Univerzita Komenského v Bratislave

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**Autoreferát dizertačnej práce**

# Exfoliácia a pokročilá funkcionalizácia dvoj-dimenzionálnych materiálov

**na získanie akademického titulu philosophiae doctor**

**v odbore doktorandského štúdia:**
4.1.4. Kvantová elektronika a optika

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4.1.4. Kvantová elektronika a optika

**Predseda odborovej komisie:**

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(meno a priezvisko s uvedením titulov a hodností
a presná adresa jeho zamestnávateľa)

## *Abstract*

The cancer treatment is a complicated process build-up from a large number of specific procedures and methods. Because of this, it is necessary to focus on the multidisciplinary research of new alternative approaches, technologies, and materials. Targeted cancer treatment using low-dimensional materials joints multiple scientific disciplines on the edge of physics, chemistry, and biology with the target on the preparation of new drug nanocarriers. A novel family of two-dimensional materials allows unprecedented possibilities in the area of bioconjugation thanks to the high surface-to-volume ratio. In this thesis, I focused on the liquid-phase exfoliation of two candidates for the drug nanocarriers, namely $MoS_2$ and graphene oxide. I studied the exfoliation process of those two-dimensional materials, resulting size of nanoplatforms and their potential for bioconjugation. The second step was the study of the possible functionalization routes of developed nanoplatforms. For each of the two, I developed a specific procedure for attaching the specific antibody to its surface.

## *Introduction*

Research of novel materials always had a firm place among other fields of physics. Their discovery opens new possibilities in all the fields of everyday life, whether its new technologies, biomedical applications, or simply basic research.

One such breakthrough was when graphene was first observed in 2004 [1]. It was the first-ever two-dimensional (2D) material observed and opened a new chapter in the material research [2]–[5]. Graphene is a single layer of carbon atoms, crystallized in a hexagonal structure. It has unique mechanical [6], electrical [7], [8], thermal [9], [10] and optical [11] properties. The discovery of graphene led to the discovery of a whole new family of 2D materials [11]–[13]. Each material has unique properties and is suitable for different applications in different fields. The largest family of 2D materials are transition metal dichalcogenides [12], [14]. Nanomaterials from this family contain one atom of transition metal (M) sandwiched between two atoms of chalcogenide (X), thus forming an $MX_2$ structure. Such a crystalline structure allows the exfoliation to monolayers, similar to the graphene.

One such promising application is in the drug delivery systems [15]. The big advantage of 2D materials is their large specific surface. In the case of graphene, all its atoms are at its surface. The 2D nanomaterial can serve as the smallest possible nanocarrier, that carries the drug into the cells.

However, the functionalization of 2D nanomaterials has not a universal procedure. The same drug will require a different binding procedure if bound with graphene or transition metal dichalcogenide.

In my thesis, I focused on the production, physicochemical characterization, and functionalization of 2D materials for bioapplications. Namely, I focused on graphene oxide and MoS2 and their use in targeted cancer treatment. I developed a reliable exfoliation method for both of the nanomaterials, which will result in the exfoliated 2D nanomaterial in the solution. The complete physicochemical characterization is presented as a solid background for bioconjugation with a specific antibody. The antibody M75 was used for its specificity towards CAIX antigen, which is expressed on the cancer cell's surface.

# 1. Two-dimensional materials in cancer treatment

## 1.1. Graphene Oxide

Graphene oxide (GO) is a successor of the first two-dimensional (2D) material, i.e. graphene. GO is obtained from graphite by oxidation and exfoliation procedure [1], [2]. Oxygen groups increase the interlayer distance and successively decrease the strength of van der Waals bonding between the neighboring graphite sheets. Followingly, the oxidized graphite is easier to exfoliate to monolayers and what is crucial to our application, it is soluble in water. The schematics of the oxidation and exfoliation process is shown in Fig. 1.

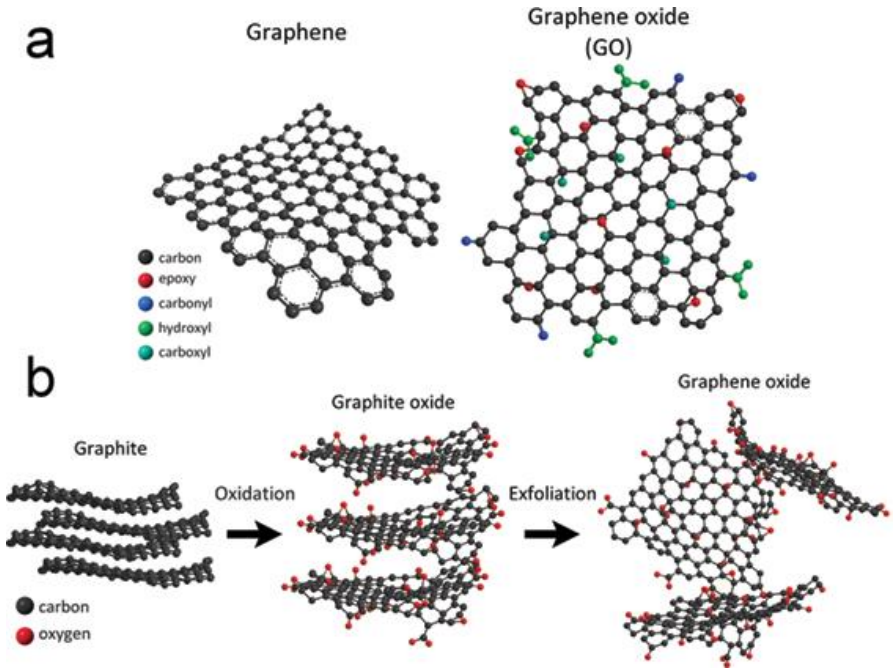


Fig. 1: a) Schematic representation of the GO structure and b) the production of the monolayer GO process[3].

Oxygen groups are used for bioconjugation. Mainly, the carboxyl groups are used for bioconjugation because of the strong covalent bonding between carboxyl and amine groups [4]–[6]. The linking between carboxyl and amine group is facilitated by crosslinking agents EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) and sulfo-NHS (N-hydroxysulfosuccinimide) [5], [6]. These two crosslinkers mediate the formation of a covalent bond and are present only during the formation of the amide bond between carboxyl and amine. After the amide bond is created, the crosslinkers EDC and sulfo-NHS are no longer active, as they are quickly hydrolyzed in the water.

## *1.2. Molybdenum disulfide*

Molybdenum disulfide is a transition metal dichalcogenide, that can be exfoliated to monolayers [7]. Contrasting to graphene, bulk $MoS_2$ is an indirect semiconductor with a bandgap of 1.2 eV, while the monolayer has a direct bandgap of 1.9 eV due to the quantum confinement effect [8], [9]. The photoluminescence (PL) of monolayer $MoS_2$ is located at approximately 650 nm so that the red PL can be observed in $MoS_2$ monolayer [8], [9]. The presence of PL and the metal atoms in the chemical structure permits the $MoS_2$ detectability in soft tissues.

The functionalization of $MoS_2$ is more complicated than in the case of GO. The lack of functional groups prevents the use of conventional bioconjugation techniques. Instead, the sulfur vacancies are utilized to functionalize the $MoS_2$ [10]–[12]. The defect site will attach a sulfur atom and thus promote the bonding. This sulfur atom can be present either in a thiol group [13]–[15] or sulfur from lipoic acid [11], [16], [17]. The linkage between $MoS_2$ nanosheet and the conjugate is often provided by the PEG [10], [15], [16], polyethyleneimine [17] or different thiolated ligand [13]. PEG is a well-known excipient [18], that is often used in medicine to increase the stability in the physiological condition and decrease the reaggregation and immunogenicity [19], [20] of the drugs. The other end of the PEG molecule is not attached to $MoS_2$ nanosheet and can be used for the attachment of a drug [16] (Fig. 3), toxin [15], or an aptamer for tracking of processes inside the cells [17].

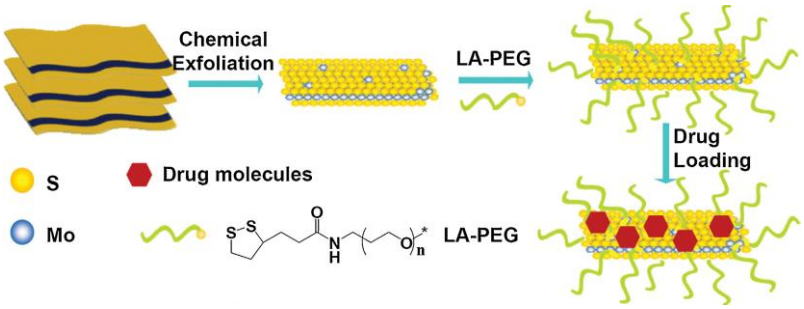


Fig. 3: A schematic representation of $MoS_2$ functionalization via lipoic acid [16].

# 2. Langmuir films

Originally, a Langmuir film is a self-assembled layer of molecules on the water surface [21]. The Langmuir films formation consists of the dropping of a solution of lipid molecules on the surface of the water subphase. The amphiphilic molecules spread across the subphase surface, and the solvent evaporates. It leaves the molecules mobile on the surface, and a pair of movable barriers slowly confine the area, where the molecules can float. The barriers movement forces the molecules on the surface to compress and form a monolayer film of the oriented molecules with their polar ends in the water subphase.

## 2.1. Langmuir films of 2D materials

### 2.1.1. Graphene oxide

It is not straightforward to produce Langmuir films from GO on the water subphase, because of its amphiphilicity. There are several ways to overcome the dispersibility of GO in the water subphase.

We can use the amphiphilicity to work against the dispersibility in the water subphase [22]. By increasing the pH of the water, the carboxyl groups at the edge of GO flakes are deprotonated. Therefore, the GO flakes get more charged, and the solubility in water increases. On the other hand, if we decrease the pH of the subphase, the GO flakes will become insoluble in the water subphase. Plus, good spreadability of our solution on the water surface is guaranteed by adding methanol to the GO dispersion. Methanol will not change the stability of the dispersion of GO, but its large difference in the surface tension will cause the droplet to spread across the water and not to sink in.

### 2.1.2. $MoS_2$

Making Langmuir films of $MoS_2$ requires similar condition as GO. The $MoS_2$ can be directly exfoliated in ethanol/water mixture, so the good spreading is guaranteed. And because the $MoS_2$ is to some extend dispersable in water, changing the pH to lower values is advisable as well.

## *3. Aim of the work*

The aim of this thesis is to study the preparation, characterization, and applications of two-dimensional materials.

Namely, the liquid-phase exfoliation of $MoS_2$ and GO to produce sufficiently small 2D nanoplatforms, that will be used as nanocarriers for specific antibodies.

In my thesis, I focused on the liquid-phase exfoliation of $MoS_2$ and GO using ultrasound. The basic optimization of the parameters of exfoliation was studied to maximize the yield of the desired size of nanoplatforms. Further size-selective sorting was be performed using a centrifuge.

The dispersions of nanoplatforms were be transferred onto substrates using the Langmuir-Schaefer approach before analysis.

The resulting size distribution of nanoplatforms was detected by imaging methods such as AFM, SEM or TEM and supported by spectroscopy methods such as Raman spectroscopy or absorption spectroscopy.

Nanoplatforms with desired size distributions were chemically functionalized to enable the bioconjugation of antibodies.

## *4. Analytical techniques for Langmuir films*

The monitoring of the surface pressure is essential for the Langmuir film formation. The change in the surface pressure of the subphase is the primary information on the forming layer during the compression. In the case of low-dimensional nanomaterials, the main interest is in the formation of a closed, homogenous monolayer suitable for further applications. Here, we define the monolayer as a film, which has the highest possible surface density of given nanomaterial. The highest density is achieved when the nanomaterial is compressed to a critical pressure $\pi_c$. If the film is compressed above the $\pi_c$, the monolayer collapses what promotes the nucleation of the second layer. The film's elastic modulus $E = -A\frac{d\pi}{dA}$ or its inverse, the film's compressibility *C*, can be employed to assess the critical pressure $\pi_c$. A numerical calculation of the Elastic modulus *E* from the π-A isotherm is utilized to determine the $\pi_c$ correctly [23], [24].

In general, imaging null ellipsometry can be used to track the formation of the molecular Langmuir films with micrometer spatial resolution [25]. Its wide-spread modification, the Brewster angle microscopy (BAM), uses the *p*-polarized light incident at the Brewster angle, which is close to 53° for the water subphase. The large difference between the optical refraction index of many low-dimensional nanomaterials and water predetermines the BAM as the method of the first choice for studying the compression of the nanomaterial films on the water subphase [26]–[33].

The X-ray scattering techniques belong to the advanced *in-situ* characterization methods applicable to the formation of Langmuir films. In a typical GISAXS experiment, an X-ray beam impinges onto the water surface at a grazing-incidence angle close to the critical angle for the total reflection. Each nanoobject in the Langmuir film acts as a scattering center and produces a partially scattered wave. All partially scattered waves interfere and are recorded in the far-field as the intensity GISAXS scattering pattern [23], [34]. By evaluating the GISAXS pattern, we can monitor the interparticle distance and surface coverage.

## 5. Analytical techniques

The resolution of optical microscopy is not sufficient for the surface characterization of nanomaterials. The resolution limit is given by the Rayleigh criterion $d = \lambda/2NA$, where $\lambda$ is the light wavelength, and NA is the numerical aperture of the used objective. For visible light, the typical resolution limit is approximately 200 nm. Consequently, there is no possibility to distinguish between the two neighboring nanoparticles if their dimensions are in the range of tens of nanometers.

The AFM technique measures the morphology of the sample surface by evaluating the forces acting between the vibrating probe and surface. The dimensions of the probing tip limit the AFM resolution. Within today's technology, the sharpness of the scanning tip can be less than a few nanometers.

The absorption spectroscopy measures the optical response in the UV-Vis-NIR range. The change in the electronic structure of material results in the change of the absorption spectra. The electrons and their energy levels are responsible for the energy of absorbed or emitted light. On top of this, the concentration of the nanomaterial in the solution can be calculated from Beer-Lambert law.

The Raman spectroscopy was employed to reveal the crystallinity of prepared nanomaterials. In Raman spectroscopy, the sample is irradiated with a high-intensity laser, and the inelastically scattered light is detected. The photons after inelastic scattering carry the information about the vibrations of the atoms in crystals or molecules in the sample.

The X-ray photoelectron spectroscopy (XPS) was used to analyze the atomic composition of samples. The XPS analyzes the kinetic energy of photoelectrons emitted from the sample surface after the absorption of X-ray photons. The binding energy can be determined as the difference between the X-ray photon energy and the kinetic energy of emitted photoelectrons.

The resolution limit of the optical microscope can be surpassed by decreasing the wavelength of the light. The electron microscopes use this fact, and instead of the visible light, they use electrons as the probing particles. The microscope resolution is given by the de Broglie wavelength of used electrons, and hence the atomic resolution can be achieved. The transmission electron microscope (TEM) uses electromagnetic lenses to create a magnified image of a thin sample on a two-dimensional detector.

# 6. Results

## 6.1. $MoS_2$

The successful exfoliation of $MoS_2$ is crucial for the functionalization. On top of that, the highest possible concentration of exfoliated $MoS_2$ is desired. However, during the exfoliation of $MoS_2$ in ethanol/water mixtures, the elevated concentration will cause the oxidation of $MoS_2$ instead of the exfoliation (Fig. 4).

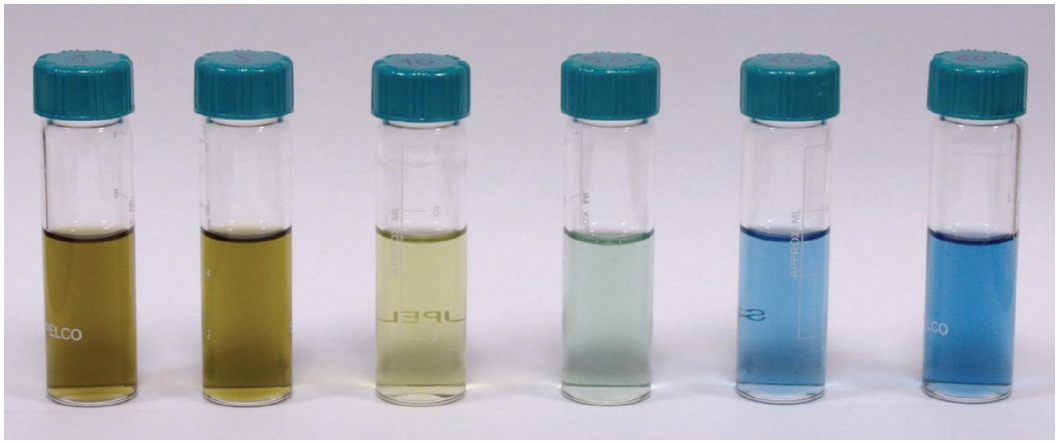

Fig. 4: The photographs of the supernatants after $MoS_2$ exfoliation and centrifugation for the initial concentrations: 1, 5, 10, 20, 40 and 60 mg/ml (from left to right).

Here, the water and dissolved oxygen act as the oxidizing agent during liquid-phase exfoliation. Along with that, the oxidation is accompanied by the release of $SO_4^{2-}$ and $H^+$ that will cause the pH of the dispersion to drop. The higher the initial concentration of $MoS_2$ in the dispersion, the more the pH drops. After the pH dropped over the stability point of the $MoS_2$ colloidal solution, all the exfoliated crystals start to sediment. Furthermore, only the $MoO_x$ nanoparticles remain the form of a colloidal solution.

Knowing the limitations of the liquid-phase exfoliation of $MoS_2$, the highest concentration of $MoS_2$ obtainable by this method was discovered.

Langmuir films of $MoS_2$ were prepared for the characterization of the $MoS_2$ nanosheets. The average thickness of $MoS_2$ was determined to be four layers and the lateral size of nanosheets was found to be in the range of 30-70 nm.

The bioconjugation of $MoS_2$ was performed via so-called biotin-avidin-biotin bridge (Fig. 5). First, the $MoS_2$ was functionalized by the lipoic acid-PEG-biotin molecules.

Secondly, avidin reacts with the terminal biotin and leaves three empty reaction sites for extra biotin molecules. These empty reaction sides react with the biotinylated antibodies to finish the process.

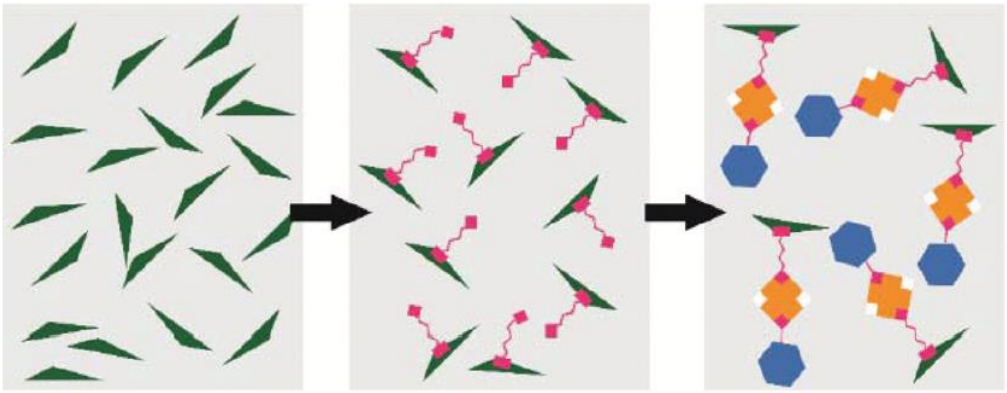

Fig. 5: Schematics of the three-step functionalization workflow: first, the MoS2 nanosheets are exfoliated with LPE in deionized water. In the second step, PEGylation proceeds and in the last step, the M75 antibody is conjugated via a biotin–avidin–biotin bridge to the nanoplatform.

## *6.2. Graphene oxide*

The main advantage of GO, when compared to $MoS_2$, is the ability to control its size. Monolayer GO with different lateral sizes ranging from micrometers to tens of nanometers can be prepared by carefully selecting the centrifugation steps.

In our work, the antibody was bonded to magnetic nanoparticles. Magnetic nanoparticles could be further used for MRI imaging or the killing of the cancer cell by hyperthermia. The magnetic nanoparticles were terminated by amino group and the bonding process relies on the formation of an amide bond between the nanoparticle and GO sheet.

First, a PBS buffer solution containing a small amount of TWEEN®20 was mixed with GO solution. TWEEN is a surfactant that opposes the agglomeration of GO during the functionalization. Second, EDC and sulfo-NHS were added to the solution. EDC and sulfo-EDC are zero-length cross-linkers, that promote the formation of an amide bond. Furthermore, in the last step, the antibody-containing magnetic nanoparticles were added. This way, the amide bond between the carboxyl group on GO sheet and amine group on the magnetic nanoparticle was created (Fig. 6).

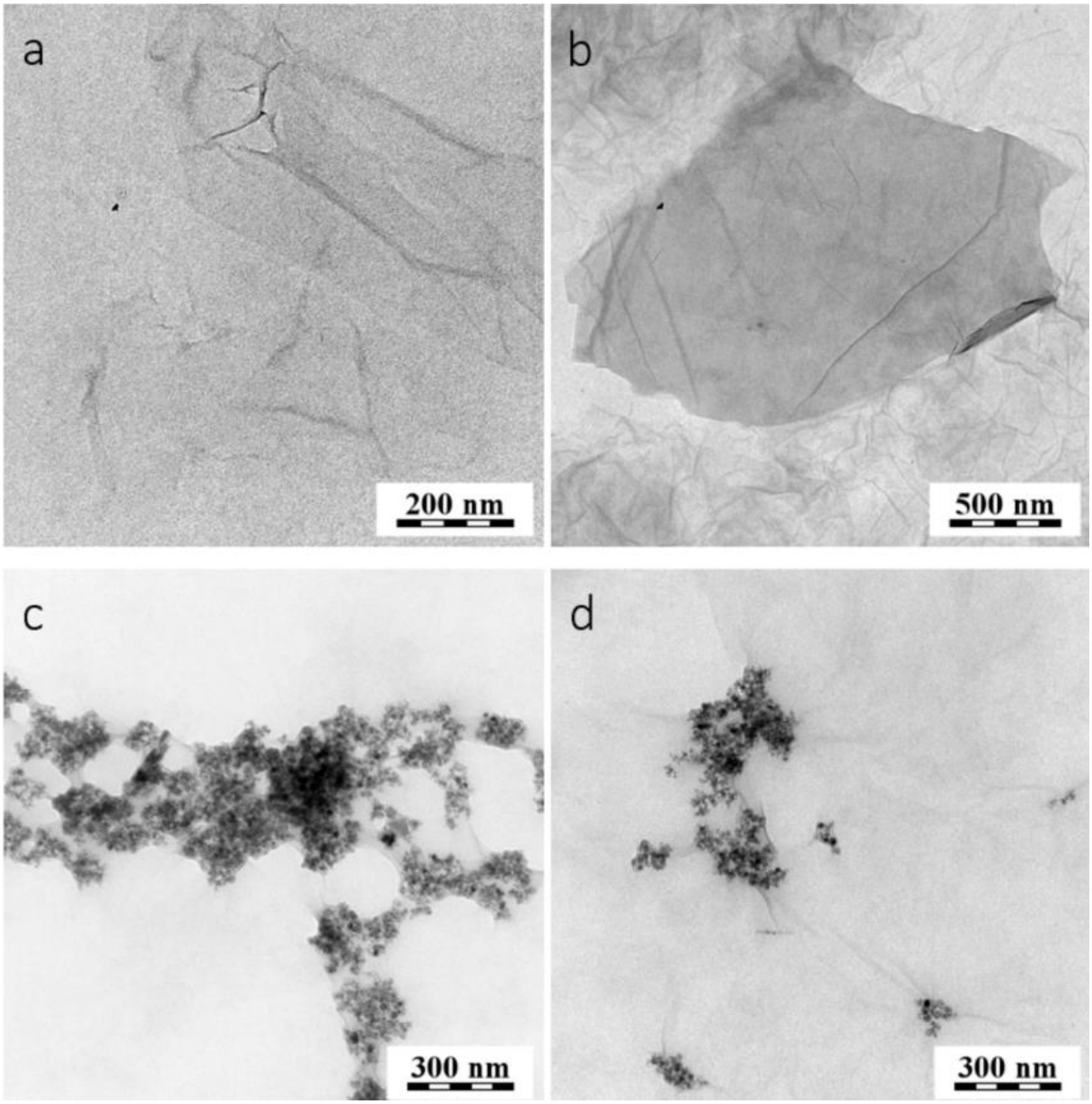


Fig. 6: Transmission electron microscopy (TEM) micrographs of the (a,b) pure GO platforms; (c) graphene oxide platforms with magnetic nanoparticles modified antibodies, and (d) graphene oxide platforms with magnetic nanoparticles modified antibodies in the presence of cross-linkers.

## *Conclusions*

The aim of this thesis was to prepare a functionalized 2D nanoplatforms, that can be used for targeted cancer treatment. In this work, we focused on the production of 2D nanoplatforms using the liquid-phase exfoliation method.

The powders of $MoS_2$ or GO were dispersed in an appropriate solvent and exfoliated using ultrasonic waves. The resulting dispersions of exfoliated 2D materials were centrifuged to remove the unexfoliated material. The supernatant contains exfoliated $MoS_2$ nanoplatforms and the precipitate unexfoliated $MoS_2$ crystals.

After the dispersions of 2D materials were prepared, the size-distribution and their crystallinity were characterized. Using Langmuir-Schaefer deposition, both $MoS_2$ and GO were transferred onto silicon or glass substrate. The characterization of $MoS_2$ and GO nanoplatforms was performed using AFM, Raman spectroscopy, SEM, TEM, XPS, and absorption spectroscopy. In this step, we analyzed whether the nanoplatforms are of the desired size and are suitable for functionalization.

The functionalization of $MoS_2$ relies on the presence of the sulfur vacancies on its surface. The lipoic-acid modified PEG binds to the $MoS_2$ surface and leaves the second end of the PEG chain ready for further functionalization. In our work, we used biotin as a terminating agent. The terminating biotin binds in the next step with avidin, which has four binding sites for biotin. In the last step, the biotinylated antibody binds with avidin and closes the biotin-avidin-biotin bridge.

On the other hand, GO has already on its surface functional oxygen groups. In our work, we used carboxyl groups on the GO to directly bind GO with antibody-coated magnetic nanoparticles. We used coupling agents such as EDC and sulfo-NHS to promote the formation of the amide bond between GO and the magnetic nanoparticle.

The functionality of such nanoplatforms was further studied on the cancer cells.

The final products of this work are two types of nanoplatforms for targeted cancer treatment. One of the nanoplatforms is based on GO, i.e., carbon-based nanomaterial and the second one is based on inorganic nanomaterial $MoS_2$.

## *Publication presented in the dissertation thesis*

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