not interrupted • It does not alter the structure and electronic properties of graphene • Electric conductivity or mechanical strength are not affected • It introduces new chemical groups on the surface.	Interactions occur by: • π-π stacking interactions • Electrostatic hydrogen-bond • hydrophobic interactions • Van der Waals • polymer wrapping • donor−acceptor complexes	• DNA [34] • Heparin • Glucose oxidase [35] • Dopamine[36] • Porphirins [37] • Quercetin [38] • Doxorubicin • Ellipticine • Enzymes, e.g. HRP, lysozyme [39]

A representation of π–π interaction types, face-to-face and slipped as well as of CH•••π like interactions, i.e., electrostatic H-bonds, occurring around the edge of graphene that may be terminated with a hydrogen at grain boundaries or with COOH groups in GO is given in Figure 4.

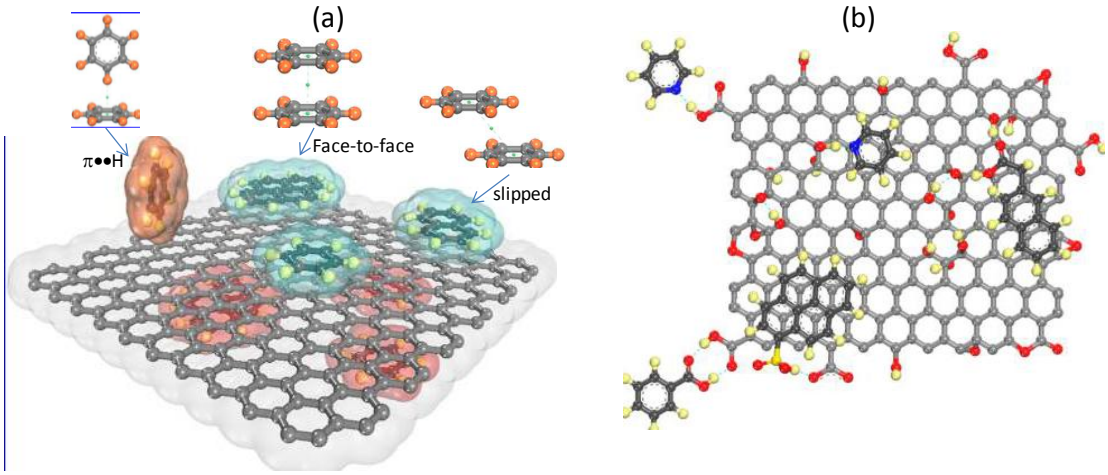


Figure 4: Face-to-face and slipped π-π interactions and π-H non-covalent interactions in (a) graphene and (b) GO

3.1 Non-covalent Functionalisation of Graphene

In the *non-covalent* functionalisation graphene can be viewed as the largest aromatic molecule that can interact with any other aromatic molecule. *Non-covalent* interaction has often been used to anchor small aromatic molecules, i.e., pyrene derivatives, which act as linkers for the connection of graphene-based materials to other bodies. As an example, Figure 5 shows a pyrene derivative π-π interacting with graphene and acting as a linker between graphene and glucose oxidase (GOx) enzyme in a bioelectrochemical sensor of glucose:

- first, aromatic molecules, such as 1-pyrene butanoic acid, which contain a function group, physically adsorbe while aligned with graphene lattice through π-π stacking, as shown in Figure 5.
- Next, biomolecular probes are attached to the functional group and stably attached to the graphene surface without disturbing the electronic structure

Other bifunctional molecules with an aromatic tail and a reactive end (e.g. perylene tetracarboxylic acid, thionine and many porphyrin derivatives) can also be employed as linker molecules [40].

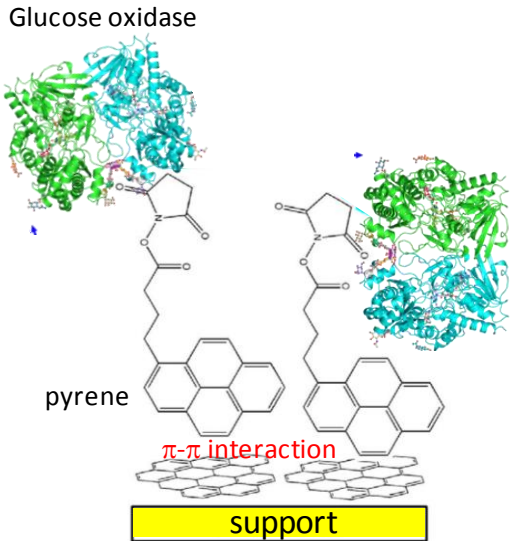


Figure 5: Scheme of the non-covalent π-π interaction between graphene and pyrene used to bind the enzyme Glucose oxidase

3.2. Covalent Functionalisation of graphene

In the *covalent functionalisation* of graphene the most attractive organic species for the reaction with sp^2 carbons of graphene are organic free radicals, such as diazonium salts and benzoyl peroxide, and dienophiles, such as azomethine ylide, all giving addition reactions to graphene. Examples of those two classes are shown in Figure 6.

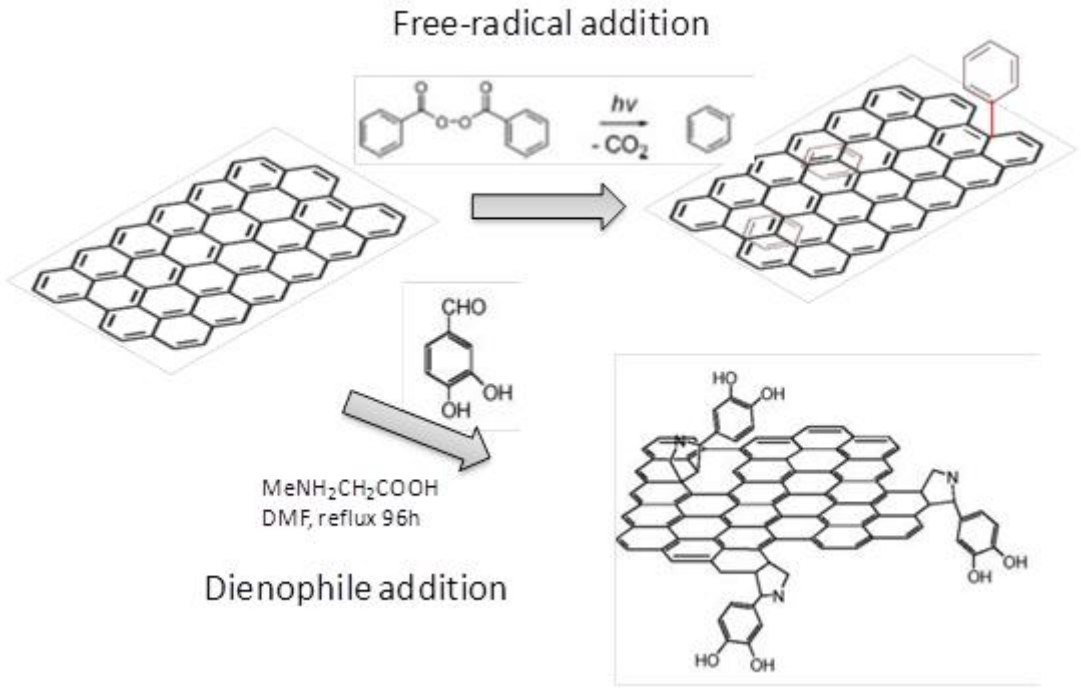


Figure 6: Schematic representation of the radical (upper reaction) and dienophile azomethine ylide (lower reaction) addition on graphene. The top panel indicates that simultaneous noncovalent functionalisation could occur on the basal plane of defect-free graphene, while covalent bonding formed on the edge of the graphene

The functionalisation with azomethine ylide is important because enables to choose among several aldehydes or substituted α-amino acids as precursors, thus yielding a variety of desirable functional groups further used to functionalise graphene with porphyrins, aminoacids, proteins etc.

Alternatively, hydrophilic groups can also be introduced onto the surface of graphene to improve their aqueous solubility and biocompatibility. For example, carboxylic (–COOH) and hydroxyl (–OH) groups, are covalently created on the graphene using strong acids and/or oxidants. Once those moieties are created on graphene, carboxylic groups –COOH can react with proteins, carbohydrates or other polymers via amide or ester linkages.

In addition, exposure to ammonia and ammonia or nitrogen plasmas treatment has been used to create amino ($–NH_2$) groups on graphene [41], which can further reacts with –COOH of aminoacids, proteins and DNA to further link those biomolecules to graphene through an amido $–(CONH_2)$ reaction.

3.3 Covalent Functionalisation of GO

In the majority of reports on graphene-based hybrid nanomaterials, graphene oxide (GO) has been used as the starting material. Its reduced form, RGO, is also applicable, since it is populated with these oxygen-containing chemical groups of hydroxyl, carboxyl, and epoxy groups that favour the formation of graphene derivatives through the covalent attachment of organic groups on its surface. In the *covalent* functionalisation of GO, the added groups are linked through the oxygen atoms of GO.

A variety of biomolecules, such as chitosan, dextran,), poly(L-lactic acid), polyaniline, poly(2-methoxystyrene), polyacrylate, poly(3-caprolactone), polyethylene (PE), poly(vinyl alcohol) (PVA), cycloamyloses, polyethylene glycol (PEG) (see Figure 7) have been covalently attached to GO [42].

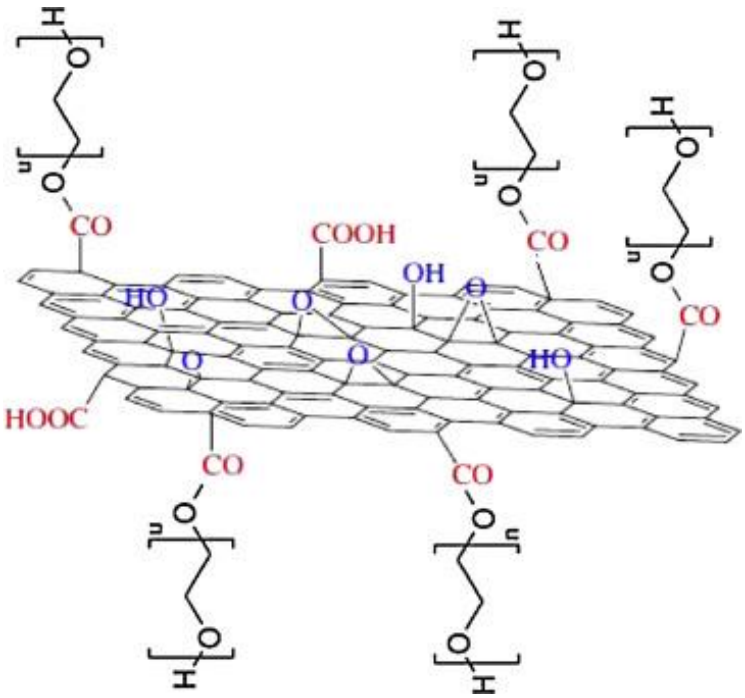


Figure 7: PEGylation of GO.

PEGylation is commonly used to render graphene-based materials more stable in water. The amino-terminated PEG acts as surfactant and a "bridge" to link functional molecules with nanomaterials by amido bond.

The PEG-GO are prepared through a covalent bond formation between polymeric chain of polyethylene glycol (PEG) and GO, which was called PEGylation. Accordingly, 0.05 g GO is dissolved in water and then was exposed to ultrasonic for 5min. After removing the solution from the ultrasonic bath, 2ml of solution is immediately added to 1 g polyethylene glycol 600 and kept under 300 rpm for 24h at 25 ◦C.

Since the importance and use of PEGylation here we report for comparison protocols used for both:

- covalent PEGylation of GO
- non-covalent PEGylation of graphene

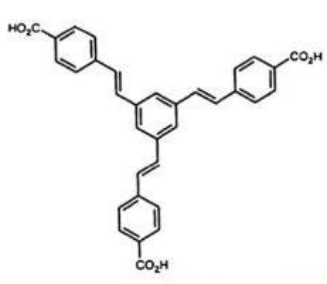
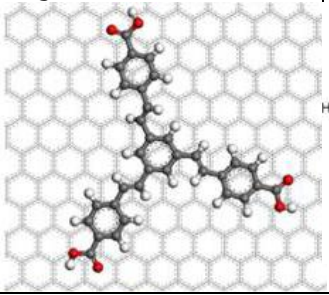
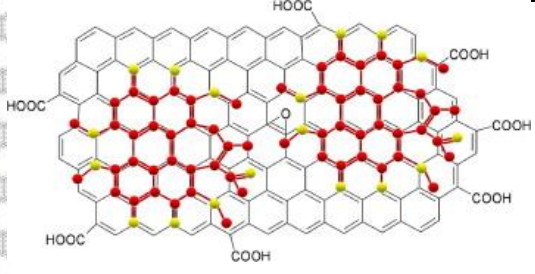
Table 3. Comparison of protocols for PEGylation of GO

Covalent PEGylation of GO	Non-covalent PEGylation of graphene
1.2 g of NaOH are addedto 10 mL of GO (1000 mg/L). This solution is sonicated for 3h, and then concentrated HCl is added until the pH=1. The solution is then washed three times with water and brought to a concentration of 1000 mg/L. Poly-(ethylene glycol) with amine termination ($6PEGNH_2$) is added to the solution at 2000 mg/L and sonicated for 5 min. N-(3-Dimethylaminopropyl-N0-ethylcarbodiimide)hydrochloride is added to the solution (0.76 mmol) followed by sonication for 1h. The GO solution is then centrifuged at 22000g for 6h in double phosphate buffer saline (PBS, 0.8% NaCl, 0.02% KCl,) to remove any aggregates	Graphene sheets are suspended in a solution of an amphiphilic polymer C18-PMH-mPEG5000 [comprised of a 9 unit poly(maleamide-alt-1-octadecene) backbone and linear PEG chains . Centrifugation is done at 22000g to remove any aggregates and washed eight times with 100 kDa MWCO Millipore centrifuge filter at 4000g [43]

3.4 Non-covalent Functionalisation of GO

Despite it has been reported that biomolecules and protein non-covalent adsorption on graphene is minimal due to hydrophobic interactions [47], non-covalent functionalisation strategies to attach small peptides and nucleic acids bind to graphene have been used to detect proteins [44], polynucleotides [45], and cells [46]. Non-covalent π−π stacking with graphene or GO is preferable in most cases (preserving the aromatic conjugation of graphene as well as of molecules) because the release of the biomolecules is easier to tailor in comparison with that of covalently bonded molecules functionalising graphene-based materials. Here, we report two examples of procedures of non-covalent interactions with graphene and GO:

Table 4. Examples of procedures for non-covalent interactions with graphene and GO

π-π of Ramizol with graphene	π-π of Hypocrellins with GO
Ramizol is an example of an aromatic organic molecule with antimicrobial and antioxidant activity functionalising graphene by π−π stacking; the three carboxylates present at the ends of the molecule are lft free and give the needed water solubility for graphene nanosheets [47].	Hypocrellins are natural pigments intensively studied as photosensitizersin photodynamic cancer therapy. Hypocrellins solubility in water required for in vivo uses can be increased by π−π interaction with a GO surface as well as hydrogen bonds between the hydrogen and the oxygen groups of the two components [48].
Procedure: Ramizol is dissolved in water at 0.1 mg/ml after the addition of 0.1M NaOH, such that the final pH=10, since Ramizol has limited solubility at pH⩽7. Graphite flakes were added to the solution at a weight to weight (w/w) ratio of graphite to Ramizol=1:1, 1:0.1 and 1:0.01 in a total of 20mL of 0.1mg/ml Ramizol solution. The solution is ultrasonicated for 2h, and then centrifuged at 453*g* for 30 min to remove residual graphite flakes. Excess Ramizol in the processed solution is removed from each reaction mixture by centrifugal washing water at 18,730*g* for 30min	**Procedure:** Hypocrellins are dissolved in 10 mL water, using traces of DMSO as solvent, and mixed with GO aqueous suspension at room temperature for 24h with slow string. Then the solution is ultracentrifuged at 16000rpm for 1h and washed for three times.
 	

The problem is that despite the non-covalent attachment of those biomolecules to graphene, the activity and selectivity of proteins whose function depends on a specific tertiary or quaternary structure have not been demonstrated and observed unambiguously [49].
This loss of function could be ascribed to conformational changes and denaturation of proteins adsorbed to graphene [50]. As an example, anti-E. coli antibody readily adsorbs onto graphene, but loses its specific recognition ability.

Additionally, GO is highly negatively charged and is able to electrostatically adsorb oppositely charged molecules.

The technique of non-covalent layer-by-layer (LbL) assembly involves the alternate deposition of oppositely charged polyelectrolytes on the GO sheets via electrostatic interactions and results in functional multilayers [51]. In addition, hydrophobic or van der Waals interaction may assist the physical adsorption. However, physical adsorption is non-specific.

An example of the non-covalent functionalisation of GO is given by two natural polysaccharides, chitosan (CS) and dextran (Dex) chosen as oppositely charged polyelectrolytes, widely used in drug delivery, due to their compatibility, biodegradability and low immunogenicity [52].

GO nanosheets have negatively charged surfaces suitable for the deposition of positively-charged chitosan and negatively-charged dextran successively, based on electrostatic forces.

The preparation in solution of this kind of non-covalent functionalised GO is very simple, as summarised in the following practice:

Firstly, to prepare chitosan-GO, 100 mg of chitosan is dissolved in 100 mL of 0.6% (v/v) acetic acid at pH=6.5 (with 0.5 M NaOH). Then, 50 mL of GO suspension (1 mg/mL) is added to the solution with stirring for 30 min. Then, 100 mg of dextran is dissolved in 100 mL of water and 50 mL of chitosan-GO suspension is added to the solution with stirring for 30 min. The samples are dispensed in vial bottles and frozen at -80°C for 6 h. Then, the lyophilization goes on in a freeze-dryer for 72 h in the 60 pa condition.

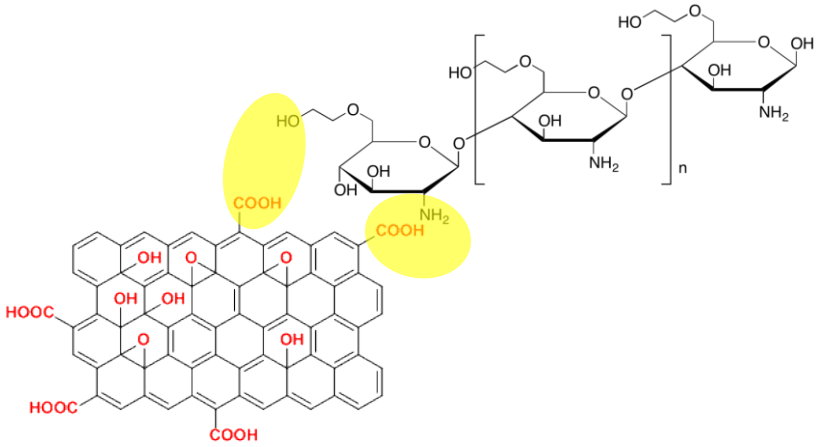


Figure 8: Schematic representation of the interaction between GO and chitosan

Indeed, although the chitosan-GO has been reported as mainly non-covalent, looking at the molecular structures of GO and chitosan shown in Figure 8, it is clear that also covalent interactions between –COOH of GO and –OH or $–NH_2$ groups of chitosan can occur, also at different positions.

This consideration also applies to graphene which usually possess oxidised groups at grain boundaries: this implies that the same graphene can be functionalised non-covalently or covalently through the formation of amide bonds between amine functionalised biomolecules and carboxylic groups at defects as well as can be functionalised non-covalently through π-π as shown in Figure 9 using the example of porphyrins.

Additionally, the role of the interaction between the π systems of the G structure and aromatic molecules in the final hybrid is so decisive that in some cases it can induce important changes in the structure of the molecules and an example is given in the Figure 9 [53].

Therefore, those example clearly emphasises the difficulty of categorising the interactions between graphene-based materials and biomolecules. Additionally, the multiple possible interactions can lead to a different geometry of the chemisorbed or physisorbed biomolecules, leading to a difficult control of the secondary structures of biomolecules and therefore of their bioactivity.

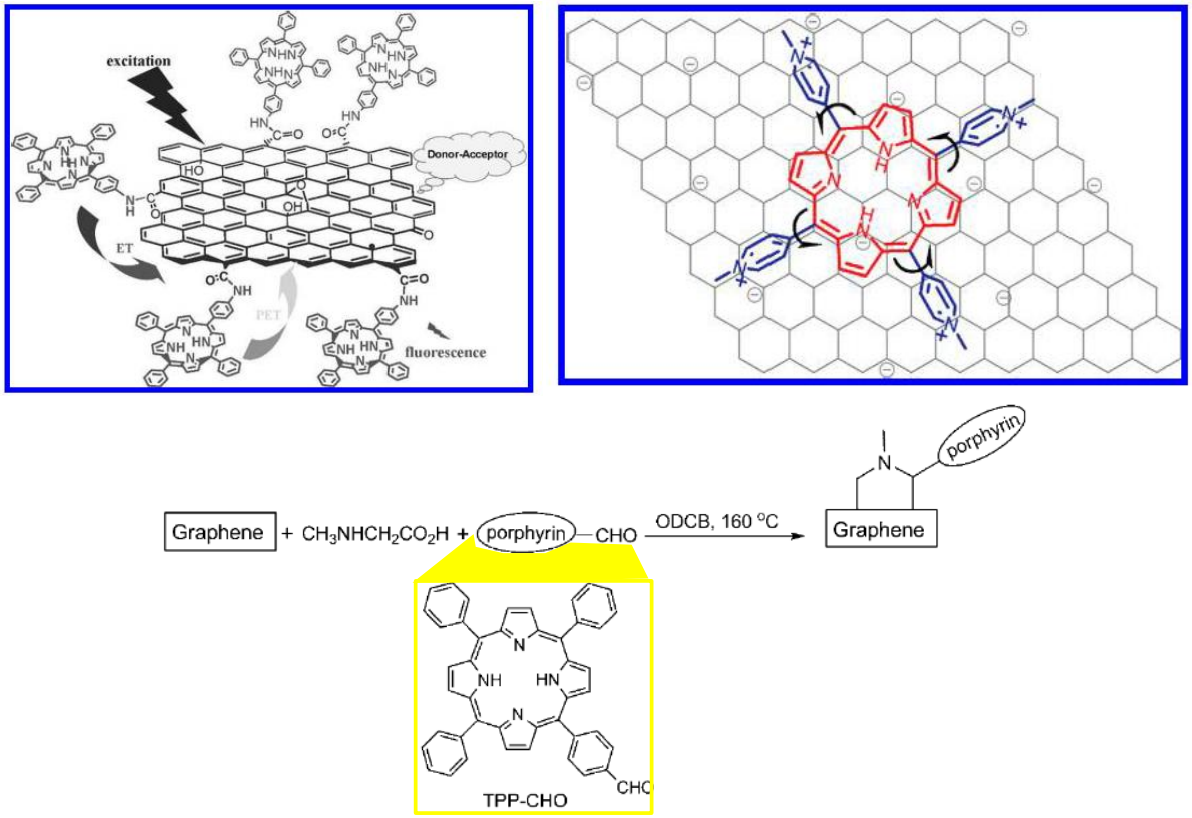


Figure 9: Schematic representation of covalent and non-covalent attachment of porphyrins to graphene and GO.

Since the excellent electrochemical activity and photochemical electron-transfer ability, good optoelectronic properties of graphene-porphyrins hybrid materials that can be used as redox probes for label-free electrochemical detection of aptamers [46]. Examples of procedures for the covalent functionalisation of graphene and GO with porphyrins are given and compared below:

Table 5. Examples of procedures for non-covalent interactions with graphene and GO with porphyrins

Covalent functionalisation of graphene with Porphyrins	Covalent functionalisation of GO with Porphyrins
A porphyrin (TPP) with an aldeide group -CHO is used as precursor for the functionalisation. The graphene in ODCB (50 mL), sarcosine (25 mg), and TPP-CHO are placed in a 100 mL round-bottomed flask and stirred at 160°C under $N_2$for 1 week. After the reaction was complete, the reaction mixture was filtered through a 0.45 mm nylon membrane. The obtained filter cake was subsequently washed several times with ODCB, DMF, and $CHCl_3$ with use of repeated redispersion, sonication, and filtration steps. The final suspension in $CHCl_3$was centrifuged at 5000 rpm for 30 min. The precipitate was dried under vacuum to afford the desired graphene-TPP.	100 mg GO is dispersed in a large excess of thionyl chloride (60 mL) containing a catalytic amount of DMF under sonication for 3 h, and stirred at 60°C for 24 h. After the removal of thionyl chloride under reduced pressure, the solid was washed with anhydrous THF and filtered. TPP (400 mg) and trimethylamine (5 mL) are added to the suspension of activated GO-COCl in anhydrous DMF (50 mL and the reaction is stirred at 80 °C for 72 h, after which the functionalised GO is isolated by washing off the unreacted TPP with excess DMF, filtered through a single layer nylon membrane (0.22 μm) several times. The generated trimethylamine salts is removed by dispersing the TPP-GO hybrid in water. Finally the hybrid was collected by filtration, and dried in vacuum at 50 °C for 24 h to give dark brown product.

3.5 Graphene-based materials functionalisation with DNA

The manipulation and assembly of DNA on graphene-based materials *via* different strategies is a skilful and challenging area that can lead to a variety of potential applications such as biotransistors, catalysts as well as electronic and optical sensitive biosensors and biochips [54].

Several strategies for the immobilization of single-stranded (ss)-DNA on graphene surfaces have been used, which can be grouped as summarised below:

Non-covalent π-π interaction	The interactions electrostatic, hydrophobic, and van der Waals between the DNA and graphene-based materials are very weak in the non-covalent approaches. [55] Controlling the amount of DNA deposited on the surface of graphene, is difficult when using this strategy.
Covalent	Covalent bonding between the carboxylic group of graphene sheets and NH_2-modified ssDNA [56]. Chemical bond with -SH thiolated ssDNA adsorbed onto GO sheets[57]

Table 6. Examples of typical procedures for the direct *non-covalent* functionalisation with DNA

DNA functionalisation of Graphene	DNA functionalisation of GO
Freeze-dried ssDNAwas added to water and sonicated for several seconds. The ssDNA solution was dropped on the graphene and incubated for 3 h and rinsed with water.	GO aqueous solution (1 mL, 0.1 mg/mL) is mixed with DNA by sonicating for 2 h and then incubated at room temperature for 24 h. To remove free unbound DNA, the solution is centrifuged at 16000g, the supernatant is discarded, and the precipitated GO-DNA is redispersed in 0.5 TBE buffer (Tris, 44.5 mM; boric acid, 44.5 mM; EDTA, 1 mM; pH 8.0).

Vice versa, the example in Figure 10 shows the use of linkers, i.e., graphene first is *non-covalent* π-π functionalised with diaminonaphthlene, whose amino groups -NH_2 can be further functionalised with glutaraldehyde to also introduce -COH functionalities, and both –NH_2 as well as –HC=O functionalities can then be used to *covalently* anchor DNA strands [58].

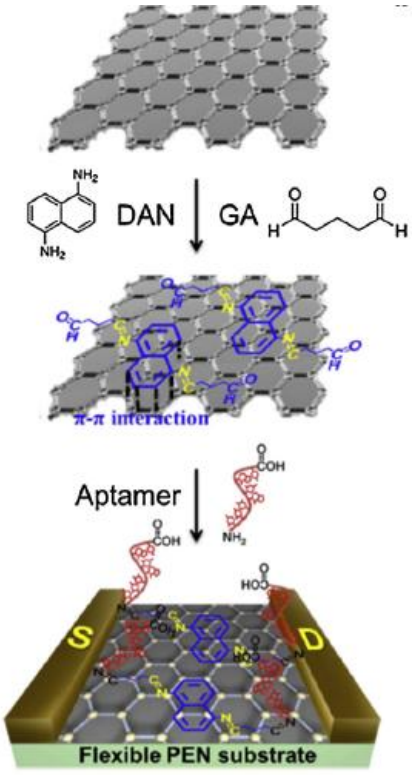


Figure 10: Example of two-step non-covalent and covalent functionalisation of graphene with DNA using linkers.

The *covalent attachment of* amino-labeled DNA to GO occurs via an amide linkage.
The procedure used in this case is:

the conjugation reaction is carried out for 3h at room temperature under magnetic stirring in a glass vial with a final volume of 500µL containing 100µg/mL GO, 2µM DNA, 10mM EDC·HCl (freshly prepared), 25mM NaCl, and 25mM MES (pH 6.0). The GO/DNA complex is purified by centrifugation at 15000rpm for 15min followed by removing the supernatant. To remove non-covalently attached DNA, the sample is washed with isopropanol followed by dispersing the sample in 5 mM pH 9.5. To remove unbound DNA, the sample is soaked in 12M urea and heated at 70 C for 10 min twice. Finally, the sample was washed by water, dispersed in PBS, and stored at 4°C.

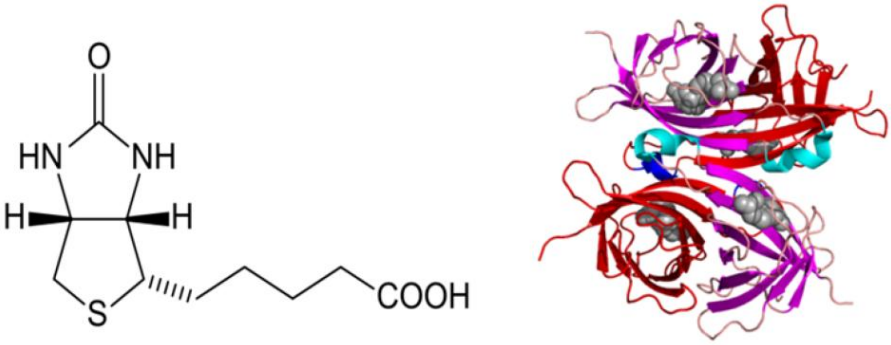
Both during the *covalent* and *non-covalent* binding the DNA can be adsorbed either vertically, with the end base stacking with the graphene-based materials surface, or horizontally, with the several end base pairs open to interact with the functional groups on the surface. This can affect the secondary structure of DNA and its hybridisation.

Therefore, controlling the binding directions still remains the crucial challenge of the functionalisation procedures.

The immobilization of biomolecules via supramolecular or coordinative interactions: Graphene functionalisation with Antibodies: The biotin–streptavidin system.

The most famous example used in the field of biosensor engineering is the biotin/avidin (or streptavidin) system. Biotinylated biomolecules can be attached to biotinylated substrates via avidin (or streptavidin) bridges. The advantage of such systems, compared to the other immobilization methods, is the reversibility, enabling the possibility to regenerate the transducer element.

The formation of avidin (or streptavidin)–biotin complexes is useful in a wide variety of applications. Biotin is a small molecule, as shown in Figure 11, which could bind to avidin or streptavidin binding sites with very high affinity. This specific binding is used to immobilize enzymes antibodies and viruses on graphene and GO. The biotin–streptavidin system, in which four biotin molecules bind to one streptavidin molecule, is one of the most stable and selective non-covalent biological binding couples known and hence it is often employed as a model system to study bio-recognition events.



(a) (b)

Figure 11: (a) Biotin structure and (b) biotin–avidine binding

Here we report an example of how the biotin-streptavidinfunctionalised graphene can be used for the fabrication of highly efficient electrochemical immunosensor detecting carcinoembryonic antigen (CEA), which is a reliable tumor marker for screening and clinical diagnosis of breast tumors, colon tumors, ovarian carcinoma and cervical carcinomas (J.D. Wulfkuhle, L.A. Liotta, E.F. Petricoin Nat. Rev. Cancer, 3 (2003), pp. 267–275]

The procedure used for this biofunctionalisation of graphene is as follows:

2 mg of graphene is dispersed in 2 wt% chitosan solution with sonication and mixed with streptavidin solution (100μg/mL) at 1:1 ratio under stirring for 2h. Then, 5.0μL of the resulting mixture is dropped on graphene and allowed to dry at 4°C overnight. 10μL of 2μg/mL biotin-anti-CEA is dropped onto streptavidin-graphene for 30min at room temperature, followed by washing with PBS buffer solution to remove physically adsorption. Finally, the antibodies functionalised graphene is incubated with 1% BSA solution for 1h to block nonspecific binding sites and washed with PBS for three times. The prepared immunosensor is stored at 4°C.

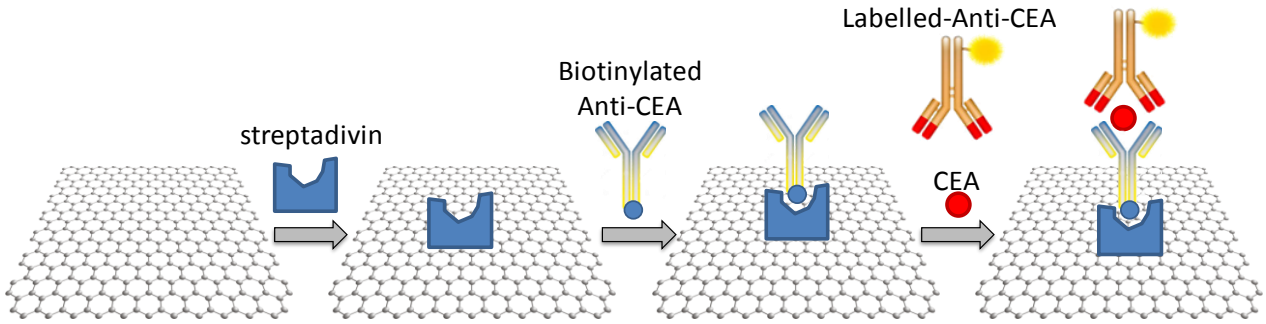


Figure 12: Schematic illustration for the fabrication of CEA immunosensor and immunoassay procedure for CEA

3.6 Graphene-based materials functionalised with gold nanoparticles

Graphene-based materials decorated with gold (Au) nanoparticles have emerged as a category of intriguing materials with promising applications in fuel cells, SERS, catalysis, and electrochemical sensors.

The most popular strategies used for the synthesis of graphene–Au nanocomposites, as shown in Figure 13, is the one-step direct chemical reduction of Au precursor (e.g. $HAuCl_4$) in the presence of graphene, GO or rGO sheets using reducing agent such as amines, $NaBH_4$, and ascorbic acid in a GO THF suspension. Specifically:

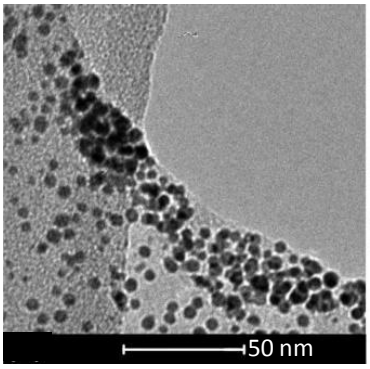


Figure 13: TEM image of GO nanosheets decorated by Au NPs

Table 7. Strategies used to anchor Au NPs on graphene-based materials

Au NPs on Graphene	Au NPs on GO
Firstly, 5 mL of 0.7 mg/mL graphene colloid is synthesized. Then, 500 mL of 1% (wt. %) $HAuCl_4$ is added. The gold nuclei can be formed on the graphene surface from the spontaneous reduction of Au^{3+} in the presence of N_2H_4. Finally, the gold nuclei grew by the reduction of 1 mL of 0.1 M sodium citrate to obtain the graphene–AuNPs hybrid. Then the solution is stirred at 60 C for 2 h. The precipitation formed dispersed in water to obtain an homogeneous solution stable for 1 h.	A 10 mg portion of GO is dispersed in 10 mL of water by sonication for 1 h. Then 20 mL of ethylene glycol and 0.5 mL of 0.01 M HAuCl4 is added to the solution with magnetic stirring for 30 min. Subsequently, the mixture was put in an oil bath and heated at 100 °C for 6 h with magnetic stirring. The GO with Au NPs on them were then separated from the ethylene glycol solution in the centrifuge and washed with deionized water five times. The resulting products is dried in a vacuum oven at 60 °C for 12 h.

Other strategies used to anchor Au NPs on graphene-based materials include:

- **Au NPs attached through a linker to graphene or GO**

Benzyl thiol (BZT) is used as linker for stabilizing the anchoring of colloidal Au NPs on the surface of CVD-graphene. While BZT is linked via π-π stacking to graphene, a strong covalent Au-S bond binds Au NPs to BZT as schematized in Figure 14. Peculiarity of this approach is the direct drop-casting of cetyltrimethylammonium bromide (CTAB) capped Au NPs on BZT functionalised graphene, without performing any additional step of Au NPs capping agent exchange reaction in solution. Specifically:

Graphene functionalisation with BZT is carried out by dipping for 30 min graphene in a 1M solution of BZT in CH_2Cl_2 and subsequent rinsing with CH_2Cl_2. Au NPs are deposited on graphene by drop-casting 0.5mL of a water solution of CTAB stabilized Au NPs diluted to a concentration of 113 μg/mL. After water evaporation, the samples were subjected to three cycles of water rinsing [60].

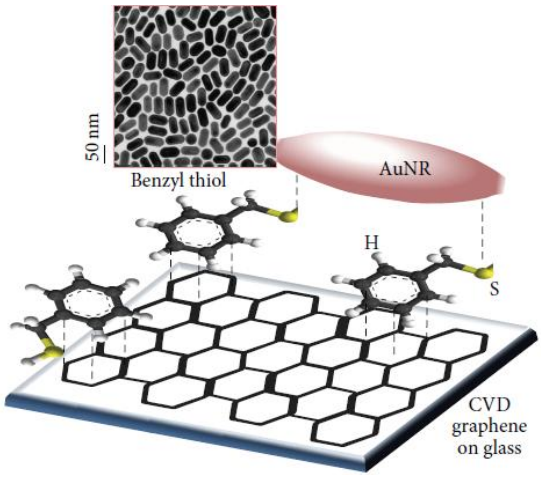


Figure 14: Scheme of BZT linkers anchoring Au NPs on graphene.The π-π interaction between BZT and graphene and the covalent S-Au bonds are indicated by dashed lines. The inset also shows the transmission electron microscopy image of the used Au NPs.

An example of FET biosensor using thermally-reduced graphene oxide sheets decorated with Au NP-antibody conjugates can be found in ref. [61] and schematised in Figure 15.

Anti-Immunoglobulin G (anti-IgG) is anchored to the GO surface through Au NPs and functions as the specific recognition group for the IgG binding. The Au NP-antibody conjugates were deposited onto the surface of GO sheets by electrospray. A blocking buffer, such as BSA, is used to prevent possible nonspecific binding events of IgC. A solution with the target proteins (IgGs) is added. This novel biosensor without any protein engineering showed a detection limit of 2 ng/mL (~ 13 pM), which is among the best of carbon nano-material (e.g., CNT, graphene, GO)-based protein sensors.

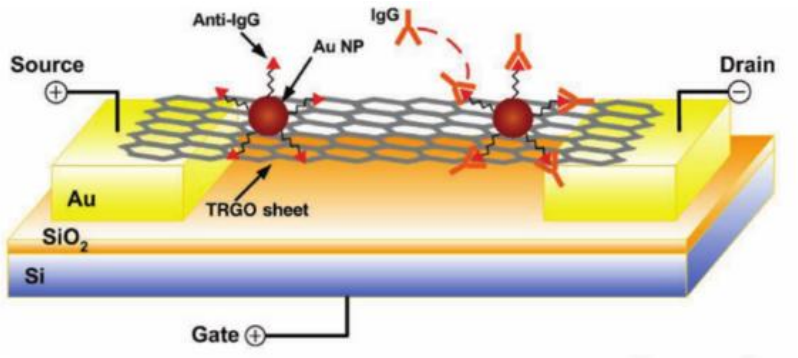


Figure 15: Scheme of a GO FET. Anti-IgG is anchored to the TRGO sheet surface through noncovalent attachment of Au NPs and functions as a specific recognition group for the IgG binding. The electrical detection of protein binding (IgG to anti-IgG) is accomplished by FET and direct current measurements. [Rearranged from source ref. [61]

More details about the biofunctionalisation of Au NPs can be found in paragraph 5.

4 BIOFUNCTIONALISATION of TMDs: MoS_2

A fascinating new class of two-dimensional (2D) layered transition metal dichalcogenides (TMDs), has emerged and gained attention over the past few years [62]. Differently from the zero-bandgap graphene, most of TMDs, *e.g.*, MoS_2, WS_2, $MoSe_2$, are semiconducting with bandgaps, as reported in Figure 16, while others are semimetals (WTe_2, $TiSe_2$) or metals (NbS_2, VSe_2).

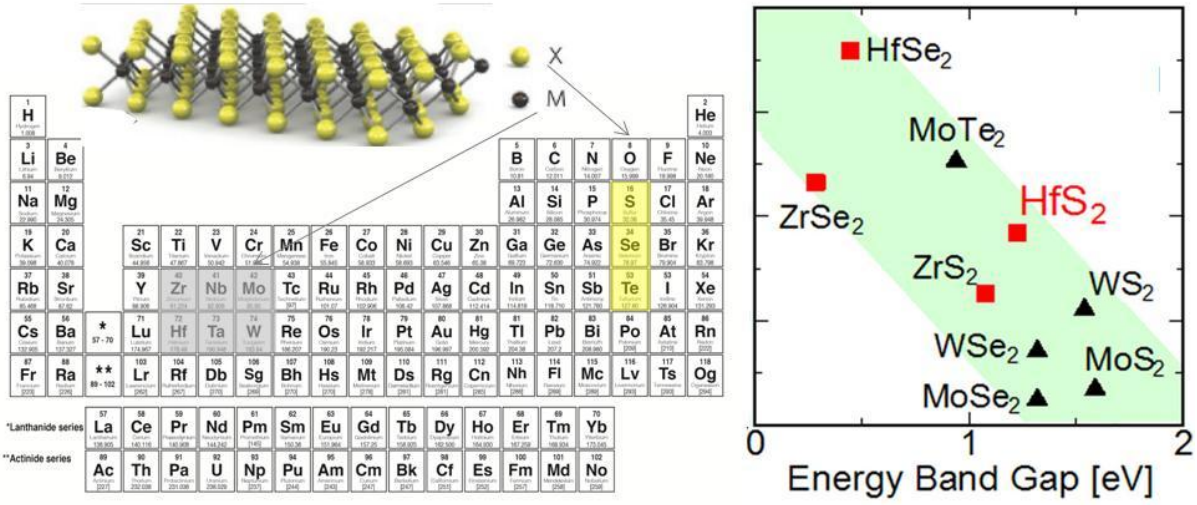


Figure 16: Main TMDs, their bandgap and structure showing that in MoS_2, each Mo is coordinated in a trigonal prismatic geometry to six S atoms.

While graphene field-effect transistor (FET) biosensors allow excellent electrostatic control of the transistor channel by the gate, and high surface-to-volume ratio, their sensitivity is fundamentally restricted by the zero band gap of graphene that results in increased leakage current, leading to reduced sensitivity. The TMDs band gap allows electrical conduction through a material to be turned on and off, which is the key point for building a transistor. Therefore, TMDs have been seen as the valid alternative to fabricate FET biosensors with high On/Off current ratios up to 10^8 [63], which, in combination with their atomically thin structures, can potentially enable higher detection sensitivities for gas, chemical, and biological sensing than graphene FETs. As an example, monolayer or few-layer MoS_2 have a band gap of 1.2-1.8 eV depending on the number of layers, which significantly reduces the leakage current and increases the abruptness of the turn-on behaviour of the FETs, thereby increasing the sensitivity of the biosensor.

Taking MoS_2 as our model TMD, we explore possible biofunctionalisation practices for TMDs, which are still in its infancy but have already provided several important breakthroughs.

The structure of MoS_2 is based on a hexagonal crystal, where Mo atom is six-fold coordinated and hexagonally packed between two trigonally coordinated sulphur atoms. One S-Mo-S quintuple-layer is weakly bonded to another S-Mo-S layer by van der Waals forces. MoS_2 sheet is terminated with sulfur atoms without dangling bonds, leading to favourable chemical stability under a variety of conditions; therefore, MoS_2 is considered to be rather chemically inert along the basal plane that forms the van der Waals gaps in the 2D layered structure.

While MoS_2 sheets with pristine surfaces are highly sought after, in practice, several imperfections may arise due to exfoliation procedures [64]. Similarly, defects characterises CVD MoS_2. Common defects are sulphur vacancies on the surface or edge. The increased reactivity at S-vacancies sites arises from unpaired electrons along the flakes edges and lattice defects in the planar surface of 2D MoS_2 [65]. These defects have been identified as potential synthetic targets for surface modification and functionalisation.

The past 2–3 years has seen multiple routes to functionalise 2D TMDs. An array of molecules react with or physisorb on MoS_2, leading to functionalisation of the material, although these methods require further exploration.

Similarly to graphene, the various TMDs biofunctionalisation practices can be grouped in:

Table 8. Non-covalent and covalent TMDs biofunctionalisation

Non-covalent	Covalent
• Such functionalisation generally involves physisorption of the desired probe onto the basal surface of the TMD, allowing maximal loading as well as interaction with the surrounding environment. • It is carried out in TMDCs when keeping the intrinsic properties of the 2D TMDC is essential	• Covalent functionalisation involves formation of chemical bonds on the TMD surface. • This can take place through specific reaction processes such as thiol (–SH) chemistry for the case of sulfur based TMDs • Such covalent bonding alters the physical/electronic properties of the TMDs.

Indeed, it has to be pointed out that the covalent functionalisation chemistry of Chhowalla [66] and Backes [67] represents the only well-characterized examples of covalent tethering of organic functionalities to TMDs. No other reports have explicitly demonstrated and showed conclusive experimental evidence to support non-covalent or covalent functionalisation on the basal planes of MoS_2 monolayers as well as not provided sufficient proof to show the functionalisation leads to bond formation. Therefore, there understanding and control of MoS_2 surface biofunctionalisation is still in its infancy. Nevertheless, we group the procedures as originally claimed by authors in the description below.

4.1 Non-Covalent Functionalisation of MoS_2

There are very few examples of non-covalent functionalisation of TMDs [66, 68-73]. One of those few examples is the basal functionalisation of exfoliated MoS_2 with phthalocyanines [73], as schematised in Figure 17, according to the following practice:

A 10 mL aliquot of the exfoliated MoS_2 is added to 10 mL of a phthalocyanine solution (0.1 g of 29H,31H-phthalocyanine in 20 mL of chloroform) and stirred overnight at room temperature. To remove the excess phthalocyanine, the mixed solution is centrifuged for 90 min at 19 000 rcf, the nanosheets are collected in the sediment, re-suspended in toluene and washed 3 times with toluene.

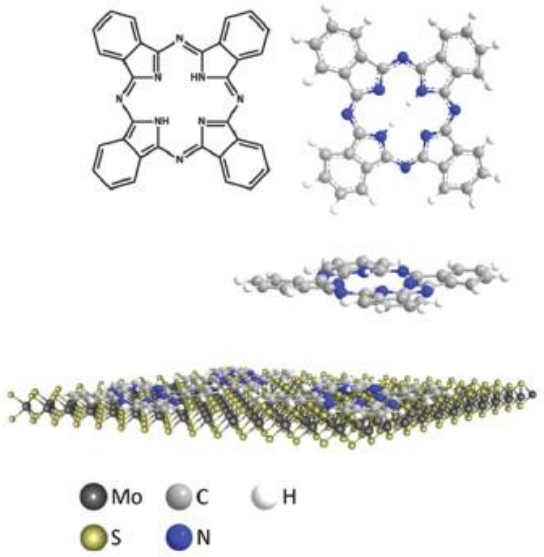


Figure 17: scheme of the non-covalent interaction of a phthalocyanine with MoS2 basal plane.

4.2 Covalent functionalisation of MoS_2

In all the covalent functionalisation procedures, a new kind of S-bond, i.e, S-C is detected.

The first example, by Chhowalla and co-workers [66] described the covalent functionalisation of MoS_2 nanosheets by reacting them with organoiodides (2-iodo-acetamide and iodomethane) or diazonium salts (also reported by Backes et al. [67]), who has recently discovered that aryldiazonium salts can also result in basal-plane functionalisation of MoS_2.

The covalent functionalisation types are summarised in Figure 18.

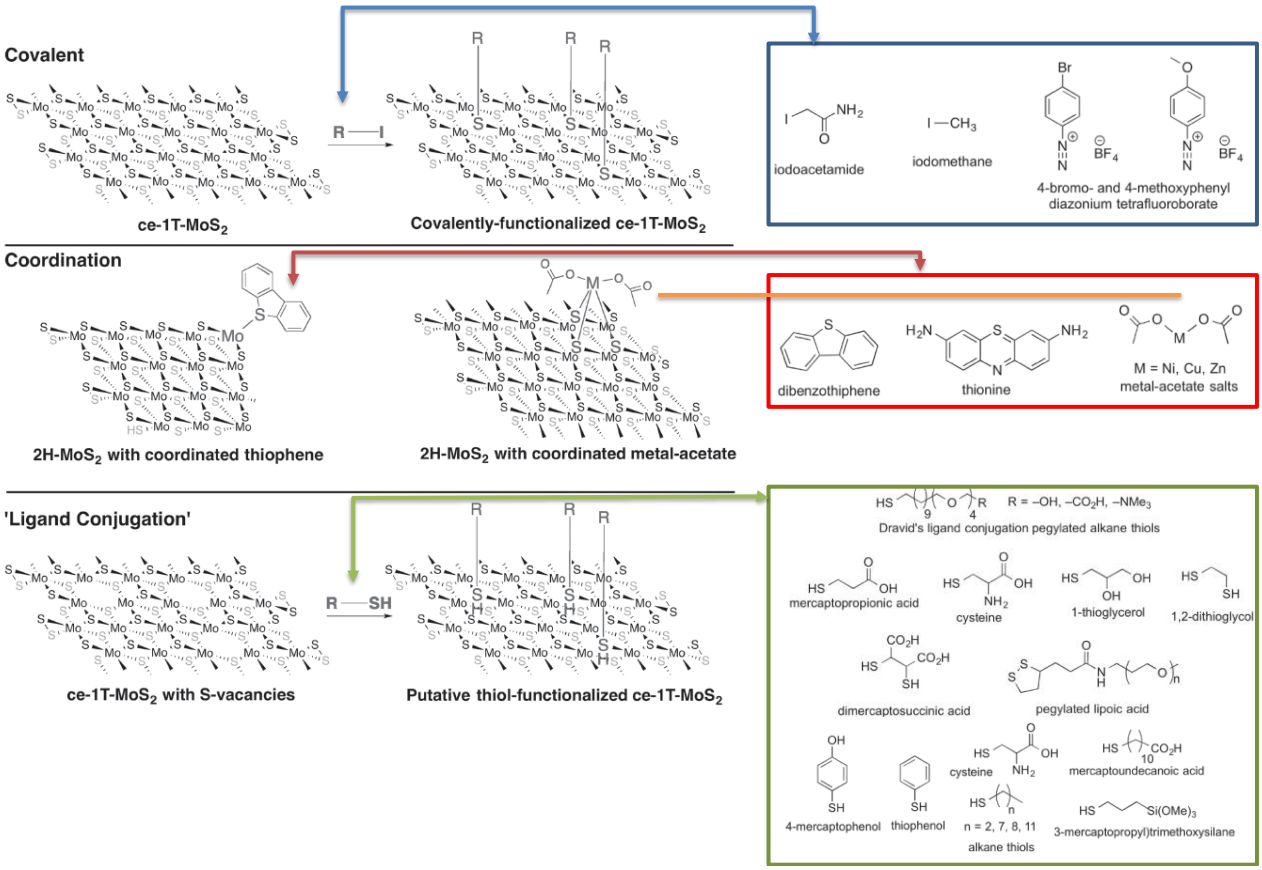


Figure 18: Scheme of the main covalent functionalisation types of MoS_2 [Rearranged from ref. [74]

Mostly, the "plug and play" strategy [75] for MoS_2 flakes and nanosheets in solution is used, which takes advantage of the presence of S-atom defect sites on MoS_2, through the binding of sulphur containing ligands with the unsaturated molybdenum sites present at the edges or basal planes, as schematised in Figure 19. This functionalisation technique has been seen as a bond-forming and, hence, covalent functionalisation method, i.e., a Mo–S bond between an S-atom in the organic functional group and a Mo-atom in the MoS_2 has been formed.

There are now many examples of organic thiols reacting with MoS_2 (see Figure 19), yielding functionalised MoS_2. This functionalisation technique has been described as a bond-forming or covalent functionalisation method, i.e., a Mo–S bond between an S-atom in the organic functional group and a Mo-atom in the MoS_2 has been formed. Indeed, MoS_2 was found to be facilitating the oxidation of the thiols during functionalisation resulting in physisorbption on MoS_2 rather than any bond-forming process, suggesting that functionalisation of 2D MoS_2 using organic thiols may not yield covalently or datively bonded functionalities. Critically, it has also to be considered that MoS_2, having free sulphur atoms, also provides an interesting opportunity to covalently attach reduced biomolecules with the thiol termination –SH through disulfide bond formation, i.e., a *molecule*–**S-S**-*MoS_2* covalent bond on the surface.

The functionalisation with different thiol-containing bifunctional linkers also provides opportunities for introducing different functional groups and biomolecules onto the basal plane of MoS_2 MLs and enabling a wider range of applications.

The X-SH functionalisation via S-vacancies at edges and basal plane uses a very simple, although not well controlled or certified procedure:

The mechanically exfoliated MoS_2 or CVD-grown MoS_2 nanosheet samples supported on Si/SiO_2 substrate are soaked in any –SH containing thiol or biomolecule solution neat 4-fluorobenzyl mercaptan liquid (or neat 1,5-pentanedithol liquid or 5- (trifluoromethyl) pyridine-2-thiol aqueous suspension) for 48 h. Afterward, the substrate is soaked in ethanol, acetone, isopropanol, and water for 10 min each and rinsed harshly with these solvents to wash off physisorbed molecules.

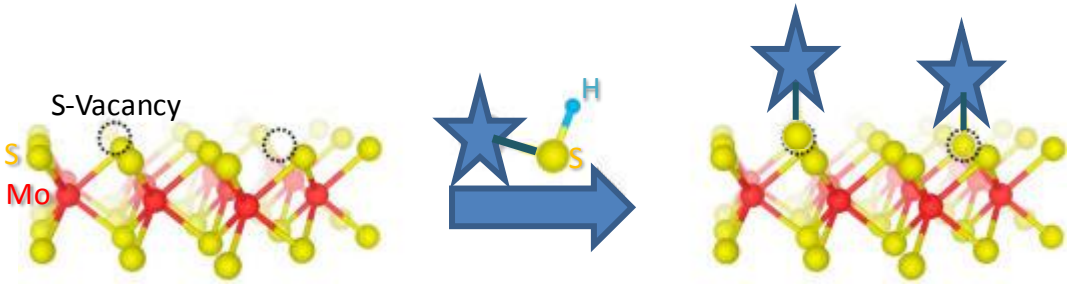
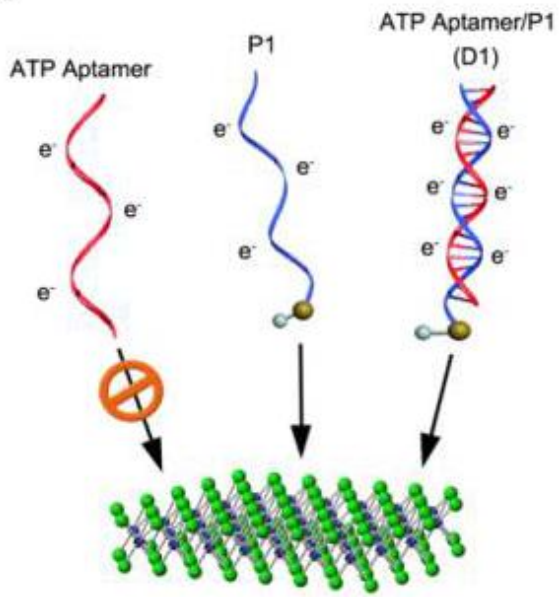


Figure 19: Scheme of the S-vacancies at the MoS2 surface that can bind covalently conjugated thiols (-SH) where the star represents any biomolecule

Examples of the use of S-defect on the MoS_2 surface is the covalent functionalisation using S-atom-terminated DNA (see Figure 20) as the anchor or the PEGylation.

MoS_2 as well as most of 2D TMDs do not form well-defined stable suspension in many common polar solvents such as water, thus making it difficult for in vivo applications. To improve the stability of TMDs in solution, especially for use in biological systems, additional functionalisation is required.



Demonstration of these capabilities has been performed by Liu et al. [76] where MoS_2 nanosheets are functionalised with polyethylene glycol (PEG) and its derivative Lipoic-acid-modified-PEG (LA-PEG), as shown in Figure 21. The PEGylation served dual functions, i.e. enhancing nanosheet stability and biocompatibility while at the same time attaching folic acid for targeted entry into cancer cells. However, Figure 21 also puts in evidence that PEG can anchor on various S-sites creating a disordered layer, which can bind subsequently proteins, antibodies etc. using the additional –COOH functional group, but with a non-defined geometry and order which can be responsible of denaturation and loss of bioactivity.

Figure 20: Covalent attachment of DNA-SH to MoS_2

Similar procedures are used for the functionalisation of MoS_2 nanosheet with thiol-labelled DNA [77].

Those procedures are summarised in Table 9 [78].

Using this thiol HS- strategy, several organic molecules such as dyes as well as bioreceptors [79], drugs such as rhodamine B isothiocyanate [80], thionin [81] guanine, and adenine [82] aminoacids.

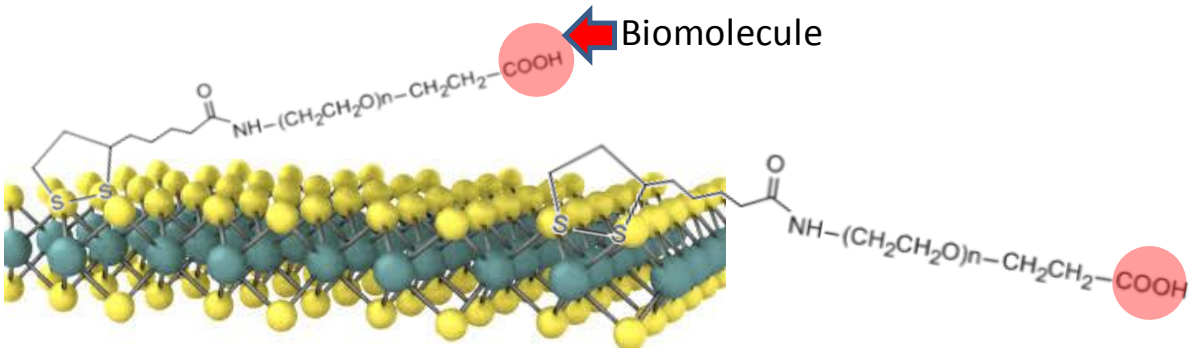


Figure 21: Scheme of the LA-PEGylation of MoS_2 using the S vacancies on the MoS_2 surface; the scheme intend to show that the LA-PEG can anchor MoS_2 at different S-sites

Table 9. Best-practices for covalent functionalisation of Mos_2 using –SH groups

Formation of MoS_2 Nanosheets	
200 mL aqueous solution with 5 mg/mL MoS_2 powders and 1.5 mg/mL sodium cholate is sonicated for 20h. Subsequently, the dispersion is centrifuged at 3000 rpm for 30min and a yellow-green supernatant containing sodium cholate and exfoliated MoS_2 is collected. The sodium cholate is then removed by centrifugating (10000 rpm, 30 min) the yellow-green mixture and MoS_2 is collected. The MoS_2 is dissolved in water via sonication and centrifuged (10000 rpm, 30 min), followed by the collection of sediments. The washing process was then repeated two times to thoroughly remove sodium cholate absorbed on MoS_2	
Thiol-PEGylation (PEG-SH)	**Thiol-DNA (DNA-SH)Functionalisation**
For the PEG functionalisation, 10 mg of LA-PEG-SH is added into 1mg of MoS_2 nanosheets dispersed in 2 mL of water. After sonication for 20 min and stirring overnight, excess PEG is removed by centrifugal filtration with 100 kDa MWCO filters (Millipore) and water washing. The obtained MoS_2-PEG is water-soluble and stored at 4°C C. For chemotherapy drug doxorubicin (DOX) loading, PEGylated MoS_2 (0.2 mg/ml) is mixed with DOX in phosphate buffer (PBS, 20 mM) at pH = 8.0. After stirring at room temperature for 24 h, unbounded DOX is washed away by filtering the solution through a 100 kDa Millipore filter and rinsing with water	1 µM thiol-labelled DNA (DNA-SH) solution is added into TAE buffer solution containing 200 µg/mL MoS_2 and sonicated for 10s and then incubated at room temperature (25 ℃) for 5 h, resulting with the attachment of thiol groups on MoS_2. The resultant is centrifugated at 10000 rpm for 30 min to remove the unattached DNA oligonucleotides. Finally, the sediments of DNA-SH functionalised MoS2 is collected and then re-dispersed into ultrapure water.

Very recently a covalent functionalisation of TMDs (MoS_2, $MoSe_2$, WS_2, and WSe_2) with thiobarbituric acid conjugates (see Figure 22) can serve as nanosheet platforms for further functionalisation in a multitude of applications using the additional function group of –OH, C=O present in the molecule [83].

This conjugated can be prepared according to the following procedure:

Chemically exfoliated MoS_2 (25 mg, 0.15 mmol) and thiobarbituric acid (30 mg, 0.20 mmol) are added to a 4 mL reaction vial with a PTFE-lined cap, suspended in 1 mL of H_2O, sonicated for 5 min at 37 kHz, and heated to 75 °C for 48 h with vigorous stirring. The grey mixture is then diluted with 3 mL of H_2O, concentrated by centrifugation, and washed 3 times with H_2O, 1 time with a 50/50 mixture of acetone: water, and 3 times with acetone. After each removal of the supernatant, the pellet resuspension and washing is aided by a 5 min sonication. Finally, the product is dried under vacuum overnight to yield 15 mg of dark grey powder.

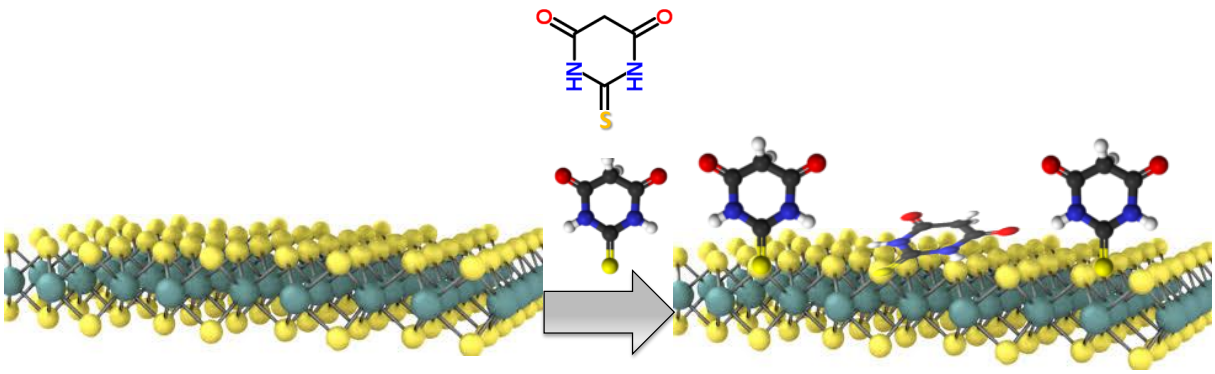


Figure 22: Scheme of the –SH covalent and non-covalent functionalisation of MoS_2 with thiobarbituric acid

Figure 23: Scheme of the antigene-antibody functionalisation of MoS_2

Both covalent and non-covalent biofunctionalisation of MoS_2 with antigen-antibody has also been reported in a classic label-free immunoassay format. And also in this case, the best-procedure for functionalisation has not yet been established and both configurations, namely antigene immobilized on MoS_2 reacting with the antibody, as shown in Figure 23 and the vice versa are still being investigated.

The sensing of prostate-specific antigen (PSA) has been demonstrated by antibody IgG, which is the most abundant antibody isotype in human on MoS_2, physisorbed non-specifically on MoS_2.

Table 8. Examples of procedures for covalent and non-covalent biofunctionalisation of MoS_2

MoS_2-physically adsorbed antibody Adsorption of Human IgG onto MoS_2	**MoS_2-covalently reduced antibody** Covalently disulfide attach reduced antibodies
The MoS_2 surface is incubated with human IgG (10 pg/mL ~ 10 µg/mL) (in phosphate buffer saline (PBS), pH 7.2) for 15 minutes, followed by rinsing with PBS for 1 minute and drying under N_2. Anti-PSA (100 µg/mL in PBS, pH 7.2) is immobilized on the MoS_2 surface for 1 hour. In order to avoid evaporation, the dispensing process was performed inside a humidity chamber. The chip was rinsed with PBS buffer for 1 minute [84].	The antibodies is reduced to expose the hinge-region disulfide –S-S- bonds through biointerfacing the sulfhydryl groups, -SH, in the antibody with the S atms of the MoS_2 surface. Polyclonal anti-PSA antibodies are vertically cleaved using 2-mercaptoethylamine-HCl (2-MEA). This reagent acts as a mild reducing agent and selectively cleaves the hinge-region disulfide bonds of IgG heavy chains. Briefly, 1 mg/mL antibody solution is mixed with PBS-EDTA (pH 6.0) reaction buffer. The buffer is prepared by mixing 30 mM sodium phosphate, 150 mM sodium chloride, and 5 mM EDTA. 2-MEA (12 mg) is added to the buffered antibody solution while the reaction mixture was kept under mild stirring conditions at 37 °C for 90 min. The antibody solution left in the column after the desalting process was separated and equilibrated with PBS-EDTA buffer. The resulting solution is centrifuged at 10 000 rpm (15 min) to remove any traces of the native antibody. The above-cleaved antibodies is stored in PBS-EDTA buffer. The media was changed from PBS-EDTA to PBS with the use of a desalting column. The above step was required to avoid any interference from EDTA during disulfide bond formation between the cleaved antibody and the MoS_2 surface [85].
Comparison of the developed immunosensor	
Linear range (ng/mL) 0.001-10	10^{-5}-75 ng/ml
Detection limit (ng/mL) 0.001	10^{-5} ng/ml

It is claimed by Kubbar et al. [85] that, unlike a simple physical adsorption approach, the covalent immobilization of anti-PSA antibodies via a covalent approach has achieved a much wider and clinically significant range of PSA detection as indicated in the table above.

Here, we mention that we have reported the above comparison only to emphasizes the concept that the functionalisation practice strongly affects the performance of the same immunosensor; it is not intended to assert that one procedure is better than the other, since no certification and validation of those reported protocols exists.

4.3 2D-TMDs functionalised by Au nanoparticles

Differently from graphene/Au-NPs whose interaction is based on chemisorption/physisorption, or anchoring through linkers, MoS_2 can also bind covalently Au-NPs forming a -S-Au- bond since the strong affinity of Au for sulphur.

The TMDs-Au metallic nanoparticles are generally synthesised by mixing $HAuCl_4$ with the TMD in solution followed by chemical reduction of these compounds to form metallic nanoparticle-TMD composites [86].

We report some of those procedures in the Table 10 below.

Table 10. Examples of procedures for 2D-TMDs functionalisation by Au nanoparticles

Covalent Au NPs decoration of MoS_2	**Microwave-assisted hydrothermal formation of AuNPs-MoS_2**	**Covalent bond formation between Au and sulphur (S)**	**Au NPs decoration on CVD and exfoliated MoS_2**
MoS_2 powder is dispersed in a 45 vol % ethanol/water mixture (20 mL) and sonicated for 12h. In a separate vial, gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H2O$) is dissolved in water. To decorate MoS_2 with Au nanoparticles, a small aliquot of the $HAuCl_4$ solution is added to an aqueous dispersion of chemically exfoliated MoS_2 at room temperature, which is then stirred for 30 min. A reaction occurred immediately, completing within a few minutes.	AuNPs-decorated MoS_2 is synthesized by microwave-assisted hydrothermal method., 5 mL (0.08 mg/mL) of MoS_2 nanosheets aqueous dispersion is added into a 10 mL quartz tube equipped with a magnetic bar. Under vigorous stirring, different aliquots of 100 mM $HAuCl_4$-$3H_2O$ are mixed with MoS_2 nanosheets solution. Then, the reaction mixture is heated to 60 °C for 5 min in the microwave reactor. Finally, the product of AuNPs-MoS_2 is purified by centrifugation [87].	Aqueously suspended MoS_2 nanosheets are decorated with AuNP by addition of aqueous gold chloride (AuCl3). AuNP-decorated nanosheets can then be drop-casted onto silicon dioxide, SiO_2, substrate or any other support [88]	$AuCl_3$ dissolved in water is used for the growth of Au NPs on MoS_2. To prepare Au NPs/CVD-MoS_2, the as prepared CVD-MoS_2 on sapphire substrates are directly immersed in 5 mM $AuCl_3$ for 20 sec, followed by rinsing with water. To prepare Au NPs/exfoliated MoS_2 samples, the MoS_2 is delaminated and several drops of 5 mM $AuCl_3$ water solution are added into the water. Finally, the Au NPs/CE-MoS_2 floating on water are collected on a support [89].

An example of the MoS_2/Au-NPs nanocomposites is shown in Figure 24, both in solution and dried.

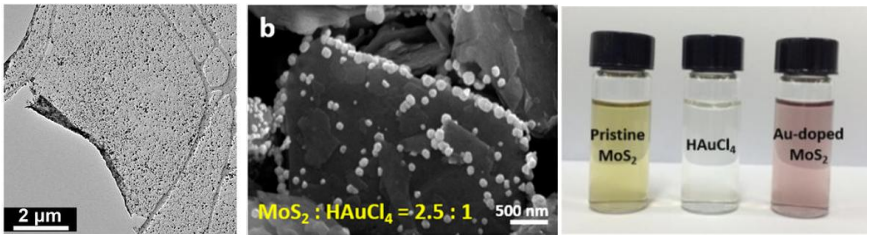


Figure 24: Low magnification TEM images of Au NPs/CE-MoS_2 sheets and photos of solutions of MoS_2 and HAuCl4 precursors as well as of the nanocomposites AuNPs-MoS2. Rearranged from refs. 89 and 90.

Recently, hemoglobin was immobilized on a gold-nanoparticle decorated MoS_2 nanosheet to serve as a hydrogen peroxide and nitric oxide sensor [86]. Wang et al.[91] utilized the catalytical activity of MoS_2-Au nanocomposite to construct a sandwich-type immunosensor for carcinoembryonic antigen (CEA) detection, able to detect as low as 0.27 pg/mL CEA.

AuNPs-MoS_2 has also been used as an electrochemical sensing platform to detect chemical/biological molecules, including neurotransmitters, ATP, thrombin and protein [92].]

5 BIOFUNCTIONALISATION OF PLASMONIC GOLD NANOPARTICLES

Within the group of noble metal nanoparticles, gold nanoparticles (Au NPs) provide a versatile platform for chemical, biological sensors and immunosensors due to their biocompatibility, their optical and electronic properties, and their relatively simple production, modification and functionalisation. Functionalised AuNPs may act as both molecular receptor and signal transducer in a single sensing motif, thereby simplifying the sensor design as improving the sensitivity. Therefore, there are numerous AuNP-based sensor systems fashioned to date of both fundamental and clinical importance, which include colorimetric sensing, fluorescence-based sensing, electrical and electrochemical sensing [93]. Furthermore, particular interesting is the optical behaviour of AuNPs where irradiation with light of one specific wavelength causes an oscillation of the electrons in the conduction band, called resonant surface plasmons [94]. This environmental dependency represents a great advantage for (bio)-analytics since the recognition event can result in a change of the oscillation frequency and therefore to a colour change of the gold nanoparticles observable with bare eye. In this context, a wide series of efficient colorimetric biosensors were developed for DNA or oligonucleotide detection, or immunosensors. Since Au NPs manifest the phenomenon of localized surface plasmon resonance (LSPR) [94] they are largely used in surface plasmon resonance sensors and surface enhanced Raman scattering (SERS)-based sensing. Gold nanoparticles have also demonstrated their advantages in bioanalysis using SPR transduction.

LSPR and SERS sensing are label-free. The advantage of a label-free sensor is that the measured signal is due only to the presence of the target molecule. Because LSPR directly detects the target's refractive index, it fits into this category, as schematised in Figure 25. This is in contrast to the vast majority of biological sensors, which rely on labels to generate and amplify the target signal. Most assays, such as enzyme-linked immunosorbant assay ELISA, detect the target antigen after specialized exposure and rinsing protocols, which execute some molecular strategy to generate a signal. In fact, the target antigen is not directly detected in an end point assay, but rather it captures a second antibody, which generates the signal. Furthermore, labels can affect interactions.

Label-free assays are inherently simpler than end-point assays because they require only a single capture antibody and provide direct detection with less extensive protocols. The use of nanoparticle LSPR for label-free assays localizes the sensing area, thus reducing the total amount of analyte needed to generate a signal.

There have been many reports demonstrating biological assays by LSPR sensing and characterizing their performance.

Here, we discuss some procedures used to biofunctionalise Au NPs.

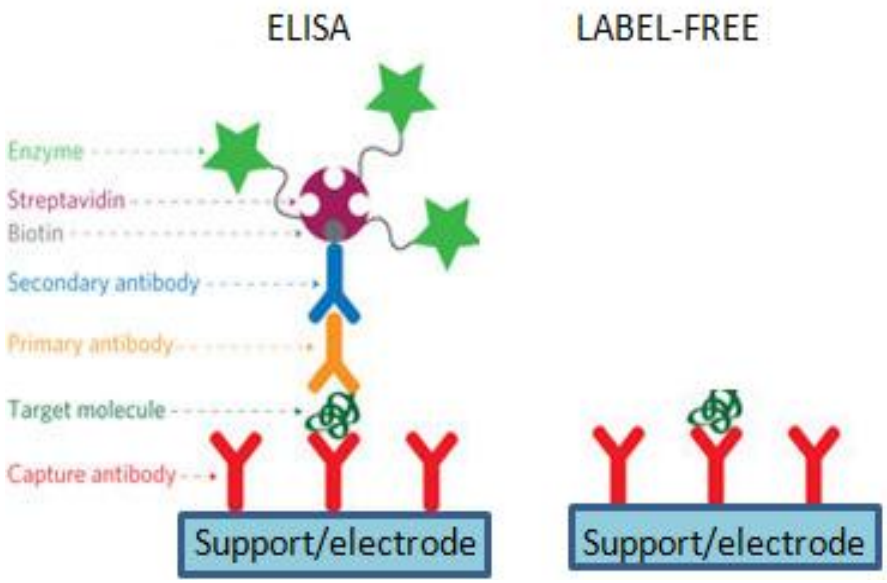


Figure 25: Schematics of a traditional immunoassay (ELISA) and of a label-free immunoassay

In general, the roles of AuNPs in biosensing can be categorized into four categories (see Table 11).

Table 11. Four roles AuNPs in biosensing

1	**The first role is immobilisation of biomolecules**
2	The second and most significant role is to enhance electron transfer. The conductivity of nanoparticles enhances electron transfer between the active centres of biomolecules and electrodes so that the particles act as electron transfer conduits or mediators.
3	The third role is acting as a catalyst. Since nanoparticles are chemically more active due to their high surface energy they can contribute to developing new electrochemical analysis systems; as an example, in electrochemical sensors; nanoparticles tend to decrease over potentials and enable the reversibility of some redox reactions. Their catalytic properties have proven to increase sensitivity.
4	The fourth role is to enhance the read-out capabilities providing new capability such as the optical surface plasmon resonance (SPR) read-out mode. Surface plasmon resonance (SPR) forms the core of label-free optical transduction technology, offering far superior sensitivity owing to the strong electric field probing the target molecules under conditions of resonant excitation of plasmons. Optical SPR transduction methods avoid expensive, time-consuming and precision-interfering labelling steps to mark analytes. Instead, they register the attachment of a ligand to its receptor via refractive index monitoring, which enables a real-time control of binding/recognition events.

Ultimately, the biofunctionalisation strategy depends on a numbers of factors such as:

- size and shape of the NP as well as of the biomolecule,
- surface chemistry,
- type of ligands and functional groups,
- type of biological molecule,
- type of transdution mechanism,
- final application.

A large variety of small organic molecules, carbohydrides, thioles, polymers, proteins, aminoacids, DNA, iRNA, antibodies, aptamers and anticancer drug molecules have been attached to Au NPs, as sketched in Figure 26.

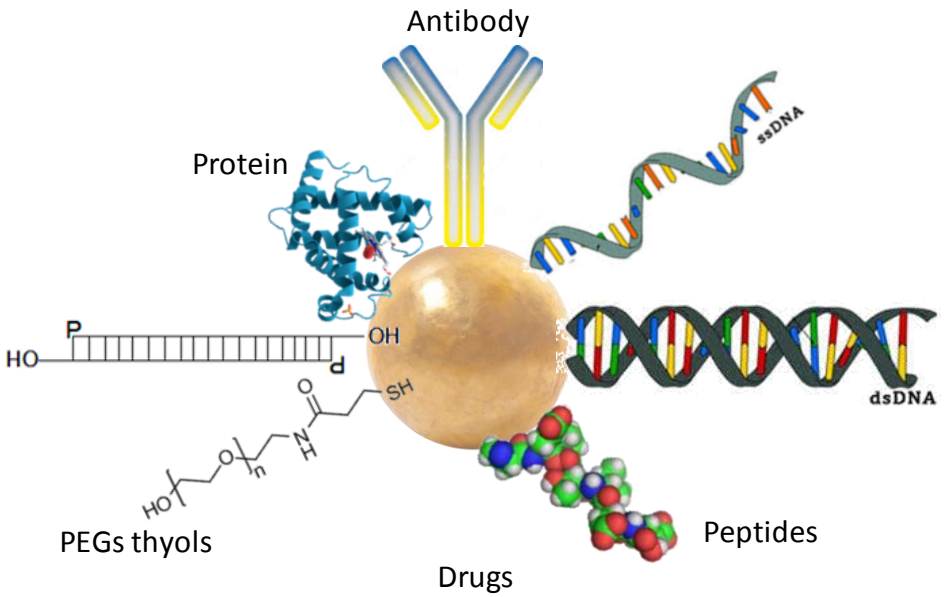


Figure 26: Sketch of a gold NP with the various molecules that have been immobilised on it

Colloidal AuNPs are the most studied and available NPs for biosensors. The most commonly used AuNPs for interfacing with materials are synthesised in solution by reducing $HAuCl_4$ in water using sodium citrate, a method that traces back to 1951 [95]:

$2\ HAuCl_4 + 3Na_3C_6H_5O_6$ (sodium citrate) $+ 1.5\ H_2O \rightarrow 2\ Au^0 + Na_2C_5H_6O_5$ (sodium ketoglutarate)

In the Turkevich method, the citrate acts as both a reducing agent and a capping agent, citrate is often added in excess and negatively charged citrate is weakly adsorbed by AuNPs as shown in Figure 27. This leads to an electrostatically stabilised suspension since the equivalent charges on the GNPs tend to repel each other. The citrate capping is loosely bound on the GNP, enabling it to be easily replaced with other functional groups.

A breakthrough in the field of AuNP synthesis was achieved by Brust and Schiffrin in 1994 [96], when a two-phase synthetic strategy, utilising strong thiol-gold interactions to protect AuNPs with thiol ligands was achieved. In this variant, $HAuCl_4$ is transferred from aqueous phase to toluene using the surfactant tetraoctylammonium bromide (TOAB) and reduced by sodium borohydride ($NaBH_4$) in presence of dodecanethiol. These thiol-protected AuNPs feature superior stability because of the strong thiol-gold interaction and they can be easily handled, characterized, and further functionalised.

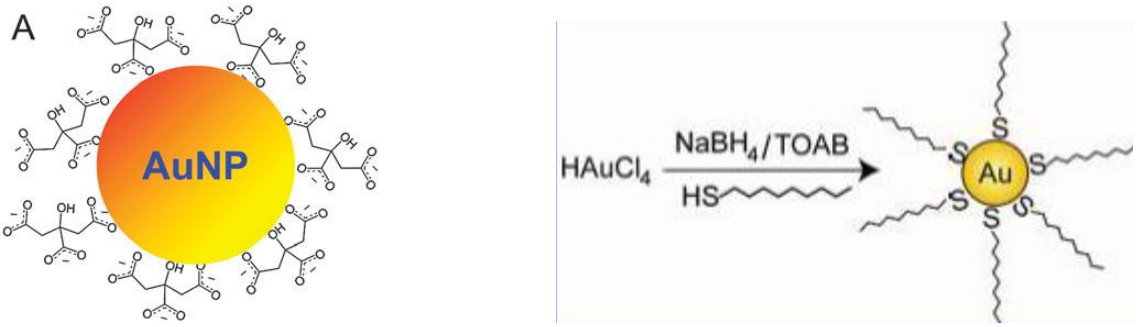


Figure 27: AuNPs functionalised with citrate and negatively charged (left) and with thiol through the formation of a covalent Au-S bond (right).

It is worthy to mention that alternative strategies of producing clean AuNPs directly on a support/substrate without any stabilising shell are being investigated and developed. Those methods rebased gold evaporation or plasma sputtering of a gold target [97]

The strategies for NPs surface functionalisation should satisfy the following criteria:

i) enabling NPs with good water solubility,
ii) providing a reduction of toxicity of NPs in some instances,
iii) providing additional functionalities for further conjugation of biomolecules for targeting applications,
iv) stabilising the physical properties of NPs.

A masterpiece of biofunctionalisation is the work by Alivisatos who reported oligonucleotide-AuNP conjugates through gold-thiol bond formation [98].

A variety of strategies for surface functionalisation of AuNPs fulfilling the above criteria have been developed, which can be grouped in the following main categories, which are sketched in Figure 28.

Table 12. The main categories of strategies for surface functionalisation of AuNPs

Direct absorption	the interaction is usually weak and nonspecific	Based on electrostatic or van der Waals interactions affected by pH and media.
Covalent coupling	specific and stable conjugation of biomolecules with NPs	Functional groups on the NPs surface including carboxylic group, amide group and thiol group can cross-link with biomolecules.
Biological approaches	highly specific interactions of biomolecules	Antibody-antigen interaction, receptor and target interaction, enzyme and substrate interaction, and complementary base pair of nucleic acid.

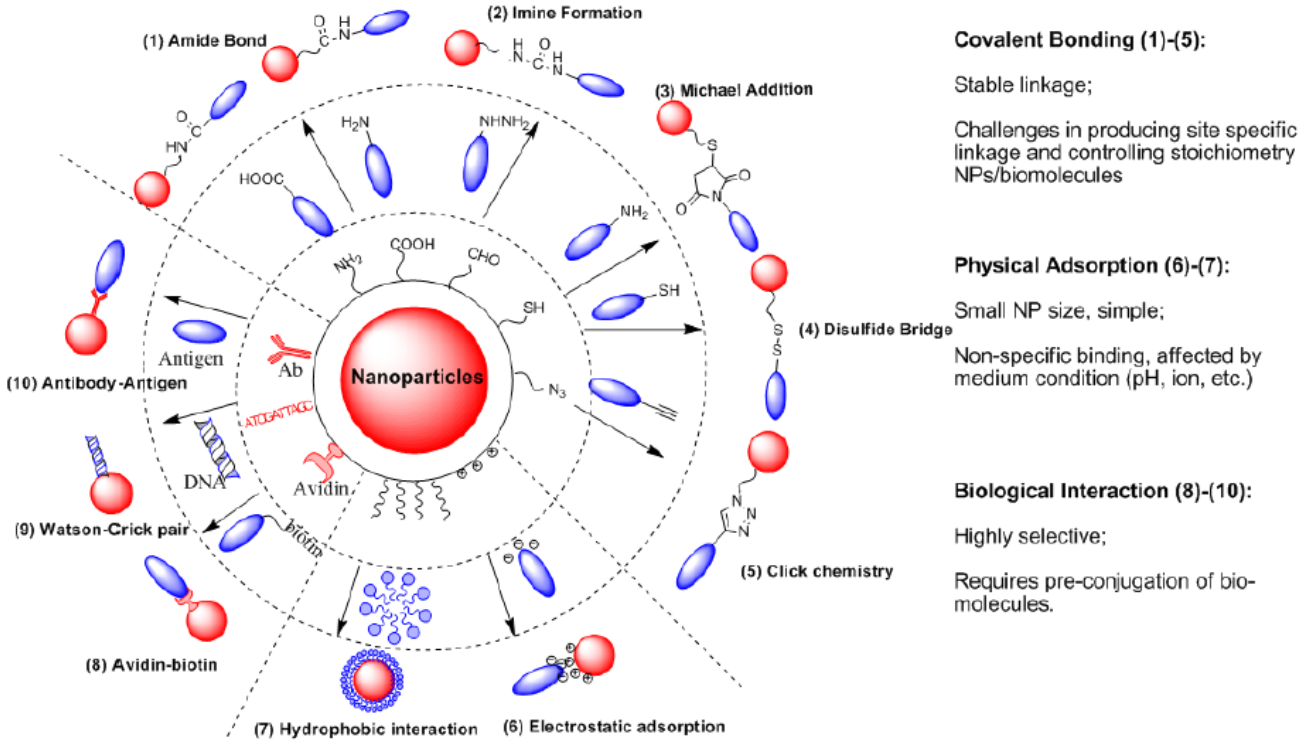


Figure 28: Representative bioconjugation protocols.

Examples of physical adsorption (6-7 in Figure 28) include:

- ***Citrate capped AuNPs***

AuNPs can be functionalised electrostatically, for example a positively charged ligand would be attracted to the negatively charged citrate capping on a Turkevich synthesised AuNPs. This type of attachment in which the ligand mixes with the citrate layer is also referred to as physisorption and

relies upon electrostatic and Van Der Walls effects. This approach can be used to form the selfassembly of nanoparticle structures and functionalisations in solution. However, the approach has several shortcomings, it is only suitable for use with appropriately charged ligands, the ligands are not attached in a particular orientation making the biological response difficult to control and the binding is affected by changes in pH.

Examples of covalent conjugation (1-5 in Figure 28) involve:

- ***Amine group involved coupling***

Probably the most commonly coupling methods for bioconjugation of NPs involve amine functional group. Primary amine can react with carboxylic group to form a "zero length" amide bonds. The linkage is usually mediated by carbodiimide agents, such as most commonly used 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC).

The conjugation strategy has been applied to coupling of various proteins, enzyme and antibodies and amine-terminated nucleic acids.

- ***Thiol group involved coupling***

The thiol group can selectively conjugate with primary amine groups. The reaction starts rapidly under the mediation of reagents such as maleimides and iodoacetamides.
Most commonly, the coupling reagent is sulfosuccinimidyl-4-(maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC). NPs can be bioconjugated with various thiolated biomolecules, including thiol modified peptide, residue thiol-teminated DNA, or peptides and proteins with free or reduced cysteine, or verse vice. Disulfide bridges can be also used for reversible coupling of NPs. Glutathione (GSH) disulfide and dithiothreitol have been used for delivering and releasing of drug via hybrid nanostructures, under the regulation of GSH.

- ***Click chemistry***

Another common approach for bioconjugation is the so called "click chemistry". The reaction basically involves coupling of alkyne to an azide giving a 1,2,3- triazole ring under catalyst of Cu (I). The process has been demonstrated to be highly versatile and great potentials for bioconjugation of NPs. The one-step click process has also been shown to give the possibility of introducing multiple functionalities onto NPs. The limitation probably is that it requires special preparation of the azide or alkyne-functional bioactive species, which is often involved a labor work and has low yielding. Fortunately, now many commercial kits have been developed to simplify the process and improve the products yield.

Examples of biological conjugation (8-10 in Figure 28) involve:

- ***Biofunctionalisation with DNA and i-RNA***

Most of the works published about NP–DNA refer to oligonucleotides. The preference for short DNA sequences (between 10- and 100mer per base pair) is due to their great versatility as probes for DNA, RNA, proteins and other biomolecules of interest. Publications in the field increased since Letsinger and Mirkin's group [99] and Alivisatos and co-workers [98] described how to functionalise AuNPs with thiolized oligonucleotides. In the same way, work from Li and Rothberg's [100] is a cornerstone demonstrating the existence of non-specific interactions with the AuNP surface and single stranded DNA.

DNA is able to binding directly to AuNPs through strong bonds with some specific positions i.e. the N3 nitrogen as well as the keto oxygen of its bases, as shown in Figure 29. On the basis of the bonds, the following strength of interaction between AuNPs and nucleosides has been established:

Cytosine > Guanine > Adenine > Thymine

The explanation to this order lies in the availability of the pair of electrons from the base that interact with the surface of the NP. C, G, and A all present an imine N, which T does not have.

The electrons of the imine group are more available than those from the amino group because the lone-pair electrons are delocalized in the aromatic ring. Further, C has less canonical structures

(causing the interacting electron pair to be less delocalized) than G and A; the same logic is applied to G respect to A in order to justify the observed affinity order with DNA.

In addition to chemical bonding, a number of other forces have been proposed to be responsible for DNA adsorption including van de Waals force, hydrophobic interactions and induced dipole interaction (a type of van de Waals force).

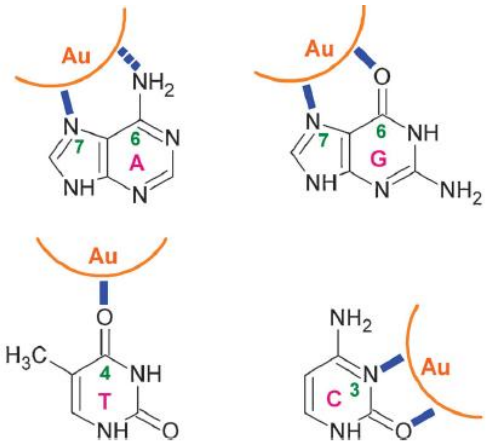


Figure 29: DNA bases adenine,A guanine,G thymine,T and cytosine,C adsorption onto AuNPs; bonding interactions are indicated by the blue lines.

Owing to the large numbers of different possible sequences of bases for a DNA or RNA strand, there also exists an even larger variety of possible conformations (in terms of secondary and tertiary structure) of these molecules. It has been found that certain sequences can strongly bind to a target molecule by molecular recognition, determined by geometric matching of the surfaces of the two molecules. This interaction is mediated by electrostatic, hydrophobic van der Waals forces or hydrogen bonds. This can be exploited to generate the so-called aptamer sequences to a given target molecule by molecular evolution, technically realized by multiple randomization, selection and amplification of strongly binding sequences, resulting in an optimized strand of DNA, RNA or peptide for the target molecule with affinities comparable to antibodies.

For ss-DNA a good affinity to Au NPs is found though thilisation.

Thiolated DNA functionalised gold nanoparticles (DNA–AuNPs) are used to develop new tools for numerous applications, including bioassays, bioimaging, and nanomedicine. The presence of a thiol group in the 5' or 3' position of DNA implies the possibility of formation of a covalent bond with the AuNP surface, which in turn causes an increase of the colloidal stability and functionalises the target AuNP. The DNA chain's exact structure upon adsorption will vary based on ssDNA concentration (with higher concentrations yielding the most upright conformations due to phosphate backbone repulsions), ionic strength, chain flexibility, and individual nucleotides and sequences' affinities to gold surfaces.

Protocols for the functionalisation of AuNPs with DNA have been published [101-103].

A salting-aging thiol-DNA/AuNPs conjugation procedure, based on the high affinity between thiol and gold, is the most commonly applied method for achieving stable DNA–AuNPs conjugates.

Many functionalisation routes make use of thiolated strands of DNA in order to exploit S–Au specific interactions. Most of those protocols using colloidal AuNps involve a ligand-exchange reaction where the citrate is replaced by a thiol, e.g. poly(ethylene glycol) (PEG) that also works as spacer between the DNA strands and increases the nanostructure stability in biological medium, as sketched in Figure 30.

PEG is a linear polymer consisting of repeated units of $–CH_2–CH_2–O–$. The ether group in the backbone of PEG chains utilizes hydrogen bonding and steric stabilization for water solubility, instead of electrostatic interactions. The inertness and non-toxic properties of PEG give rise to a number of applications in medicine, chemistry or biotechnology PEG-modified nanoparticles are more stable at

high salt concentrations and in biological environments; they show less non-specific binding to proteins and cells. PEG with functional groups is coupled to certain amino acids, most commonly lysine and cysteine, exhibiting amino and cysteine groups, respectively. Bifunctional PEG molecules can be used to introduce new functional groups and DNA on the surface, like with bifunctional crosslinkers in conjugation chemistry.

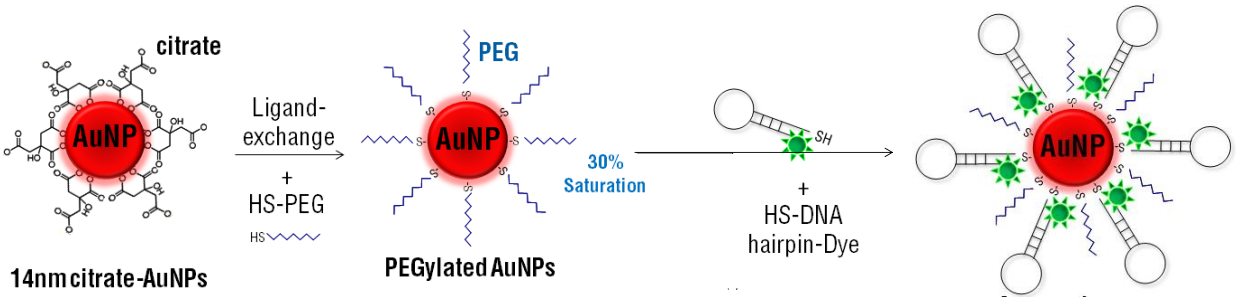


Figure 30: PEGylation of AuNPs and thiolated HS-DNA covalent binding to AuNP.

For double-strand, dsDNA, due to its double-helix structure, which exposes the negative charges of the phosphate groups to the outside presents a bigger rigidity than the ssDNA, which hinders the interaction with the surface of anionic AuNPs. In the case of cationic AuNPs, the electrostatic forces favour the interaction. Although cationic NPs causes partial denaturation of the DNA helix. The electrostatic interactions cause DNA bending while the hydrophobic interactions with the bases in the grooves deform the DNA structure.

- ***Functionalisation with Antibodies***

Immobilization of antibodies (Ab) on AuNPs is essential for developing immune-based assay nanosystems ranging from biosensors and antibody arrays to cellular targeting systems.

Among the five major classes of antibodies, immunoglobulin-G (IgG) is the most abundant in human serum and therefore the most widely used for the biofunctionalisation.

The basic unit of IgG consists of two identical light chains and two identical heavy chains that are held together by noncovalent interactions as well as disulfide bonds (Figure 31). The four protein chains are assembled into a specific Y-shaped geometry, with two identical antigen-binding sites localized at the end of the arms of the Y and called Fab fragments (antigen binding fragment, in green). The stem of the Y is known as the Fc fragment and ensures that the antibody generates an appropriate immune response for a given antigen by triggering effector functions. The position of the antigen binding sites clearly indicates that the final molecular orientation of the Ab on the surface of a nanomaterial affects antigen binding. Thus, there are four possible spatial orientations upon conjugation to the surface of a nanostructure (Figure 31).

Antibodies immobilized through their Fc region (*end-on* orientation) would be properly oriented with their antigen binding sites well exposed to the solution phase.

Also *flat-on* orientations (all three fragments attached to the surface) also preserve the antibody's antigen-binding activity.

Thus, strategies that allow *end-on* or *flat-on* orientations of the Ab upon immobilization would yield Ab-functionalised nanomaterials with higher antigen binding capacities compared to that of random (*head-on* and *sideways-on*) orientations.

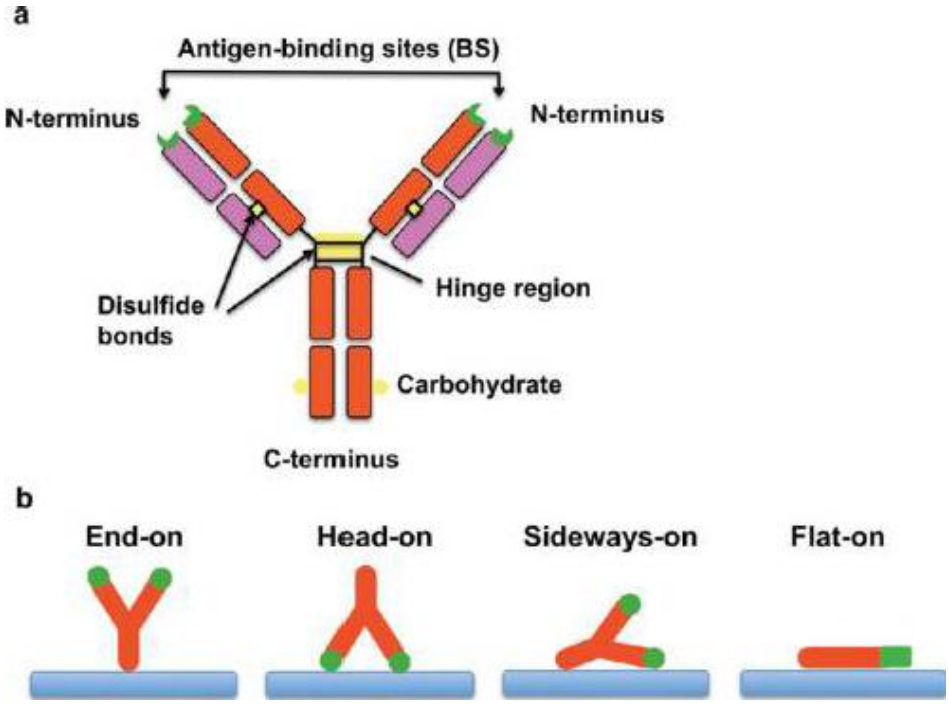


Figure 31: (a) the Y-shaped structure of an antibody. The light chains (variable regions) and the heavy chains (constant regions) are colored in violet and red, respectively. The antigen-binding sites are drawn in green, while groups that can be used for attachment to NPs are drawn in yellow. (b) Possible orientation of antibodies immobilized on surfaces

Several strategies for conjugation of antibodies to the gold particles, both covalent and non-covalent immobilization modes have been used for the site-specific oriented immobilization of antibodies on Au NPs, which exploit the site-specific location of carbohydrate chains or disulfide bridges of antibodies far from the antigen-recognition sites as shown in Figure 32.

Physical interaction between antibodies and AuNPs depends on three phenomena: (a) ionic attraction between the negatively charged gold and the positively charged antibody; (b) hydrophobic attraction between the antibody and the gold surface; (c) dative binding between the gold conducting electrons and amino acid sulfur atoms of the antibody. Chemical interactions between antibodies and nanoparticle surface are achieved in the number of ways like (i) chemisorption via thiol derivatives; (ii) through the use of bifunctional linkers (iii), and through the use of adapter molecules like Streptavidin and biotin

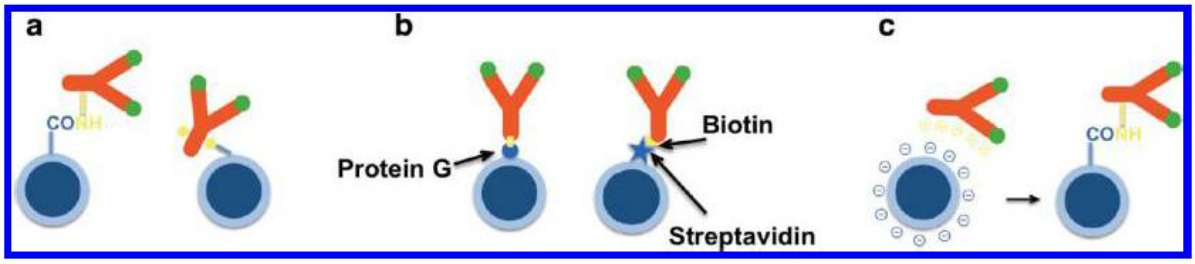


Figure 32: Different strategies for the functionalisation of nanoparticles with antibodies: (a) covalent binding via amine groups on the Ab; (b) use of adapter biomolecules (protein G, streptavidin−biotin); and (c) two-step strategy involving ionic adsorption plus covalent binding. [Adapted from ref 104].

Despite the advantages of oriented binding strategies, protocols that result in a random Ab attachment are still used. For example, a direct covalent binding strategy involving the primary amine groups of the Ab is still used. This requires the introduction of functional groups onto the AuNP surface and/or the use of chemical linkers to activate them in order to achieve the covalent binding of the amine groups of the antibody. Another aspect to take into account is the high reactivity of all of the amine-

binding linkers on the NP surface, which makes them very unstable at alkaline pH values. This imposes the requirement of mild pH conditions (below pH 8.0) for the coupling step. At this pH value the most reactive amine moieties are the terminal amine groups of each of the four polypeptide chains belonging to the Ab, located within the antigen recognition sites. Consequently, using this strategy can result in interference with the antigen detection capability if the antibody is attached via amine groups or adjacent to the antigen-binding site.

A flat-on orientation of Abs can be obtained via a two-step immobilisation process, consisting of ionic preadsorption followed by covalent attachment rather than direct covalent binding through amine groups.

- ***Biotin - (strept)avidin and derivatives attachment***

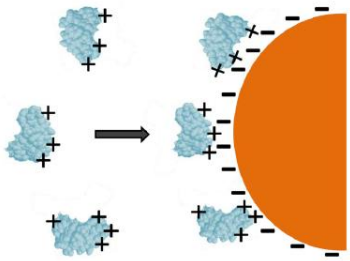
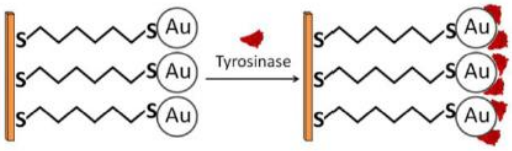
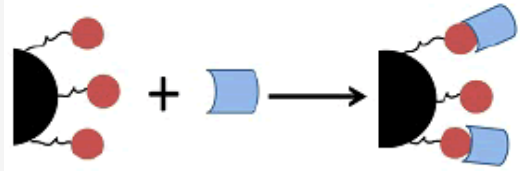
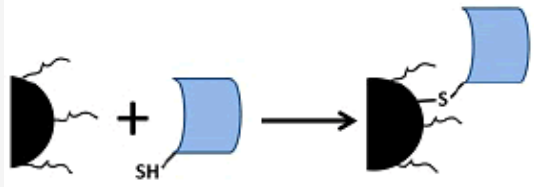
One of the most common used biological interaction is the (strept)avidin-biotin interaction.
The biotin–avidin system consists of a small molecule (biotin) and a protein (avidin), either with or without carbonhydrates (streptavidin, neutravidin and other derivatives).
Biotin as a small molecule with one free carboxylic group, also readily available with a number of modifications such as –NH2 or –NHS, is covalently bound to the nanoparticle by conjugation chemistry or directly to the inorganic particle surface by accordingly modified ligand molecules

- ***Enzymes immobilisation***

However, the immobilisation on solid surface induces structural changes which may affect the entire molecule. The physico-chemical properties of the support may induce different conformational state in immobilised enzyme. Enzyme 3D structure is essential to its activity. Any structural alteration, namely unfolding of the protein, is responsible for the loss of activity. Among the various nanostructures, AuNPs are very attractive to be used as host matrixes. AuNPs can act as conduction centres to facilitate transfer of the electrons. Immobilisation of the redox enzymes together with AuNPs is thought to aid the protein to assume a favorable orientation or to make possible conducting channels between the prosthetic groups and the AuNP. Because the AuNP surfaces permit absorption of protein molecules, AuNPs have been used as a matrix for enzyme immobilisation where the activity of enzymes is retained. Enzymatic immobilisation on AuNPs use both whole cells and isolated enzymes, which include lysozyme, glucose oxidase [105], aminopeptidase [106], as well as alcohol dehydrogenase [107].

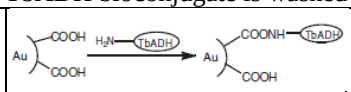
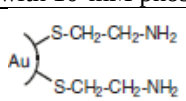
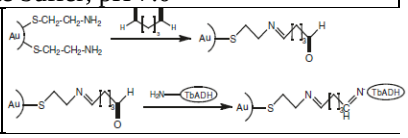
There are four basic methods of enzyme immobilization: physical adsorption, chemical adsorption, self-assembling monolayers (SAMs), and direct conjugation of the NPs, as summarised the Table 13.

Table 13. Four basic methods of enzyme immobilization

Physical Adsorption 	It involves reducing the Au NPs with a negatively charged ligand such as citrate. The reduced gold nanoparticles are then allowed to associate with the ligand, insulating the GNPs from electrostatic repulsion and offering it stability. The resulting citrate layer imparts a negative charge onto the colloidal particle surface. Positively charged amino acid residues allow enzymes in solution to be electrostatically adsorbed on the surface by merely dipping the modified electrode into the solution. Although this method has the benefit of speed and simplicity, unfavourable orientations and decreased functionality are likely.
Chemical Adsorption (covalent) 	Chemical adsorption involves direct covalent binding between the enzyme and the electrode surface – the colloidal gold surface. Chemisorption is achieved via covalent interaction between the SH groups of the cysteine residues and Au on the GNP surface. However, as with all adsorption methods, chemisorption is a non-specific immobilization procedure. Indeed, unregulated covalent binding of an enzyme to a surface can potentially restrict the active site or denature the enzyme.
Self-Assembling Monolayers (SAMs) or conjugation using specific affinity of protein 	Covalently tethering the gold nanoparticles with surface functional groups (–CN, $–NH_2$, or –SH). Short-chain molecules such as 3-mercaptoproprionic acid and cystamine can be self-assembled.
Direct conjugation to the NP surface 	A direct reaction of a chemical group on the protein without the use of a linker is usually desired if the particle is used as a biosensor where FRET or electron transfer is used. For AuNPs, this can be achieved by the Au-thiol, where a protein with a cysteine covalently bonds to AuNPs. The conjugation requires incubation of the protein and the nanoparticle together as the Au–S bond is strongly favored.

Here, as an example we show the effect of different immobilisation, i.e., physical, covalent, and cross-linking on enzyme activity and structure of alcohol dehydrogenases from Thermoanaerobium brockii (TbADH) that are used in industry as catalysts for catabolic processes.
To activate the different immobilisation, the AuNPs are functionalised as described in Table 14 [108].

Table 14. Protocols for the basic methods of enzyme immobilization

Physical Immobilisation Au-citr(CTAB) NPs	**Direct covalent adsorption by carboxyl groups of the enzyme Au-COOH(CTAB)**	**Direct covalent adsorption by amine groups of the enzyme (Au-NH2(CTAB)**	**Covalent through crosslinker**
Au-citr NPs stabilised by cetyltrimethylammonium bromide (CTAB) are obtained by adding 20-μL (0.1 M) CTAB to a 15-mL Au-citr NPs.	Au-citr NPs functionalised with carboxyl groups (Au-COOH) are obtained by adding 6.3-μL MPA (MPA - 3-mercaptopropanoic acid) to 100-mL solution of Au-citr(CTAB). The resulting solution is left for 3 days in the dark at 26°C. Then carboxyl modified NPs are treated with 1.5-mL (0.1 M) CTAB	Au-citr NPs functionalised with amine -NH_2 groups and stabilized with CTAB are prepared mixing 15 mL of Au-citr(CTAB) solution with 20-μL (5.8 mg mL^{-1}) cysteamine and left for 2 days in the dark. Then, thiol modified NPs are treated with 1.5-mL (0.1 M) CTAB.	The cysteamine is adsorbed on Au-citr(CTAB) NPs' exposing the amine group. Then TbADH is bound to Au-NH_2(CTAB) utilizing Schiff-base reaction.
After immobilization on Au-citr(CTAB), the enzyme activity decreased by 90%, revealing that this direct immobilization method has undesirable effect on TbADH activity	The covalent immobilization through direct adsorption of the enzyme on Au-COOH, Au-COOH(CTAB), causes loss of 46% the initial enzyme activity.	The covalent immobilization through direct adsorption of the enzyme on Au-NH_2(CTAB) shows loss only of 28% of the initial enzyme activity.	TbADH retained more than 70% of its native activity
Preparation of TbADH bioconjugates 3-mL Au-citr NPs are mixed with 3-mg enzyme solution under continuous stirring (300 rpm) for 7 h at 26°C. Finally, Au-citr(CTAB)-TbADH bioconjugate is washed with 10-mM phosphate buffer, pH 7.0			
			

The presence of glutaraldehyde may be useful in the creation of additional covalent bonds between the enzyme and the nanoparticles. This usually result in a stabilization of the enzyme by preventing the enzyme leaching.

5.1 Comparison of 4 Biofunctionalisation practices for AuNPs for the enzyle linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a well consolidated technique based on the specific binding of antibodies to an antigen, commonly used for routine analysis [109]. ELISA advantages are its detection limit, specificity, reproducibility and the possibility of high throughput screening, with the disadvantage that the assay takes several hours to develop the response [109].

Significant improvements in signal have been rendered by gold nanoparticles (AuNPs) [110].

It has been demonstrated that various parameters, including surface chemistry and morphology, pH, stabilizing agents, as well as procedures strongly affect the final coverage and recognition efficiency of enzymes.

A recent study has compared different biofunctionalisation strategies to improve ELISA sensitivity [111].

Here we provide an example of guiding principles in the comparison to select appropriate biofunctionalisation procedures using the common proof-of-concept case-study of AuNPs for detection of rabbit IgG by AuNPs functionalised with goat anti-rabbit IgG (Ab).

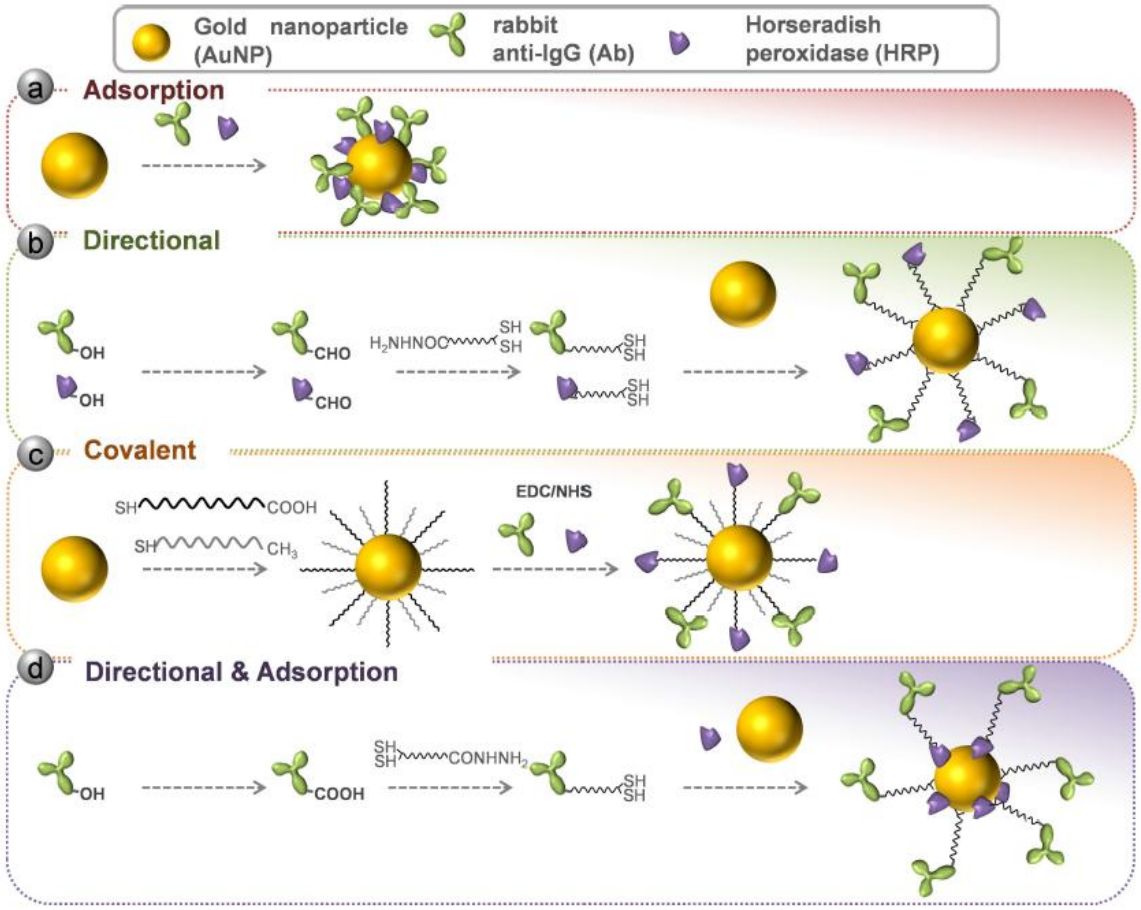


Figure 33: Schematic representation of the four different functionalisation methods explored in this work. (a) Direct adsorption. (b) Directional conjugation with control of antibody and HRP orientation. (c) Covalent conjugation through antibody and HRP amine groups. (d) Combination of directional and adsorption strategy for antibody and HRP [Source: ref. 111: *P. Ciaurriz et al. J. Nanotechnol.* **2017,** *8,* 244–253]

The four different AuNP biofunctionalisation methods are summarised in Figure 33 as:

a) adsorption of biomolecules on nanoparticles by electrostatic/hydrophobic interactions,
b) adsorption of biomolecules on nanoparticles by directional binding by means of a linker,
c) covalent conjugation, where antibodies and HRP are covalently bound to the surface by the means of a PEG linker through its free amine groups using the EDC/NHS carbodiimide method [112],
d) a merge of the previously assayed procedures, combining the directional binding of the antibody with the adsorption of the HRP to the AuNP surface (directional and adsorption procedure).

For the adsorption in a) a well-known protocols (details are in ref. 113) has been used. Specifically:

133 µL of 15 mM borate buffer pH 8.7 were added to mL of AuNPs synthetized as described above to adjust the pH. The appropriated amount of goat anti-rabbit IgG and horseradish peroxidase were added and allowed to react under agitation in a carrousel for 30 min. Afterwards, sucrose was incorporated to a final concentration of 5% and incubated for 30 min. Finally, 160 µL of 3% bovine serum albumin (BSA) were added and shaken for 10 min. Thereafter the sample was centrifuged (7,500g 30 min) to remove unbound protein and AuNPs were re-suspended in 1 mL of 2 mM borate buffer pH 8.7 containing 5% sucrose, 2% glycerol, 0.5% BSA, and 0.01% Tween. The washing step was repeated once and the AuNP probe was re-suspended in 100 µL of the mentioned borate buffer.

For directional adsorption in b) details of the protocols are in ref. 114. Shortly, a hetero-bifunctional linker, hydrazide-polyethylene glycol-dithiol, is used to control the orientation of the molecules on the surface of the NPs. Hydrazide is able to react with aldehyde groups that can be generated by oxidizing the carbohydrates of glycosylated proteins, such as antibodies; antibody and HRP carbohydrates are oxidized with periodate in order to attach the mentioned linker at the Fc region of the antibody. Modified HRP and antibodies are mixed with the AuNPs, triggering a covalent binding. Specifically:

Antirabbit goat IgG (Ab) and HRP were oxidised with periodate and incubated with the linker hydrazine dithiol. Briefly, 100 µL of Ab 1 mg/mL was incubated with 30 µL of 100 mM phosphate pH 7.4 and 10 µL of periodate 100 mM protected from light for 30 min. In the case of peroxidase, 200 µL of HRP 3 mg/mL were incubated with 20 µL of periodate 100 mM protected from light for 20 min. After these incubation times, 500 µL of PBS were added respectively to quench the reaction. Thereafter, 1.97 µL of 23.5 mM linker hydrazine dithiol were added and mixed for 2 h at room temperature protected from light. The proteins were buffer exchanged against phosphate buffer 10 mM pH 7.4 using a Hi-Trap desalting column using an Äkta Prime apparatus (GE-Healthcare, Upsala, Sweden). The Ab-linker and HRP-linker concentrations were measured by absorption at 280 nm and 403 nm, respectively, as well as by Bradford assay (data not shown). Afterwards, the appropriated amount of Ab-linker and HRP-linker were mixed with 1 mL of AuNPs and incubated for 20 min. Thereafter, 100 µg of m-PEG thiol were added and mixed again for 20 min. Subsequently, 100 µL of 1 mg/mL of BSA were incubated for 10 min more.
Samples were centrifuged (5,000g 30 min) and re-suspended in 1 mL phosphate buffer 10 mM pH 7.4 containing 0.5% BSA and 0.01% Tween 20. This step was repeated twice but after the last wash, the complex was re-suspended in 400 µL.

For the covalent conjugation in c) where antibodies and HRP are covalently bound to the surface by the free amine groups of a PEG linker using the EDC/NHS carbodiimide method [112] and the conjugation procedure of previous works [115].

AuNPs were incubated overnight with methyl-PEG-thiol (mPEG thiol, n = 6) and PEG-thiol acid (n = 7) in order to create a mixed monolayer of linker on the nanoparticle. 1 mL containing 0.075 M of mPEG thiol and 0.025 M of PEG-thiol acid was added to 100 mL of synthetised AuNPs and maintained overnight under stirring. Subsequently, the AuNPs were washed by centrifugation at 18,000g for 30 min and the obtained pellet was re-suspended in a smaller volume of water to arrive at a concentration factor of approximately ×30. The conjugation to antibody and peroxidase was achieved by applying the carbodiimide method to carboxylic groups of PEG-thiol acid [30]. Accordingly, 750 µL of AuNPs-PEG where added to 750 µL of a mixture of EDC/NHS 40/20 mM and incubated for 30 min at room temperature. Thern, AuNPs were centrifuged at 18,000g for 30 min and re-suspended in 1,500 µL of a solution containing 25 ppm Ab and 440 ppm HRP in borate buffer pH 8.7 and incubated for 4 h at room temperature. Finally, AuNP-Ab-HRP complexes were washed twice at 18,000g for 30 min and re-suspended in 300 µL Tris-HCl 20 mM pH 8.8 20% glycerol and 1% BSA.

For the merge of the previously assayed procedures, combining the directional binding of the antibody with the adsorption of the HRP to the AuNP surface (directional and adsorption procedure) the protocol for directional Ab loading and concentration of HRP from adsorption method is briefly:

To 1 mL of synthesized AuNPs, 133 µL of 15 mM borate buffer pH 8.7 were added to adjust the pH. Then, the appropriate amount of Ab-linker was added to reach a final concentration of 2.25 ppm and the solution was mixed for 20 min at room temperature. After antibody incubation, the sample was mixed with nonmodified HRP to a final concentration of 144 ppm and shook for 20 min. Thereafter, sucrose (5%), BSA (0.5%), and Tween 10 (0.01%) were added to assure complex stability. The mixture was allowed to react for 10 min and purified by centrifugation at 7,500g for 30 min. The AuNP probe was re-suspended in 1 mL of borate buffer pH 8.7 containing 5% sucrose, 2% glycerol, and 0.01% Tween. The washing step was repeated once and the complex was re-suspended in 100 µL of mentioned buffer.

The 4 biofunctionalisation strategies are compared evaluating the signal/noise (S/N) which represents the absorbance at 450 nm of samples in the presence and absence of IgG. The data are summarised in Figure 34, as an example of the importance of applying the appropriate Au NPs biofunctionalisation strategy.

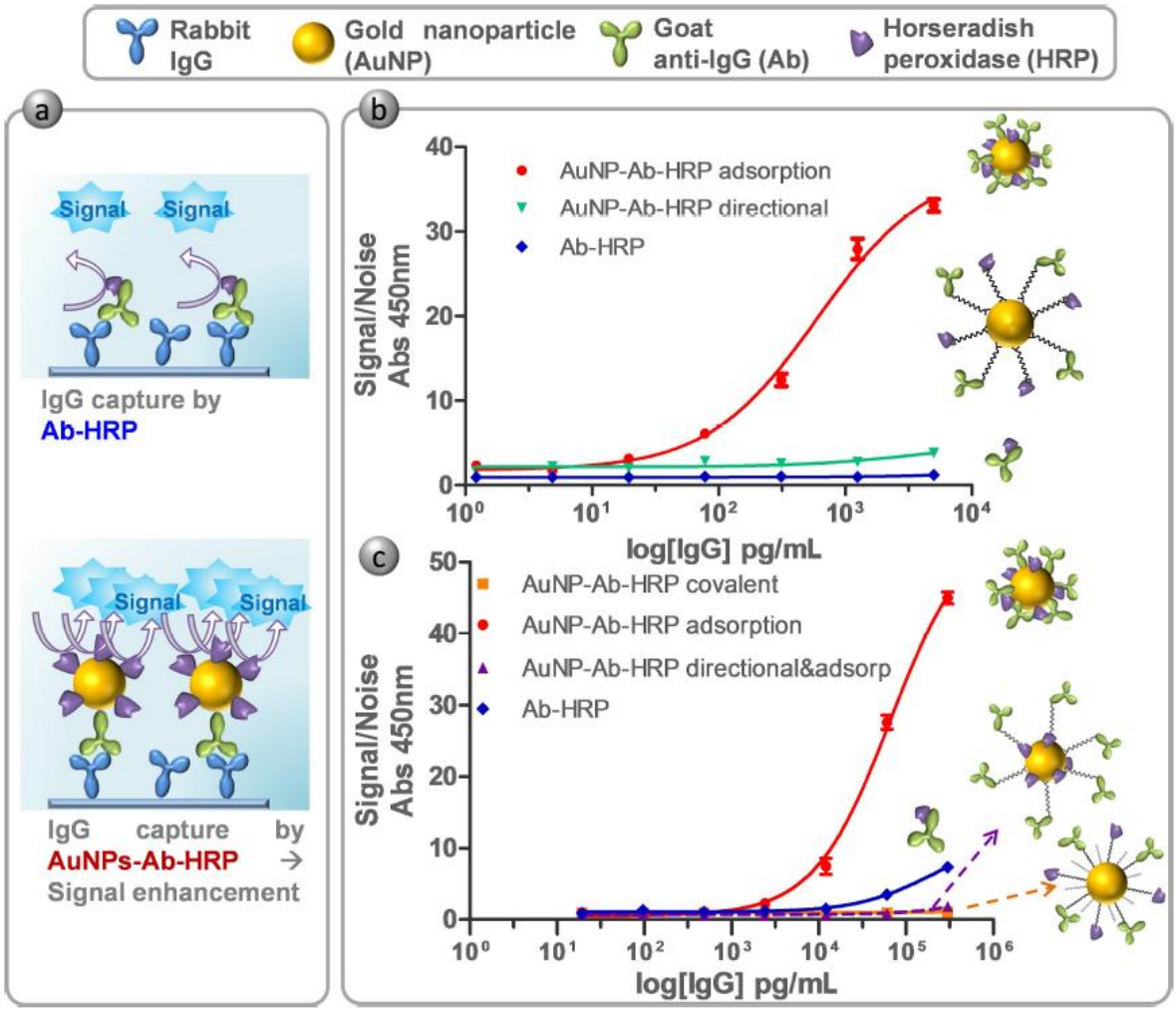


Figure 34: (a) Schematic representation of model ELISA and the basis of enhancement by means of AuNP probes. (b) Result of ELISA comparing AuNP probes prepared by adsorption (red curve), directional conjugation (green) and labelled secondary antibody (Ab-HRP, blue). (c) Result of ELISA comparing AuNP probes prepared by adsorption (red), covalent (orange), directional and adsorption (purple) and Ab-HRP (blue). Results are represented as the S/N ratio which represents the absorbance at 450 nm of samples in the presence and absence of IgG, respectively.[Source: ref. 112: *Beilstein J. Nanotechnol.* **2017,** *8*, 244–253.]

The figure shows that the S/N ratio of samples conjugated by adsorption showed a higher response than directional conjugates or Ab-HRP. Covalent and directional/adsorption biofunctionalisation strategies resulted in worse S/N values than direct adsorption and even more than Ab-HRP, although it is described that covalent and site specific immobilization leads to more stable and better defined composition conjugates [115].

This can be explained considering that direct adsorption can lead to protein multilayers, as biomolecules have numerous groups which can non-specifically adsorb on AuNP surfaces, and adsorption of protein in multilayers allowed the conjugate to recover activity and remain stable.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

A plethora of chemical and bio species are compatible with innovative materials. Here we have presented the cases of graphene based materials, emerging transition metal dicalchogenides (TMDCs) and gold nanoparticles that can bind from small organic molecules to large molecules such as polymers and even to biomolecules like DNA and enzymes. Currently functionalisation of those innovative materials for biomedical applications is still in its nascent stages but has shown much potential as a new nanomaterial for drug delivery carriers, therapeutic agents, diagnostic platforms as well as biosensing, synergistic therapy, multi-modal imaging, highly sensitive biosensing, and externally controlled in vivo drug release agent which is difficult to be replicated by current biodegradable materials like liposomes, nanocrystals, micelles, and hydrogels.

However, certain key obstacles are still present in the proliferation of their application in biological systems. Firstly, consistency in the synthesis of these materials has not been completely achieved yet. There is currently no standard synthesis technique that can produce reproducible graphenes, functionalised TMDCs, and gold nanoparticles with well controlled dispersity, material properties, and dimensions, as well as uniformity in functionalisation across the material surface.

No universal biofunctionalisation strategy exists to cover the wide variety of surfaces and materials as well as of biomolecules available for this purpose. A functionalisation protocol that works well for one type of material and nanoparticle does not work well for another, since they could be very different in terms of surface termination, defects, charge, surface area, stability, and type of reactive groups, etc. Furthermore, biomolecules vary significantly in terms of size, chemical composition, 3D complexity, and location of its biological active site.

In absence of standard functionalisation protocols, each particular case material/biomolecule demands optimisation. Therefore, the development of “smart” multifunctionalisation strategies is vital focus and there exists both great promise as well as additional work to be carried out on the functionalisation of graphenes and TMDCs to make it a truly viable nanomaterial for biomedical applications.

Also, in most cases, the interaction between DNA and those materials has not been identified clearly, which should be addressed properly, so that the hybrid materials can be specifically selected for the appropriate application depending on the nature of interactions. Hence, considerable attention has to be paid to understand the reaction mechanism or chemistry of these hybrid materials.

The coming years should address what functionalisation can facilitate: can we produce large quantities of monolayers of graphenes and TMDs through functionalisation? Can we tune their electronic properties through functionalisation? Using biofunctionalisation, can we couple graphenes, TMDs and AuNps with other materials to develop multifunctional devices at the industrial level? Ultimately, after addressing these questions, we expect the exploitation of the biofunctionalisation of these bioconjugated will contribute to the widespread their application in biophotonics, drug delivery, and medical devices.

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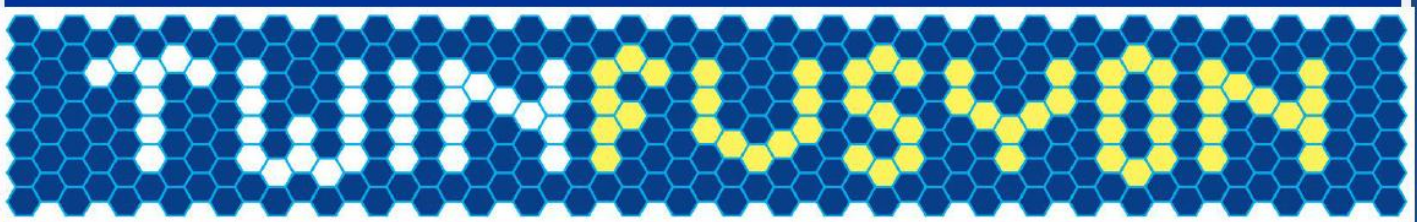

ISBN 978-3-200-05312-0