Cost-Effective Synthesis of 2D Molybdenum Disulfide (MoS₂) Nanocrystals: An Exploration of The Influence on Cellular Uptake, Cytotoxicity, and Bio-Imaging

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Research Article

Keywords: MoS₂, 2D, cells, nanoparticles

Posted Date: December 23rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-130126/v1>

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Abstract

Ultra-small MoS₂ nanocrystals possess unique optoelectronic and catalytic properties, which have earned significant recognition and encourage their application in so many areas. Herein, we suggest a simple and cost-effective method to synthesize luminescent MoS₂ nanocrystals using the hydrothermal technique. Moreover, as-synthesized MoS₂ nanocrystals show size-dependent tunable photoluminescence. MoS₂ nanocrystals have many advantages, such as stable dispersion, low toxicity, and luminescent feature, offering them encouraging applicability in biomedical disciplines. In this study, the A549 cells are employed to assess the fluorescence imaging of MoS₂ nanocrystals. MTT assays, trypan blue assay, flow cytometry, and confocal imaging results revealed that the MoS₂ nanocrystals could selectively target and ruin lung cancer cells, especially drug-resistant cells (A549).

Introduction

Few-layered MoS₂ nanocrystal, one of the typical two-dimensional (2D) transition metal dichalcogenide materials, show the unique mechanical, optical, electrical, and chemical properties correlated with their ultrathin atomic layer structure, tendering them an appealing alternative to fluorescent dyes, and have attracted particular attention in the scientific uses. MoS₂ nanocrystals are flexible and operable for further modification, drug loading and controlled release because of the weak van der Waals interaction between its layers. It has been widely applied in medicine, drug delivery, diagnostics, and outstanding biocompatibility in living organisms.¹⁻²

Cancer is one of the most significant hurdles that endanger human health, among which lung is the most general reason for cancer-related mortality. Chemotherapy is still one of the commonly practiced healing modalities for cancer therapy over the former decades. However, chemotherapy yields some remedial obstacles, such as harsh adverse effects, low solubility, and the trend to cause drugs resistance. However, the nanotechnology trade is rapidly advancing, and nanoparticles spot applicability in various areas of our real world applications. Hence, the possibility of the communication of individuals to different kinds of nanoparticles is also proceeding. For this purpose, it is essential to study how nanoparticles can influence human bodies, as they can get there by breath, dermal touch, or ingestion. Although nanocrystals have been produced and applied for a few decades, their impact on health and the environment has not been deeply studied due to the complicatedness of the interplays of nanocrystals and their ingredients with cells.

The challenges in producing synthesis and the versatility of nanocrystals compositions and a broad spectrum of available of surface ligands are still there. Several nanoparticles such as carbon nanotubes, graphene, fullerenes, or quantum dots, have been synthesized as they manifest the encouraging potential to defeat the shortcomings of chemotherapy medicines for cancer treatment. Correlated to specific nanoparticles, two-dimensional (2D) nanoparticles occupy unique chemical, optical, and electronic characteristics and are therefore granted unique curative tools for biomedicine, particularly cancer

therapy. The bulk MoS₂ contains multilayered arrangements with weak van der Waals forces between layers and strong S-Mo – S interlayer covalent bonding. The inadequate van der Waals forces let peeling of the layered bulk crystals to top-down prepare mono or few-layer MoS₂. Hence, mechanical split, electrochemical intercalation, liquid-phase exfoliation, ultrasonic route, and hemolysis process have been investigated to produce mono or few-layer MoS₂. These methods also lack low productivity, complicated process, time waste, and severe restrictions. In addition, control over MoS₂ production through scalable route is needed to unravel its full potential. Thus, it is severe to produce new approaches to manufacture layer MoS₂ with the necessary size to study the consequent emerging optoelectronics properties.

Some compelling reports demonstrate that 2D nanoparticle remedy's promising potential in the practical section and targeted control of cancer healings ²⁷. More extra effort has been paid to search for other similar 2D materials relative to distinct unique properties. MoS₂ nanoparticle, as a variety of transition metal dichalcogenides (TMDCs), illustrated potential applications for nanoelectronic, energy storage devices, and electrochemical storage, catalysis, and sensing applications. Notwithstanding the remarkable progress with MoS₂ nanoparticle synthesis, it is necessary to produce a simplistic path for producing MoS₂ nanocrystals with steady fluorescence. MoS₂ is an excellent material having high dielectric constant, thin and large surface area that steadily increases the propagation path of light inside the sample. It also shows considerable formation of surface defects having Mo and S vacancies during synthesis process and acts as dipoles under the irradiation of light to boost interface polarization and defect dipole polarization for more attenuation of light. Over the past few years, the synthesis and use of atomically thin MoS₂ nanocrystals have extensive research attention in material science. ^{3–5}

Here, we show the synthesis and treatment of MoS₂ nanocrystals for the viability of A549 cancer cells. This work intends to show an eco-friendly, facile, and reproducible synthesis method based on hydrothermal process for the scalable production of MoS₂ nanocrystals (2–10 nm). This work explains preparing few-layer MoS₂ nanocrystals with a small size pattern through a one-step hydrothermal method utilizing sodium molybdate and thioacetamide as sodium and sulfur source materials, respectively. Cost-effective and facile approaches for MoS₂ nanocrystals controllable synthesis are quiet under essential demand, and the potential biomedical application of these MoS₂ nanocrystals should be further investigated. Excitation-dependent PL spectra are observed, and the red shifting of emission spectra is mainly founded due to the size effect. As the study on TMDs, nanomaterials' toxicity is still in its origin with hardly a few assessments conducted on a mono or few-layer TMDs (e.g. MoS₂, WS₂), it is not unexpected that no conforming researches have been carried to discover the toxicity of TMDs yet. Still, with the entrance of research and potential standardization TMDs in the prospect, it is necessary to begin studying the toxicological consequences of this assembly of nanomaterials to notify the health risks they may pretend.

This article details a straightforward, low-cost approach that employs an aqueous hydrothermal method for synthesizing two-dimensional molybdenum disulfide (MoS₂) nanocrystals and their potential

applications to explore cytotoxicity, bioimaging, and cellular uptake of A549 cancer cells. The high-resolution transmission electron microscopy (HRTEM) and atomic force microscopy (AFM) results revealed that the sizes of the as-grown polydispersed MoS₂ nanocrystals range between 2 and 5 nm; their corresponding thicknesses were verified to lie between 2 and 1 nm, a shred of clear evidence that a few-layer of MoS₂ nanocrystals had been synthesized. Photoluminescence (PL) and time-resolved PL spectra for the MoS₂ nanocrystals exhibited a strong emission in the blue region with a further slow decay constant.

Hence, in this report, the human lung carcinoma epithelial cell line (A549) after 24 h exposure to the MoS₂ nanocrystals was estimated and interpreted by applying the methyl-thiazolyl diphenyl-tetrazolium bromide (MTT) and water-soluble trypan blue assays. A549 cell line was favorably preferred for this research because the lungs are expected to be the first place in which TMD occupies and communicates with the whole body when breathed into the respiratory tract. MTT and trypan blue assays are founded cell viability assays that act in the same way. The number of cells surviving viable after nursing with the MoS₂ will be comparable to the formazan product's color intensity. By using both MTT and trypan blue assays in our research, we could be convinced that the cytotoxicity results are assured if the order collected from each assay were consistent and complemented each other. In this direction, we analyzed the sensitivity of A549 cells to the tested nanomaterials. We monitored cell viability with trypan blue and MTT assay tests. Reactive oxygen species (ROS) formation produced by MoS₂ nanocrystals was also studied. Our research is also based on morphological studies with the usage of microscopic study. The MoS₂ nanocrystals produced have excellent dispersal, small size, and PL features in aqueous suspension and encouraged biomedicine applications.^{6–8}

Results And Discussion

MoS₂ nanocrystals have been prepared using a one-step hydrothermal method utilizing sodium molybdate and thioacetamide as sodium and sulfur source materials, respectively (Fig. 1). The overall synthesis method is represented in Fig. 1. The synthesis process is addressed in detail in the experimental segment. To probe the optical properties, UV-vis absorption spectra of the nanocrystals and bulk MoS₂ were investigated (Fig. 2a). The excitonic peaks at positions A and B show the direct band-to-band transition at the K-point of the Brillouin zone. Furthermore, C and D's peaks show the direct transition from the split valence band to the conduction band at the Brillouin zone's M-point. The energy splitting within different absorbance peaks ("A & B" and "C & D") in bulk MoS₂ results from spin-orbit coupling and interlayer coupling. The energy splitting rises steadily with the reduction of layers number starting from the bulk sample. The absorption spectra of MoS₂ nanocrystal with a strong excitonic peak near the ultraviolet regime at 224 nm confirms the synthesis of a few nanometer particles.⁹ The crystal structure of bulk and nanocrystal MoS₂ was studied using the X-ray diffraction (XRD) technique, as shown in Fig. 2(b). The XRD spectra of bulk 2H-MoS₂ show an intense peak at $2\theta = 14.4^\circ$, which is assigned to (002) plane, along with other diffraction peaks, respectively (JCPDF-00-037-1492). Notably,

the peak of the (002) position of MoS₂ nanocrystals is weakened, symbolizing the lateral size reduction.¹⁰⁻¹¹ The reduction of intensity at (002) peak along the c-axis shows that the nanocrystals are few layers and too thin to be identified by XRD, harmonious with the AFM and TEM results. Figure 2(c) reveals the characteristic Raman spectra of bulk powder and as-synthesized nanocrystals. The E₁^{2g} Raman mode appears due to in-plane vibrations of S atoms corresponds to Mo atom, and A_{1g} mode results from out of plane vibration of S atoms only. The frequency difference (Δk) between the two Raman modes gives an idea about layer thickness. It has been seen that the Δk value for synthesized nanocrystals decreases to 24 cm⁻¹ as corresponded to bulk molybdenite powder having a Δk value of 26 cm⁻¹.^{4,11} However, the quantum size effect is accountable for tuning 2D TMDs nanocrystal's optical properties. The PL emission spectra of as-synthesized MoS₂ nanocrystals were studied under different excitation wavelengths ranging from 300 to 420 nm shown in Fig. 2(d). PL in MoS₂ nanocrystals arises due to the excitation recombination at the electron or hole trap formed by uncompensated positive or negative charge at the dangling bond. An intense emission peak is observed at 460 nm under an excitation wavelength of 320 nm, while the intensity of emission spectra is continuously reduced and redshifted with a further increase of excitation wavelength. Here, the excitation-dependent PL measurements prove the poly-dispersive nature of MoS₂ nanocrystals. The excitation-dependent spectra indicate polydispersity of the MoS₂ nanocrystals distributions, which is vital of excitation recombination at the electron (hole) trap formed by the uncompensated positive (negative) charge at the dangling bond from as-grown MoS₂ nanocrystals. This excitation-dependent PL response of fluorescent nanocrystals is useful for multicolor imaging purposes. As in earlier reports, the photoluminescence features of MoS₂ nanocrystals proportional to their particle dimension, which is related to the quantum size-effect of semiconductor few nanometers crystals. PL recombination dynamic of as-synthesized nanocrystals is ultimately measured to understand the mechanism of defect formation. The PL decay curve of MoS₂ nanocrystals is exhibited in Fig. 2(e). The photoluminescence decay of the as-grown sample is performed using a 321 nm laser LED excitation source. Instrumental response function (IRF) (shown by the green dotted line) was recorded using dilute Ludox colloid to maximize Rayleigh scattering and decrease the scattering effect from impurities, cuvette, and solution. The emission monochromator was fixed to the same wavelength as the excitation source (321 nm), and both polarizers were set to perpendicular to measure the IRF. It has been fitted with a third-order exponential equation

$$I = I_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + A_3 e^{-t/\tau_3}$$

with an average reduced weighted residual value of < 1.2, and the fitted curve (solid red and pink line) convoluted with IRF is shown in Fig. 2 (e).

The curve is furnished with a third-order exponential function, appearing in three governing excitonic phenomena accompanied by nanosecond luminescence lifetime. The average PL lifetime of the as-prepared MoS₂ nanocrystals was determined to be 5.615 ns. The increase in nanocrystals' average PL

lifetime is due to the defect state formation during synthesis.^{9, 13} The collected lifetime (τ_1, τ_2 and τ_3) and amplitude of i_{th} lifetime components (A_i) are shown in table 1.

Fig. 3(a) shows the HR-TEM image of MoS₂ nanocrystals presenting the hexagonal lattice structure. The MoS₂ nanocrystals with diameters of 2–10 nm are uniformly distributed; the size distribution of MoS₂ nanocrystals is sketched and shown in figure 3(b). The high-crystalline nature of the nanocrystals with lattice spacing of 0.2 nm matching to the very clear lattice fringes along with the (006) directions (shown in inset of figure 3(c)), this is indicative of the high crystalline order of the nanocrystals (shown in figure 3(c)). The atomic force microscopy (AFM) image was obtained to identify the morphology and the thickness of the MoS₂ nanocrystals, as shown in Fig. 3(e). The size distribution of as-synthesized nanocrystals lies between 2-10 nm (shown in figure 3(d)). The nanocrystal thickness ranges between 1 and 2 nm, indicating that the synthesized nanocrystals are of few-layer. The composition of as-prepared MoS₂ samples was studied by Energy-dispersive X-ray spectroscopy (EDAX). Fig.3f exhibits the EDAX image of MoS₂ nanocrystals. The EDAX study proved that the as-synthesized MoS₂ nanostructure comprises Mo and S elements, including oxygen atoms. Further, no other elements were found, which verified the purity of as-prepared samples.^{10, 13, 1}

Effect of MOS₂ on cell viability

To examine the cytotoxic effect of MOS₂, we have used trypan blue cell exclusion dye and MTT colorimetric assay. After incubation of 5,10, 20 µg/ml of MOS₂ for 24 hours, in the trypan blue method, we observed MOS₂ did not produce any significant cytotoxic effect on the A549 cell viability up to 10 µg/ml of concentration. However, there was a difference in the viable cell numbers (Fig. 4a) in control and the highest concentration used, i.e., 20 µg/ml of MOS₂. For further confirmation, we checked the effect of MOS₂ on the cell viability of A549 cells by MTT assay. The result showed a concentration-dependent toxic profile with maximum toxicity observed at the higher concentration used, a 60% reduction in cell viability, which corresponds to the decrease in the absorbance measurement, as shown in Fig. 4b. Therefore, both the cytotoxic assay confirmed the toxic response of MOS₂ at higher doses on the cell viability of A549 cells.^{15–19}

Effect of MOS₂ on production of ROS in A549 cells

Several studies suggested that ROS generation and oxidative stress production could be one of the underlying mechanisms, which leads to nanoparticle induced cytotoxicity in various types of cells. We analyzed the formation of reactive oxygen species in response to MOS₂, and we observed significant production of ROS inside the cells at higher doses of MOS₂ (Figure 5a-c). These results also correlate with our result of the particle's cytotoxic effect and could be the reason for cell death observed at higher doses.²⁰⁻²³

Cellular Uptake Of Mos By A549 Cell

To study the uptake of MoS_2 by the A549 cells, we employed flow cytometry after incubating the cells with MoS_2 . The flow cytometer can detect the light scattering signal and provide the cellular changes following the nanoparticle's incubation. The side scatters (SSC) value reflects the measurement at a 90° angle and correlates with the cell's concentration of bound or internal granularity. It has been studied that the SSC value correlates with the complexity and of internalized nanoparticles. Therefore, we compared the SSC value of both control and MoS_2 treated cells and observed an increase in the SSC value of nanoparticles treated cells for the control cells (Fig. 6a-d and Table 2). A notable variation in the SSC signal was recorded in treated cells in corresponding to the control (Fig. 6e-h). This increase in the SSC value is significantly higher (8.4% for 20 $\mu\text{g}/\text{ml}$ concentration) than control (3.2%) at the highest doses used in the study. These results indicated that MoS_2 nanoparticles could be taken up by the A549 cells and internalized inside the cells, which cause the ROS generation and influences cell viability. However, further study is needed to confirm a detailed picture of the internalization and localization of MoS_2 inside the cells and how exactly it is interfering and modulating the cell behavior.^{24–27}

Conclusion

This study used a one-step, bottom-up, hydrothermal route to synthesize blue luminescence MoS_2 nanocrystals using sodium molybdate dehydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) and thioacetamide (CH_3CSNH_2) as precursors. The as-prepared MoS_2 nanocrystals show a small lateral size distribution and good water solubility. Complete microscopic and spectroscopic techniques, including TEM, EDAX, AFM, XRD, UV-Vis, PL, TRPL, and Raman spectroscopy, were employed to confirm the morphology and composition of the MoS_2 nanocrystals. The PL properties, linked with the adequate biocompatibility and physiological stability of MoS_2 nanocrystals, directed to suitable bioimaging performance. Finally, cell viability measurements were performed with MTT and trypan blue assays after exposing human lung epithelial cell (A549) culture to varying amounts of MoS_2 nanocrystals for 24 h. Treatment of A549 cells with MoS_2 nanocrystals caused a dose-dependent increase in ROS formation up to 20 $\mu\text{g}/\text{ml}$. Eventually, as the toxicity studies of MoS_2 nanocrystals are still in its start, much labor will be needed from the scientific societies to resolve their health impacts in the long period and assure that the potential hazards are estimated before incorporating the MoS_2 nanocrystals into commercial goods.

Experimental Details

Materials and Reagents

Sodium molybdate dehydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) and thioacetamide (CH_3CSNH_2) were purchased from Sigma Aldrich. A549 Cells (a human alveolar epithelial cell line) was procured from American Type Cell Culture (ATCC).

Synthesis of MoS₂ nanocrystals

All the chemicals applied in the study were scientific-grade without additional refinement. The synthesis procedure details are as follows: 0.8 g of sodium molybdate dehydrate (Na₂MoO₄·2H₂O) was added into 50 ml of deionized water, and then 0.7 g of thioacetamide (CH₃CSNH₂) was mixed into the aqueous solution while stirring at room temperature. The solution mixture was carried into a Teflon-lined stainless steel autoclave loaded with the aqueous solution up to 60% of the full capacity, then sealed and kept at 200°C for 24 h. The collected black precipitates were centrifuged, cleaned with distilled water and ethanol five times, and then dried inside a vacuum oven at 60°C for 12 hours.

Characterization details

The UV-2401(Shimadzu Corporation) spectrophotometer was used to study the absorption spectra of as-synthesized nanocrystal and bulk powder. The crystal structure of the bulk powder and as-grown nanocrystal was examined through the Rigaku Miniflex diffractometer with typical X-ray tube (Cu K α radiation, 40 KV, 30 mA) and Hypix-400 MF 2D hybrid pixel array detector (HPAD) and the corresponding structure obtained from the analysis by High-score plus software. The size distribution and morphology of MoS₂ nanocrystals were checked in the non-contact mode by Park XE-70 atomic force microscope (AFM). Structural analysis was carried out using a transmission electron microscope (TEM) (Model JEOL JEM-2100F) performed at accelerating voltage 200 kV. TEM analysis was made by drop casting the diluted MoS₂ dispersion over the carbon-coated copper grid, followed by proper drying. The Raman spectra of the MoS₂ nanocrystal was taken with the Renishaw Raman microscope's help using 532 nm (0.3 mW) laser, 10-second scans acquired with the laser 20-x objective of an Olympus microscope. PL spectra were obtained with Fluoromax 4C HORIBA Scientific Spectro-fluorometer upon excitation of a spectrum of wavelengths using 450 W Xe lamp. The lifetime analysis was carried using the same HORIBA equipment with a PPD detector and nano led-320 excitation source (peak wavelength: 321 nm, pulse duration < 1.0 ns), and the result was interpreted using Data Station software.

PL decay profile was obtained by time-correlated single-photon counting (TCSPC) method to know the recombination mechanism of photo-excited charge carriers.

Cell culture

A549 cells was cultured in a humidified incubator at 37°C and 5% CO₂ and maintained in RPMI 1640 culture medium supplemented with 2 mM glutamine, 4.5g glucose per litre, 10 mM HEPES buffer pH 7.2, gentamycin (10 µg/ml), and fetal bovine serum (10% V/V).

In vitro cell viability assay

A549 Cells [0.3×10⁶/ml] were seeded in triplicate in 24 well culture plate, treated with 5,10 and 20 µg/ml of MOS₂ for 24 hrs, cells were harvested by trypsinization, and recoveries of trypan blue excluding viable cells proportion in the cell population was analyzed by cell counting using a hemocytometer. For MTT

assay, cells [10^4 /well] were seeded in a 96 well plate in triplicate for 24 hours and grown to 70 to 80 % confluence. The cells were then incubated with fresh media containing 5, 10, and 20 $\mu\text{g/ml}$ of MOS_2 for 24 hours. Cells with only RPMI media served as negative control. Following treatment, the cells were incubated with MTT (20 μL /well of 5 mg/mL stock) for 4 h. Mitochondrial dehydrogenases of viable cells reduce the yellowish water-soluble MTT to water-insoluble formazan crystals, which were solubilized with the addition of DMSO. The medium was then removed, and 150 μL of DMSO was added into each well to dissolve formazan crystals. Cell viability was measured by MTT assay, and absorbance was recorded at 560 nm.

Reactive oxygen species (ROS) measurement

To detect the production of ROS, A549 cells [0.5×10^6 /ml] were culture in each well of a 6 well plate, treated with or without 10 and 20 $\mu\text{g/ml}$ of MOS_2 for 24 hrs. Cells were washed with PBS twice and incubated with 3 μM of H2DCFDA dye in PBS for 30 minutes in the dark at 37⁰ Celsius. Production of ROS inside the A549 cell in response to MOS_2 was analyzed by NIS-Nikon fluorescence microscopy at 10X magnification.

Cellular uptake MOS_2 by A549 cells

To study the uptake of MOS_2 , cells [0.3×10^6 /ml] were cultured in each well of 12 well plates; after 24 hours of incubation, the cells were treated with or without 5, 10, and 20 $\mu\text{g/ml}$ of MOS_2 for 24 hours respectively. Then cells were trypsinized, washed twice with PBS, and the uptake was evaluated on a flow cytometer, BD FACS Verse.

Statistical Analysis

All experiments were executed in triplicate, and results were expressed in Mean $\pm$ SEM.

Declarations

Conflict of Interests:

The authors declare that they have no conflict of interests.

Data availability:

The data that confirm the findings of this research are accessible from the corresponding author upon reasonable interest. The data that carries the findings of this investigation are available inside the manuscript.

Acknowledgements

DS acknowledges the financial support by Shiv Nadar University, Dadri, Gautam Budha Nagar, Uttar Pradesh, India for the financial support through the scholarship.

Author contribution

D.S. imagined and designed the details of experimental plans. D.S handled experiment and characterization.

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Tables

Due to technical limitations, table 1,2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Schematic illustration of the steps for synthesis of spherical MoS₂ nanocrystals.

Figure 2

(a) UV-vis spectra of the as-prepared MoS₂ nanocrystals and bulk MoS₂. (b) XRD spectra (c) Raman spectra (d) Emission PL spectra of the as-prepared MoS₂ nanocrystals under different excitation wavelengths (e) Fluorescence lifetime of MoS₂ nanocrystals. The data are fitted using a tri-exponential decay model (black line) (f) Table shows PL lifetime (τ_1 , τ_2 and τ_3), and amplitude corresponding to different lifetime.

Figure 3

(a) HRTEM image of MoS₂ nanocrystals (b) shows the size distribution (c) show the lattice fringes (d) Corresponding AFM image of nanocrystals (e) Height profile corresponding the line in d and (f) EDAX spectrum of MoS₂ nanocrystals.

Figure 4

Comparative cell viability analyses on different cell lines using (a) Trypan blue and (b) MTT assay.

Figure 5

Effect of MoS_2 nanocrystals on ROS production in A549 cells (a) Control (b) 10 $\mu\text{g/ml}$ and (c) 20 $\mu\text{g/ml}$.

Figure 6

(a) Side scatter (SSC) of from control (a), 5 µg/ml (b), 10 µg/ml (c) and 20 µg/ml (d) subjects under flow cytometry. Flow cytometry analysis of the cell cycle distribution with different concentration of MoS₂.

Supplementary Files

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- [Table1andTable2.jpg](#)