ON THE FEASIBILITY OF APPLICATION OF BIO-CONJUGATED MOS_2 BASED NANOPLATFORM FOR TARGETED CANCER TREATMENT

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Abstract

Surface functionalization of 2D MoS_2 materials offers a convenient way to tune their properties and facilitate their application in the field of bio-sensing and nanomedicine. In this study, three different liquid phase exfoliation (LPE) methods were utilized for MoS_2 exfoliation: LPE in 45 % ethanol solution, LPE in ultrapure water and amino acid intercalation assisted LPE. The three products were bio-conjugated with PEG molecules and through a biotin-avidin-biotin bridge they were functionalized with specific antibody M75 having exclusive affinity towards cancer cells expressing CAIX protein on their membrane. We are aiming to achieve high selectivity towards cancerous cells utilizing an antigen-antibody based cancer detection scheme. This study uses CAIX antigen tagged MDCK cells simulating the cancerous environment and pure MDCK cells as control. Cellular intake studies were performed by confocal Raman microscopy imaging and confocal laser scanning microscopy before and after binding the specific antibody. The preliminary results indicate higher density of internalized nanoplatforms in the case of MDCK cells expressing CAIX compared to the control cells. However, further studies are required to validate these results.

Keywords: MoS_2 based nanoplatform, bio-conjugation, antigen-antibody based detection

1. INTRODUCTION

The idea of producing a cancer detection platform based on 2D nanomaterials originates from the achievements of bio-nanotechnology over the last decade. Biocompatible graphene derivates [1] were used to produce nano-systems that can be utilized in biomedical applications. Several studies promoted systems for drug delivery mediated cancer treatment [2,3] or cancer treatment via photothermal therapy [4,5]. In 2013, Chou et al. [6] surveyed the photothermal performance of 2D TMD (transition metal dichalcogenide) materials and showed that MoS_2 nanosheets were better than graphene. Subsequently, different approaches of photothermal therapy were demonstrated [7-10]. Several studies described PEGylated (poly(ethylene glycol) terminated) MoS_2 nanosheets as nontoxic or with negligible toxicity. Teo et al. [11] showed that exfoliated MoS_2 and other TMDs have lower cytotoxicity than graphene and its analogues and they considered it as a biocompatible material. Another study by Appel et al. [12] stated both mechanically exfoliated and CVD grown MoS_2 as biocompatible, evaluating not only their cytotoxicity but also genotoxicity. Liu et al. [13] reported that lipoic acid (LA) conjugated PEG containing a disulfide group at the PEG terminal enables a strong binding to MoS_2 and the PEG greatly enhances the physiological stability. Furthermore, Liu et al. [8] reported that the PEG not only stabilizes the MoS_2 solution but also increases the cellular uptake of the PEG-MoS_2 sheets loaded with specific drug when compared to the case where the sheets were not PEGylated.

Taking into account these findings, the nanoplatform model that we are considering consists of a MoS_2 nanosheet base, which is covalently functionalized with LA terminated PEG. This PEGylated nanoplatform needs to be able to bind the proper antibodies that can attach to cancer cells expressing specific antigens. Therefore, this nanoplatform design uses a PEG molecule with LA at one end and biotin on the other. Biotin is a water-soluble vitamin which can be bonded to avidin (avidin is a glycoprotein which has very high affinity to bind biotin). The avidin-biotin non-covalent bond is widely used in biological sciences due to its high specificity and strength [14,15]. Moreover, an avidin molecule can bind up to four biotin molecules which can be conjugated to several different molecules. This feature allows to introduce biotinylated molecules, antibodies, cytotoxins, fluorescent dyes, etc. to the system.

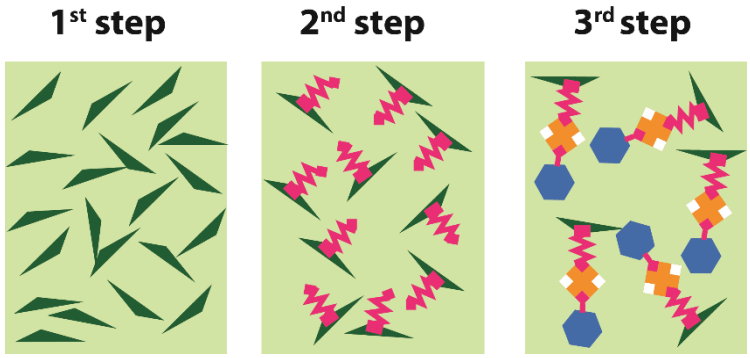

Figure 1 Step by step scheme of the nanoplatform preparation process: exfoliation, PEGylation and attachment of the specific antibody

In this study, we describe the functionalization process of the MoS_2 nanoplatforms (**Figure 1**) and demonstrate the internalization process utilizing a Madin-Darby Canine Kidney (MDCK) mammalian cell line, which is frequently applied in various biochemical models as it provides an ideal representation for epithelial tissues. We discuss the successful internalization within the MDCK cells expressing carbonic anhydrase protein (CAIX), which is associated with tumor cell growth and survival [16].

2. EXPERIMENTAL

2.1. Liquid phase exfoliation

LPE in 45% solution

The MoS_2 exfoliation starts from 1 % powder dispersion in ethanol/water in 7:9 ratio (ethanol purchased from Merck, grade: ACS, ISO, Reag. Ph Eur). The pristine MoS_2 powder was purchased from Alfa Aesar (99 % metals basis purity, approx. 3 μm of size). The selected ethanol/water ratio has the most favorable Hansen solubility parameters for an increased concentration of exfoliated product [17]. The MoS_2 dispersion was sonicated in ultrasonic bath (Sonorex RK 510 H, 35 kHz, Bandelin) at the temperature of 10°C for 48 hours. Subsequently the solution was centrifuged for 1 h with RCF 41.000 g (Model 3-30K, Sigma centrifuge) at 20°C, then it was carefully decanted to separate the exfoliated part from the sediment and stored at room temperature (sample A). The concentration of sample A is 100 μg/ml.

LPE in DI water

As in the previous case, the MoS_2 exfoliation starts from powder MoS_2 (Alfa Aesar), 50 mg was dispersed in 20 ml deionized (DI) ultrapure water. The MoS_2 dispersion was sonicated in ultrasonic bath (Sonorex RK 510 H, Bandelin) at the temperature of 10°C for 48 hours. Subsequently the solution was centrifuged for 30 min

with RCF 21.000 g (Model 3-30K, Sigma centrifuge) at 20°C. The supernatant was collected by micropipette and stored at room temperature (sample B). The concentration of the sample B nanoflakes are 40 µg/ml.

Amino acid intercalation assisted LPE

In this case we used an amino acid - arginine (Sigma Aldrich) - as an exfoliating agent [18]. 100 mg of arginine was dissolved in DI and sonicated for 30 min. 50 mg of pristine bulk MoS_2 powder (Alfa Aesar) was added to arginine-DI water mixture and stirred at 560 rot/min for 24 h utilizing a laboratory shaker. Subsequently, 10 ml of DI water was added to the mixture and sonicated for 20h at 10°C (Sonorex RK510H, Bandelin). Then the mixture was centrifuged for 30 min at RCF 21.000 g (Model 3-30K, Sigma centrifuge). The precipitate is dissolved in 20 ml of DI water, sonicated again for 20 h and centrifuged in a similar manner as before. The solution is decanted to separate the exfoliated arginine functionalized part (sample C) from the sediment. The sample is then stored at 6°C in a refrigerator unit. The concentration of sample C is 20 µg/ml.

2.2. MoS_2 functionalization

First, all three types of exfoliated MoS_2 were functionalized by PEG. In the case of sample A, the nanoflakes were transferred into water after drying out 20 ml of the product in a desiccator. Subsequently, the dried-out nanoflakes were dissolved in 10 ml of DI water and sonicated for 30 min. From this step the PEGylation process was identical for sample A and B and took place in aqueous environment. We used PEG molecules terminated with LA at one end and with biotin at the other (LA-PEG-biotin, MW 2000, Nanocs). 10 mg of LA-PEG-biotin dissolved in 5 ml of DI water was added to 10 ml of the nanoflakes. The mixtures were stirred for 1 h on a laboratory shaker, then dialyzed for 72 h with a dialysis membrane of molecular weight cut-off (MWCO) 12-14 kDA (Standard RC Tubing Spectra/Por®2, SpectrumLabs). In the case of sample C, the PEGylation process required modified environment: 10 mg EDC (98+%, Acros Organics) and 10 mg of sulfo-NHS (98 %, Sigma-Aldrich) were dissolved in 5 ml of DI water. Additionally, 5 mg of poly(ethylene glycol) 2-aminoethyl ether biotin (NH_2-PEG-biotin, MW 5300, Sigma-Aldrich) was dissolved in the same amount of DI water. 5 ml of sample C was placed into sonicating bath. First, the EDC/sulfo-NHS was pipetted into the sample, and then the PEG solution was added. Subsequently, the mixture was left in the sonication bath for 2 h. Afterwards, it was dialyzed for 72 h with dialysis membrane of MWCO 25 kDA (Pre-wetted RC Tubing Spectra/Por®6, SpectrumLabs).

Second, a biotinylated anti-human carbonic anhydrase IX (M75 [19]) was attached to the nanoplatform through a biotin-avidin bridge. 1 mg of avidin (GBiosciences) was diluted in 1 ml of DI water. 1 µl of the biotinylated antibody solution (160 kDA, 0,98 µg/µl) was mixed into 10 ml of PEGylated nanoplatform solution. The mixture was placed into a laboratory shaker (180 rot/min) and 1 µl of the avidin solution was dropped into this mixture. In the case of the samples that were prepared for confocal laser scanning microscopy (CLSM) measurements without M75 antibody, fluorescently labelled streptavidin (ATTO532 streptavidin, ATTO-TEC) was added in the same concentration instead avidin.

2.3. Cell preparation

MDCK epithelial cell line (ATCC No. CCL-34) was cultivated using VLE Dulbecco's MEM medium containing 2 mM L-glutamine (Biochrom) with 10 % fetal bovine calf serum (FCS, Biochrom) and gentamicin 80 µg/ml (Lek). MDCK cells permanently transfected with full-length human carbonic anhydrase (CAIX) cDNA in the pSG5c plasmid (marked as MDCK-CAIX) were used in our experiments, mock-transfected cells (marked as MDCK-neo) being a negative control [16]. 200 000 cells were incubated in humidified atmosphere at 37°C in the presence of 5 % CO_2 attached to a glass coverslip (0.17 mm, Zeiss) in a 3.5 cm culture dish (Greiner Bio One). After 24 h, the solution of bio-conjugated MoS_2 nanoplatforms was added to the cells (500 µl to 1 ml of medium) and incubated further for 24 h. Viability (BioTek, Synergy H4 microplate reader) was tested on the MDCK epithelial cell line with 3 different concentrations (250 µl, 500 µl and 750 µl to 1 ml of the medium)

of exfoliated nanoflakes after 24 h, 48 h and 72 h exposure. The results show no cytotoxicity, suggesting that the MoS_2 nanoflakes are not affecting the cell cycle of the examined samples.

In the case of samples that were prepared for CLSM measurements with M75 antibody, the MoS_2 nanoplatforms were first incubated with donkey anti-mouse IgG (H+L) secondary antibody conjugated with fluorescent tag Alexa Fluor 488 (Thermo Fisher Scientific) for 1 hour at 37°C and then they were added to the cells and incubated further. After the incubation phase the cells were fixed with methanol (5 min at -20°C), and then they were mounted on a glass slide with mounting medium Fluoroshield with DAPI (Sigma-Aldrich).

2.4. Cellular intake study

Confocal Raman Microscopy

Confocal Raman microscope (CRM, Alpha300 R+, WITec) complemented with an immersion objective (W Plan-Apochromat 63×, NA=1, Zeiss, Germany) was utilized on living cells in PBS (pH=7.4, Oxoid). The samples were excited at the wavelength of 532 nm and laser power of 5 mW (Spectra-Physics Excelsior 532-60 Multi Mode). The Raman spectra were acquired with a spectrometer (UHTS 300, WITec) equipped with a 600 gr/mm grating (blazed at 500 nm) and coupled to an EMCCD camera (Newton DU970N-BV-353, Andor).

Confocal Laser Scanning Microscopy

The CLSM (LSM 510 Meta, Zeiss) measurements were carried out on fixed cells using an oil immersion objective (Ec Plan Neofluar 40×/1.30 Oil DIC M27, Zeiss). The CLSM scanning unit was mounted on a Axiovert 200M inverted microscope (Zeiss). Images were scanned at the scan speed 6, 1024 x 1024 pixels, 12bit data depth with average mode 4x line.

3. RESULTS AND DISCUSSION

We examined the internalization of MoS_2 nanoplatforms with and without M75 antibody in MDCK-neo and MDCK-CAIX cell lines. All three samples A, B and C were terminated with either M75 or fluorescently tagged streptavidin.

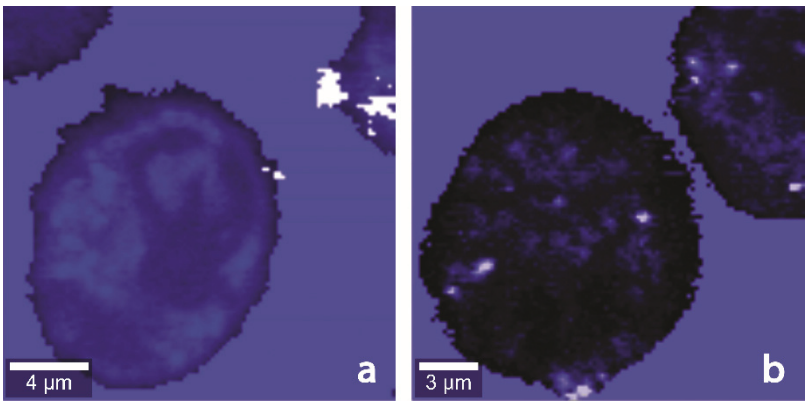

Figure 2 Localization of MoS_2 nanoplatforms (marked by white color) of sample A without M75 antibody on MDCK-neo cells (a) and with M75 antibody internalized inside MDCK-CAIX cells (b) using CRM

By means of CRM imaging, we tracked the location of MoS_2 nanoplatforms. The MoS_2 Raman modes (E^1_{2g} and A_{1g}), the CH_2 stretch mode (2854 cm^{-1}) of lipids, fatty acids and proteins and the symmetric CH_3 stretch mode of proteins (2930 cm^{-1}) were used to determine whether the examined nanoplatform is localized inside the cell, on the cell membrane or outside the cell. For this purpose, we studied the z-stack composed of several cross sections through the inspected cell. **Figure 2** represents a typical CRM image showing a cell cross

section with the MoS_2 nanoflakes highlighted in white. The nanoflakes shown in **Figure 2a** were found to be stopped on the cell membrane while the nanoflakes in **Figure 2b** passed through the cell membrane and were internalized. Our CRM measurements suggest that if the nanoplatforms are internalized (with or without M75), they are found deeper inside the cell rather than close to the membrane.

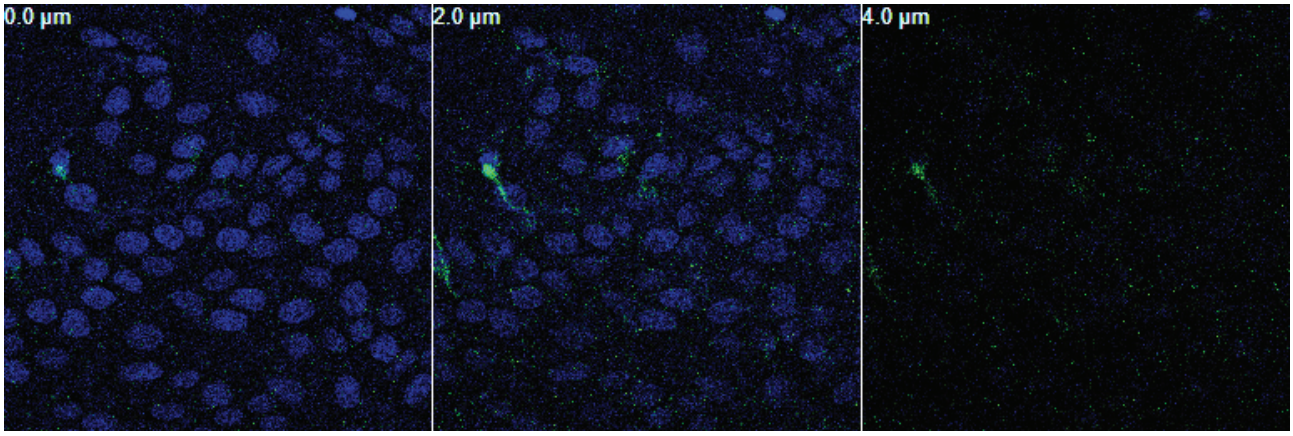

Figure 3 Selected CLSM z-stack images of PEGylated sample A scanned from the top (z=0 µm) of the cell to the glass slide (z=4 µm) with M75 in MDCK-CAIX cells. Cell nuclei and MoS_2 nanoplatforms are marked with blue and green color, respectively.

As mentioned in the introduction, other articles reported the successful internalization of PEGylated MoS_2 nanoflakes. The focus of our study is whether the specificity of the antigen-antibody bonding increases the number of internalized nanoflakes in cells expressing CAIX. Based on the CLSM z-stack images (**Figure 3**) we determined the ratio of the cells with internalized nanoplatforms to the cells without them. The internalization results are displayed in **Table 1**: for all three samples, we observed an increased number of internalized M75 bio-conjugated nanoplatforms when incubated with MDCK-CAIX compared to MDCK-neo. The results also show an increased probability of internalization in the latter for the ethanol and arginine assisted LPE produced samples without M75 (samples A, C).

Table 1 Evaluation of the nanoplatform internalization into MDCK-CAIX and MDCK-neo in case of nanoplatforms with and without M75 based on CLSM

Nanoplatform	CAIX	With M75	Without M75
Sample A	✓	12 %	0 %
	✗	3 %	5 %
Sample B	✓	7 %	3 %
	✗	3 %	3 %
Sample C	✓	12 %	5 %
	✗	4 %	9 %

4. CONCLUSION

In conclusion, the application of MoS_2 based nanoplatform for the next generation of highly selective anti-cancer drug nanocarrier was demonstrated. The nanoplatform internalization was tracked and validated on cellular level by the label-free Raman imaging and CLSM. The increased selectivity of the antibody conjugated nanoplatforms was confirmed by the increased internalization in CAIX expressing cells. Further studies are required to improve the selectivity to cancer cells and validate the observed internalization in different cell lines.

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