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Utilization of Detonation Nanodiamonds: Nanocarrier for Gene Therapy in Non-Small Cell Lung Cancer

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Utilization of Detonation Nanodiamonds: Nanocarrier for Gene Therapy in Non-Small Cell Lung Cancer

by

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of the requirements for the degree of
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Dedication

To my late mother and amazing little sister,

Without whom I would not have the determination and drive to strive for the best.

Acknowledgments

The thanks I have is immeasurable!

I would like to thank my mentor, Dr. Allen-Gipson whose wisdom has allowed me to reach great heights and achieved many great accomplishments.

I would also like to thank my committee members, Dr. Kumar, and Dr. Tian, without their help I would not be where I am standing and where I am now.

Lastly, I would like to extend my thanks to the MSPN department,
whose assistance has helped this project take off and shore into great heights.

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Abstract

Lung cancer is one of the most prevalent and high mortality cancers in the world. Two different categories of lung cancer are non-small cell lung cancer which makes up 85% of cases, and small cell lung cancer which makes up the remaining 15% of cases. A significant risk factor that can lead to lung cancer is cigarette smoking (CS) however non-smoking cases i.e., secondhand CS, exposure to certain toxins and family history are on the rise. Both the main types of lung cancer have different system of stages, different ranges of treatment, and consequently low survival rates. Unfortunately, detection and confirmation usually are determined at stage four (IV) where the current treatments used are invasive with poor patient outcomes. Combined chemotherapy and radiation therapy are the primary treatment however have severe side effects as delivery of anticancer compounds to neoplastic tissue is important to limit toxicity as a result to targeting healthy cells. The demand for alternative treatment with minimal side effects is needed. Nanotechnology plays a role in enhancing efficient delivery of anticancer drug to the affected tissues by increasing the efficacy and reducing side effects. Presently, nanocarriers (NCs) have gained huge attention for their structural ability, good biocompatibility, and biodegradability. NCs fundamental goal is to tackle the delivery-related problems associated with standard chemo or radiation therapy and to carry medicines to the intended sites of action while avoiding undesirable side effects. Evidence suggest nanodiamonds (NDs) are potential new NCs for treatment for lung cancer. The objective of this study is to functionalize NDs as potential nano-drug carrier for gene therapy with minimized side effects and injury to health normal lung cells as well as to evaluate the functionalized properties of NDs as potential NCs for cancer gene therapy. We plan to purify

commercial-graded NDs via acid wash and conduct functionalization studies utilizing Fourier transform infrared spectroscopy (FTIR). The A549, human epithelial cancer cell line will, be used to demonstrate NDs potential for safe and effective NCs that can be used for future gene therapy targeting the tumor microenvironment

Chapter 1: Introduction

1.1 Lung cancer

Lung cancer is one of world's leading causes of death, affecting over 2 million people worldwide in 2020 [19]. It is estimated approximately 90% of lung cancer incident is correlated with cigarette smoke (CS) and is considered the main risk factor [10]. According to the World's Health Organization (WHO), approximately 11 trillion people are expected to engage in CS by 2025 predicting substantial increase in lung cancer cases [11]. As long as lung cancer cases are on the rise, the need to develop new, safe, and innovative treatment would be needed to combat the damage that lung cancer has inflicted on the general population. Nanomedicine based on nanotechnology is a new and innovated area of study that has gotten a reasonable amount of attention in the last few years as alternative treatment for anticancer agents.

1.2 Nanotechnology

Nanotechnology, also known as nanotech, is a branch of science and engineering devoted to the use of small size particles in nanometers (nms) ranging between 1 to 100 nm [1]. This miniature-size product possess ideal properties, including reduced degradation time and decreased toxicity, yielding application of pharmaceutical chemistry for the production of nanomedicine to treat many diseases, including cancer.

Different types of nanoparticles and formulations have been discovered, including gold nano shells, nanocrystals, carbon nanotubes, and polymer nanoparticles. [2]. Nanoparticles are ideal vehicles in delivering drugs to the cancer cells without damaging healthy cells. Though there

are many types of nanoparticles, an emerging diamond nanoparticle has been catching interest within the medical community for its versatility and functionality. Nanodiamonds (NDs), can bind to chemotherapeutic agents, possess excellent mechanical and optical properties, and have high and tunable surface area. Furthermore, these NDs have been employed in many different fields [14,1516]. Evidence suggest, NDs have exhibited excellent mechanical and optical properties, while maintaining their tunable surface structure, bio compatible and non-toxicity [3] this means that NDs are suitable carriers for treating lung cancer.

1.3 Nanodiamonds

NDs first made their appearance during the Cold War as a byproduct in the race for nuclear power [4]. NDs have different formulations and exist in many shapes and forms making them a diverse product to use. NDs can be grown using a ND seeding technique which involves coating a substrate with a large quantity of diamond particles until it reaches epitaxial growth, which is characterized as the process of depositing a thin layer of single crystal material over a single crystal substrate usually through chemical vapor deposition. NDs have been utilized in biological reconnaissance, mainly fluorescent NDs which has been used in several new innovative batteries [17,18]. NDs possess several properties that define it as one of the better choices for biological explorations, for example they are highly biocompatible, low in toxicity, as well as able to adhere multiple groups to its surface. They are also able to be synthesized at a relatively low cost and in large quantities, these properties allows it to be one step ahead of its other nanocarrier (NCs) [20]. One of the NDs greatest properties lies in its ability to serve as drug carriers, NDs are chemically inert and can enter the human body without prompting an immune response making them ideal vehicles/carriers for gene modulating agents and are capable to target specific cell type without

causing harm to other cells. My thesis proposal will focus on the innovative properties of NDs as a potential gene therapy agent and as a novel drug carrier.

1.4 Gene therapy

Gene therapy is an innovative treatment approach for many diseases which focuses on genetic modification by transplanting normal genes into cells in place of missing or defective one in order to correct genetic disorder or produce a therapeutic by repairing/reconstructing defective genetic material. Gene therapy was first described in 1928 by Frederick Griffith, a British bacteriologist who reported the "Griffith's experiment" in which mixed non-virulent R form of type 1 pneumococcus and heat inactivated form virulent S form of type 2 pneumococcus were put into mice. The end result was that the mice died due to the S form suggesting the R form of pneumococcus was converted to the S form and thus transforming from type 1 to type 2 [5]. Gene therapy is now the focal point of many research papers in medicine due to its relative infancy and potential in curing diseases, resulting in many discoveries. There have been studies that focus on the inherited bone marrow disorders as described by Pamela Becker [7], which states that there has major advances in the experimental process of gene therapy in bone marrow, with the most successful case of this is with children with x-linked severe combined immunodeficiency disease, the children were then treated with CD34+ cells that were coated in CH-296 fibronectin peptide and retroviral supernatant, this treatment has showed great success in that the children were able to improved T-lymphocyte and natural killer cell counts [6] . While gene therapy with Parkinson's disease [8], has assisted in producing therapeutic results in clinical trials consistently in recent years in the non-primate models, furthering the understanding of Parkinson's disease and developing potential treatments too [8]. Although gene therapy have many potential uses there are limitations particularly, utilization of viral vectors resulting in poor clinical trials outcome and

survival [14], thus halting gene therapy drug discovery. Therefore, attention is needed to find suitable vehicles/carriers, with the evolution of nanotechnology, NDs have proven to be potential (NC) for drug delivery eliminating the use of viral vectors.

1.5 Current Treatments and Drawbacks

Current existing treatments for lung cancer are chemotherapy (chemo and radiation) as well as other targeted therapeutic drugs (TTD) that include but not limited to Epidermal Growth Factor Receptor (EGFR), Kirsten Rat Sarcoma Viral homolog (KRAS), and Anaplastic Lymphoma Kinase (ALK) [11]. However, there are limitations with these current therapies due to drug resistance which have been observed in Tyrosine Kinase Inhibitors (TKIs) making treatment ineffective and due to the lack of new drug discovery to promote improved efficacy studies such as anti-KRAS [11]. Even though these are limitations with chemotherapy, the most common side effect is that these drugs also target healthy non-cancerous cells [13]. Therefore, it is imperative to utilize new treatment methods that have the capability of NDs to serve as a potential NC without evoking any immune responses while targeting cancer cells. In this proposal we plan to demonstrate the physical and chemical properties of NDs. We plan to characterize our commercially available NDs for purity and generate formulation to utilize as potential NC for application in gene therapy for lung cancer. Our findings will further support the use of nanomedicine to improve health outcomes and survival ship for patients with lung cancer.

1.6 Nanodiamonds and gene therapy/ rationale

The scope of both NDs and gene therapy has yet to be fully explored, this presents a great chance to utilize both areas in combination to test their capabilities in cancer research. It has already been proven that NDs do not educe cytotoxicity to cells when in a purified state, as such

it can be a very versatile material to work with in medicine. Gene therapy has been shown to help stop the progression of cancer by infecting cancer cells with genes that help the affected cells regain control of their functions. We propose to evaluate the utilization of NDs as potential targeting tool for gene therapy in cancer treatment.

1.7 Hypothesis and aims

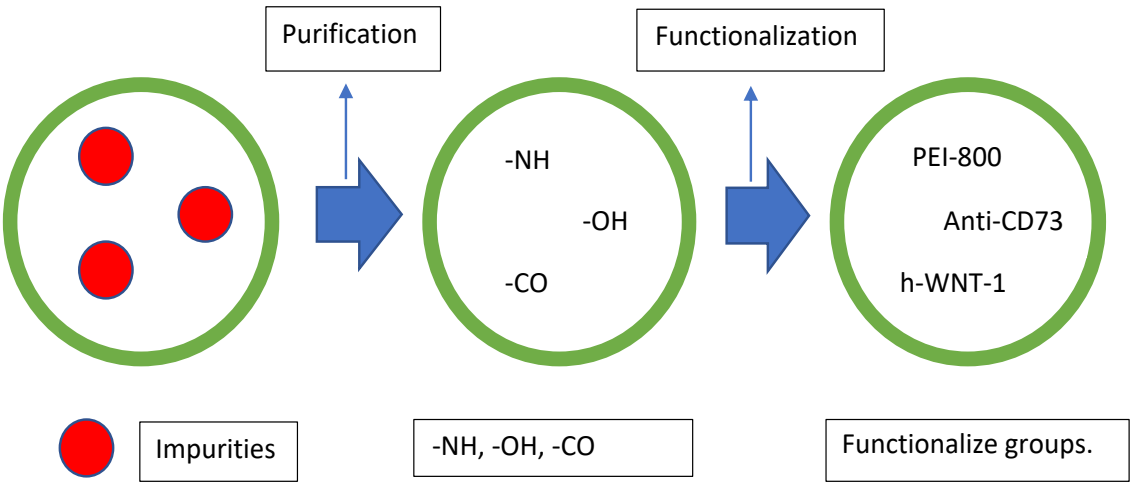

Figure 1: Experimental Illustration

Central Hypothesis: To Functionalize NDs Surface to Enhance Delivery of h-WNT-1 by Targeting CD73. This can be illustrated by Figure 1.

To address the central hypothesis, we proposed two aims:

Aim 1: To Determine Purification and Characterization of NDs

Aim 2: To Functionalize NDs Surface to Enhance Delivery of h-Wnt-1 by Targeting CD73

Chapter 2: Materials and Methods

2.1 Materials

Pre-purified nanodiamonds (P-NDs) were purchased from Advance Medical Supplier (La Mesa, CA). Unpurified nanodiamonds (UP-NDs), were provided as a gift from Dr. Ashok Kumar, and were from International Technology Center (Raleigh, NC). CD73 antibodies, and nitric fuming acid were purchased from Fisher Scientific (Hampton, NH). Hydrofluoric acid (48-52% concentration) was purchased from Grainger (Lake Forest, IL). Polyethyleneimine branched (MW: ~800 kDa), FBS, glutamine, penicillin, streptomycin, fetal bovine serum (FBS), 96 MTT tissue culture plates, MTT assay kit were purchased from Sigma Aldrich (St. Louis, MO). Human Epithelial Lung Cancer Cells, A549/ATCC®CCL-185TM, were purchase by American Type Culture Collection (Manassas, VA). Passage 25-32 grown in mammalian cell culture growth medium (Gibco, DMEM/F-12, no glutamine; Thermo Fisher Waltham MA) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin, at a temperature of 37 degrees Celsius (°C) until they reach a confluency rate of 85%-95%. All of machines are located at the University of South Florida (USF), Raman spectroscopy (Jasco Inc.) located at USF Nanotechnology Research and Education Center, Ultrasonic processor (Qsonica Q125 sonicators) located in Shriners Children Hospital at USF, Bransonic ultrasonic baths (Thermo Fisher) located in Shriners Children Hospital at USF, Sorvall RC-6 plus high-speed floor centrifuge machine (Thermo Fisher) located in Shriners children hospital at USF.

2.2 Methodology

Nanodiamond purification: Purification of the NDs were conducted following instructions using the methodology outline by V. Pichot et al. [21]. NDs were submerged in an acid bath consisting of hydrofluoric acid (HF) and fuming nitric acid (HNO3) for 6 hours.

2.2.1 Purification of Detonation Nanodiamonds

Purification of detonated NDs were conducted at room temperature. UP-NDs were acid wash as the first purification step. First, fuming nitric acid (HNO3; 0.75 ml; Fisher scientific, Hampton, NH) and hydrofluoric acid (HF; 0.25 ml; Grainger, Lake Forest, IL) were mixed along with the UP-NDs. To ensure that the NDs will go to the bottom of the beaker, deionized water (diH_2O) was added, constantly mixed for 30 minutes, and then allow to rest for 10 mins, this will cause the NDs to sink to the bottom (Figure 2), once the NDs have sank to the bottom of the beaker, the liquid layer was removed and resubmerge alone with the NDs and fresh diH_2O, to rise the pH level to 6-7. As the pH level start to increase from its initial level, the NDs solution will undergo a process known as turbidity, which is characterize as a cloudy solution that allows NDs to be suspended in solution resulting in loss of product. In order to separate the water and the NDs, we centrifuged the NDs solution at a speed greater than 10,000g force allowing NDs to stick to the bottom, we continued this process until a pH level of 6-7 was achieved. Once the pH level was verified, the solution was placed a heated water bath at 45°C to remove any residual water in NDs. NDs were allowed to be dried, crushed into a fine powder and were sealed. Ramon Spectroscopy was used to verify the purity of NDs, and surface characteristic spectroscopy was utilized in order to determine the surface characteristics of the NDs.

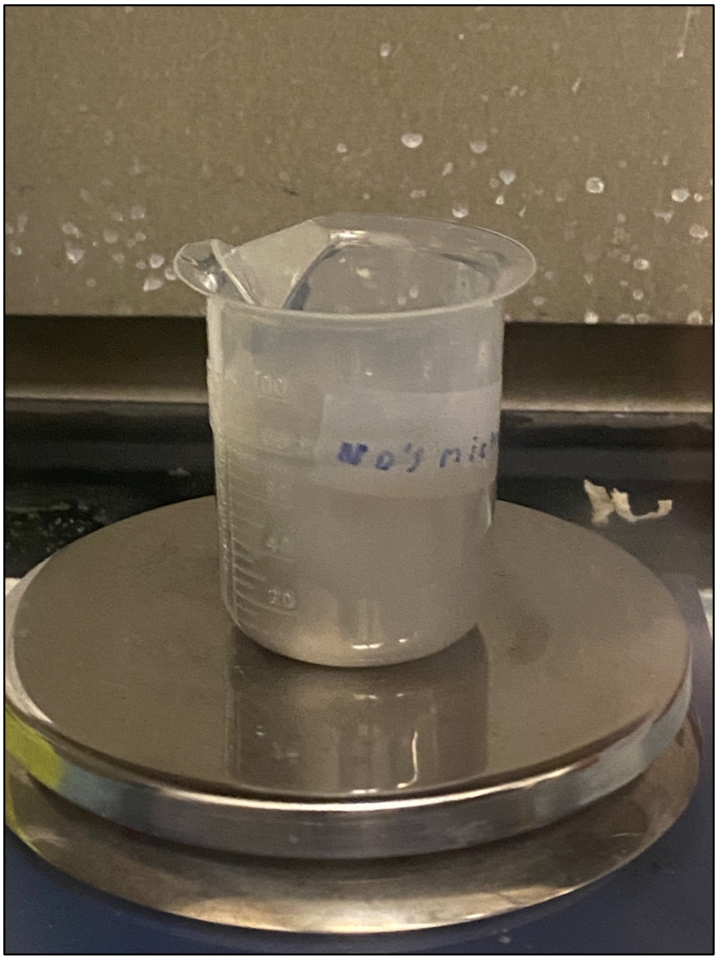

Figure 2*:* NDs Submerge in $HN0_3$, HF and diH_2O Solution.

2.2.2 Validation of NDs Purification via Raman Spectroscopy

After the NDs were dried, they were verified using Raman spectrometry, which is a non-destructive method of characterizing the surface of any samples (carbon, metal etc.). The NDs were placed on a slide and then in the Raman machine, after closing the door of the machine, a laser was shot into the NDs resulting in Raman scattering particles that were recorded, producing a graph which revealed the surface characteristics of the NDs.

2.2.3 MTT assay

In order to ensure that the purified NDs do not induce cytotoxicity, MTT was conducted to confirm the concentration of NDs that would be safe for our *invitro* cellular model, the human

bronchia; epithelial cancer cell line, A549. The A549 cells were seeded at 4x10^4 cells/ml in a 96-well plate. Concentration and time course experiments were conducted to determine optimal NDs concentration to used and treatment time. For our 72-hour study a cell count 2x10^4 cells/ml was used, 48-hour study is 2x10^4 cells/ml, and a 24-hour study is 4x10^4 cells/ml were used respectively. We used 0 - 400 ug/ml of NDs for each timeline to test the ability of ND uptake into the cell. Each well will have the pre-determine number of cells as indicated above based on their specific experimental time. Each experimental time (24, 48, and 72 hour) cells were allowed to adhere within the initial 24 hours to safeguard cells were properly attached to the cultured plates. Each plate was placed into an incubator and maintained at 37° C in the cell incubator for the appropriate amount of time. At the end of the experimental run time, the medium was removed and MTT solution was added 100 uL/well. At the end of each respective timeline, plates were read using Spectramax 190 and analyzed at 570 nm per vendor instructions.

Determination of ND concentration (MTT): We created a stock solution of 4 grams of NDs with 40 mL of Phosphate-buffered saline (PBS) to have a concentration of 4 mg/mL. We diluted the stock solution to obtain a 1:10 dilution of our working concentration by utilizing 1 mL of the stock (400 ug/ml) dilute with 9 mL of cell growth medium.

2.2.4 Functionalization of the NDs

Functionalization is the process of attaching groups to the surface of the NDs in order to implant a desired role, and for our purposes, we were implanting a target system while maintaining its ability as a gene delivery vehicle. Functionalization of NDs was carried out within a sanitized fume hood to avoid any contaminations from outside sources, 0.03 g of NDs were suspended in 30 mL of diH_2O, however, to avoid NDs cluster formation we combined use of a standard sonicator and ultra-process sonicator to break up these clusters. NDs and water

mixture were sonicated for 10 minutes, followed by the ultra-possess sonicator for 5 mins. The visible ND cluster(s) disappeared and NDs were ready for functionalization. In order to test the proposed vehicle, we tested all of the functionalized groups that were attached to the surface of the NDs by themselves as well as possible combination that could affect cell proliferation. In order to ensure proper testing, 10 ug in a 1:10 weight ratio each condition (ND only, Anti-CD73, h-WNT-1, Anti-CD73+h-WNT-1, NDPEI800, NDPEI800+Anti-CD73, NDPEI800+h-WNT-1, and NDPEI800+Anti-CD73+h-WNT-1) was used and conducted in quadruplets with initial seeding of 4x10^4 cells/ml per well and treated for 24 hours. After 24 hours, 100 ul/mL of the different test groups were pipetted into each well, making a grand total of 200 ul per well. 100 mL of the medium was removed and then were incubated with MTT reagent for 4 hours followed by an acid treatment in order to remove the phenol red in the medium.

Functionalization of NDs: Work was done in sanitized fume hood alone with ice since some agents required low temperatures, the ND-PEI800 was vortexed in order to ensure even distribution. Utilizing the (MW) of each functional group, we made samples that that had a weight value of 10 ug/ml each to ensure consistency. Then after sample were combined, they were vortexed for 1 min. to ensure proper distribution. All samples were then stored in a freezer at -4 ° C for future testing.

Preparation of NDs and PEI-800: Utilizing the (MW) of NDs and PEI800, we used this to determine the molar ratio at a 1:10 (respectively). Before then we had created a stock of NDs and diH2O (1mg/mL). After calculating, we had found that 0.150 mL of PEI800 alone with 1 mL of the ND+diH2O will be needed to create the molar ratio.

2.2.5 Statistical Analysis

Statistical Analysis: Statistics performed were a determine by conversion of known values into required values. For the determination of the P-value, utilizing the Microsoft Office Excel. Statistical t-test was determine using paired two samples for mean by comparing the control group to a test group.

Chapter 3: Results and Discussion

3.1 Purification of NDs

3.1.1 Raman spectroscopy

Raman spectroscopy was used to scan the surface of the NDs in order to prove a detail report of them. Three groups were scanned, the commercialized purified NDs which would serve as our positive control group, the un-purified NDs which would then serve as our negative control group, and the group of NDs that underwent our purification step will serve as our experimental group. All three of these groups will have sample pictures displayed on the top of the graphs to show how they look within the Raman spectroscopy. machine (Figures. 3, 5, 7, respectively).

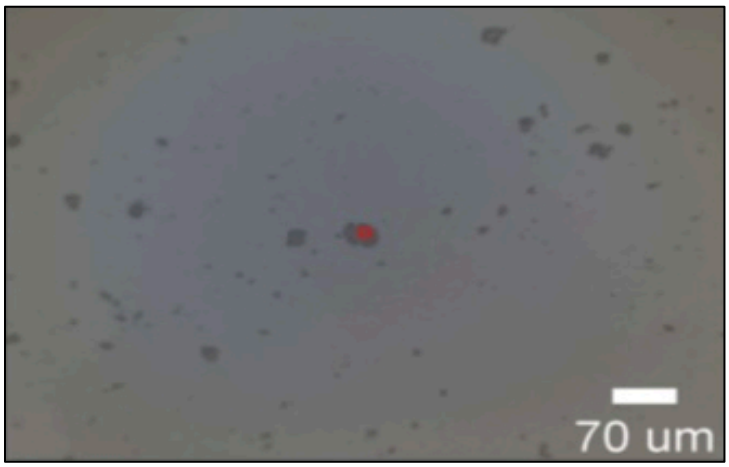

Figure 3: visual representation of inside the Ramen Spectroscopy machine with the commercialized purified NDs at 70 um.

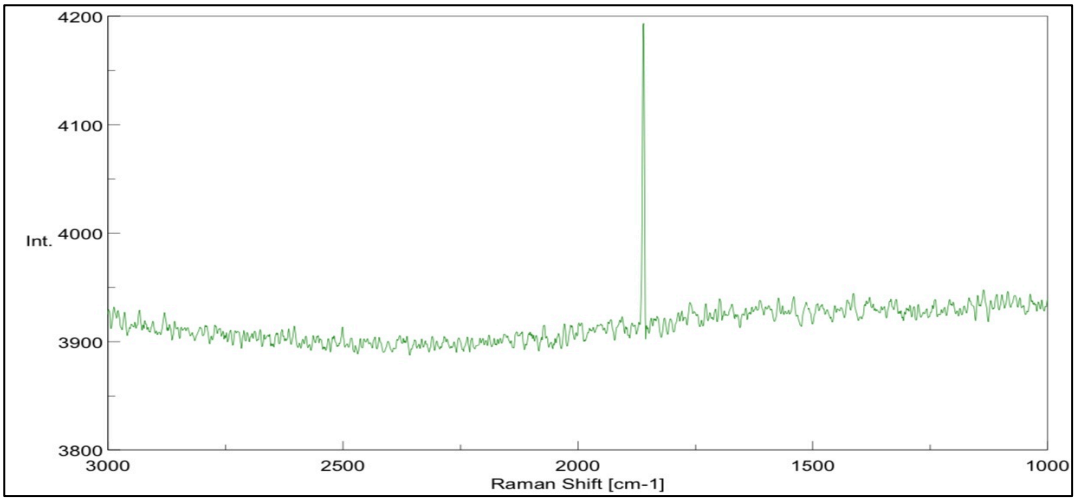

Figure 4: The purified NDs (positive control group), shown here is the Raman spectroscopy graph.

As defined by the graph Figure 4 this sample displayed a prominent peak with little noise. This shows that the surface has only one group present during the time of scanning. The NDs were bought pre-purified as to serve as our control group and will be used as our goal. Figure 3 represents what the positive control group NDs look like while inside the Raman spectroscopy instrument, they appear as small black dots.

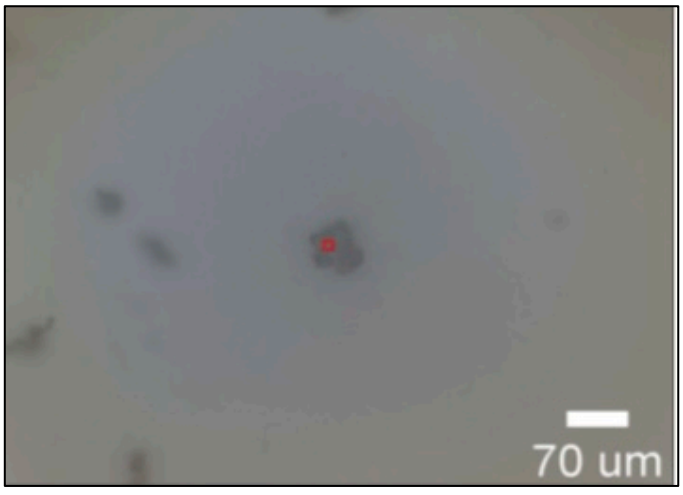

Figure 5: Visual representation of inside the Ramen Spectroscopy with unpurified NDs (negative control group) at 70 um.

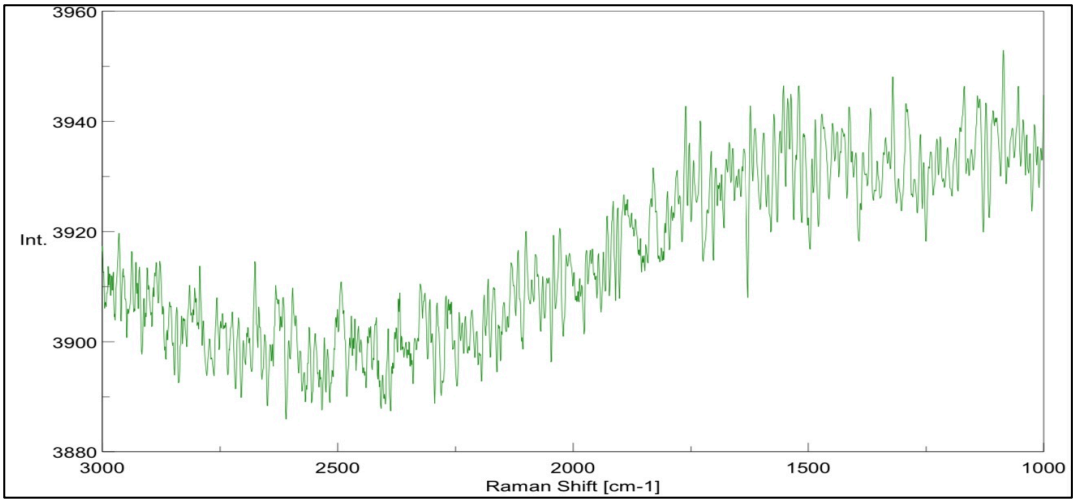

Figure 6: The unpurified NDs (negative control group) taken by the Raman Spectroscopy machine.

Figure 6 represents NDs which have not undergone any purification and as such are littered with impurities that can cause stress to the cells when introduced. The graph is characterized by jagged peaks, these interferences are due to numerous groups on the surface of the NDs as such no clear peak can be interpreted by the Raman spectroscopy. The purpose of the purification is to reduce the number of impurities on the surface of the NDs in order have them biologically safe.

Figure 7: Visual representation of inside the Ramen Spectroscopy with purified NDs (experimental group) at 70 um.

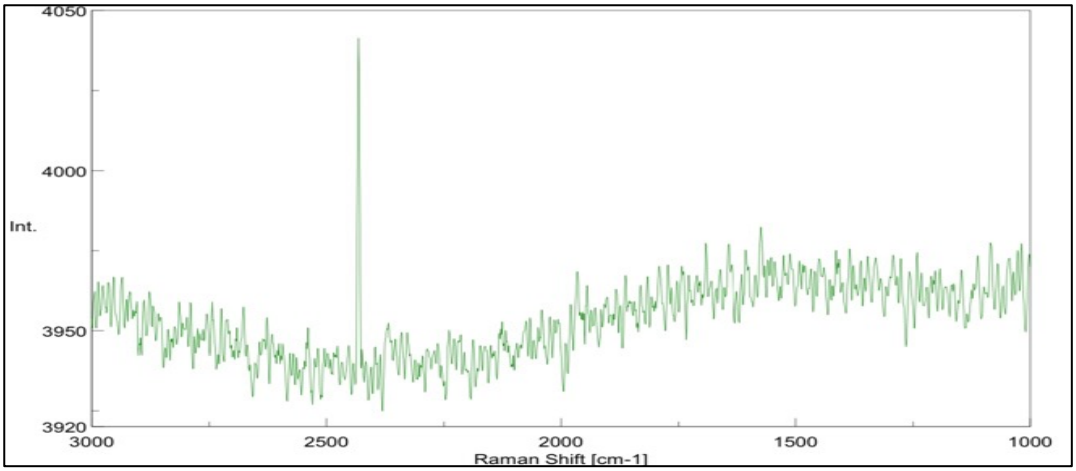

Figure 8: Unpurified NDs after the purification step, (experimental group) taken by Raman Spectroscopy.

As seen by the Raman spect graph Fiure.8, the jagged peaks were decreased, and a new prominent peak is visible with the reduction of noise the Raman spectroscopy graph has picked up. Given the information from the graph, the acid-wash was successful in reducing the level of impurities on the NDs surface to the point that a clear and define peak can be interpreted, but further purification will be required to see if impurities level could further decrease. Then looking at figure 7, as compared to both figure 3 and 5 respectively, these NDs appear larger and could factor in how the Raman Spectroscopy interpreted the results.

3.1.2 MTT assay

In order to understand and test whether or not the purified NDs can affect the proliferation of the cells, MTT was performed using these pre-purified NDs:

Figure 9: Time and Concentration study graph. A549 cells were treated with concentrations ranging from 0- 400 ug/ml of NDs for 24, 48, and 72 hours. These results demonstrated that there no significant toxic effect were observed in the cell proliferation in the different NDs treatment groups.

In our time and concentration study, as illustrated in graphs above, we had a range of concentrations from 10 ug/mL to 400 ug/mL for three different time ranges, 24 hrs, 48 hrs, and 72 hrs. observing our 24-hr. study we saw that the concentrations of 10 ug/ml, 75 ug/ml , 100 ug/ml 150 ug/ml, and 200 ug/mL had relatively the same level of proliferation as each other with all of them falling slightly below our control group. Concentrations of 50 ug/ml, 250 ug/ml, 300 ug/ml, 350 ug/ml, 400 ug/ml all had relatively the same proliferation with all of them roughly sharing the same level as the control group. The concentration of 25 ug/ml was the only one that displayed lower proliferation values then the rest, a possible explanation to this are that the cells were not seeded correctly and as such had affected their growth. The 48-hr. study and the 72-hr. study both displayed similarities between values, from the range of 25 ug/ml to 150 ug/ml showed same proliferation values while 10 ug/ml, 200 ug/ml-400 ug/ml all share similar proliferation values with the control.

3.2 Functionalization of NDs

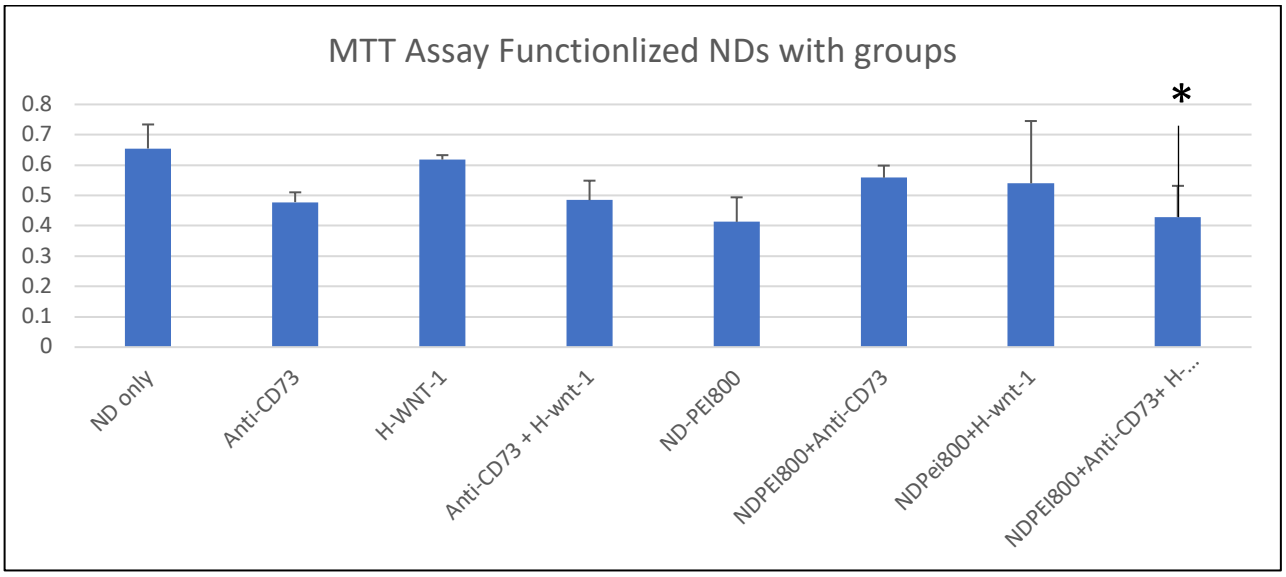

Figure 10: MTT assay of functionalized NDs at a concentration of 10 ug/ml per well, * p value < 0.05

To test if our targeted NC system could deliver the h-WNT-1 to the site where CD73 is over expressed, resulting in a better anti-cancer agent. We tested the A549 cell proliferation rate after treating with the functionalized NDs. The control group consisted of only NDs and the A549, while Anti-CD73 and h-WNT-1 were incubated with the A549, with one group dedicated to combining both agents, Anti-CD73+h-WNT-1, in our model cell. In comparison of the first three test groups, there is a notable decrease in cellular proliferation from the control ND-only group. Moving on to the next set of test groups, these were groups that had NDs that were functionalized with PEI-800 in addition to the different functional groups NDPEI800, NDPEI800+Anti-CD73, NDPEI800+h-WNT-1 and finally NDPEI800+Anti-CD73+h-WNT-1. While comparing the functionalized groups to the control group, we can tell that, just like in the case of the other groups, that cell proliferation had decreased. MTT assay results, shown on

figure 10, showed that by comparing our control group and our targeted vehicle group (NDPEI800+AntiCD73+h-WNT-1), the p-value came out to be 0.01. As the value is less than 0.05, the data is significant.

Chapter 4: Conclusion

The purpose of this experiment was to create a nanocarrier capable of targeting a specific biomarker while also able to deliver drugs. The targets of this study was CD73 which has been shown to be a prognostic biomarker as well as promoting the proliferation of cancer cells [22-23], and h-WNT-1 which is used to inhibited the WNT pathway that has been found cause militant transformation of mammalian cells .The use of the A549 cell line as our cancer cell model, which have been characterized to have an abundance of CD73 [22]. We have used the NDs as a base vehicle given its many properties including low cytotoxicity, low cost, great binding surface, and ability to carry therapeutic agents [15, 24-25]. Although the NDs themselves are non-toxic the impurities caused by the impact event to create them coats them in impurities that can cause oxidative stress to the cell, as such a purification is needed to remove the impurities from the surface. There are different approaches when it comes to the purification of NDs but most of these consists of specialized equipment that many may not have ready access to, as such, a lower cost and accusable method has been used to purify the NDs. Acid-washing of NDs has been found to remove carbon impurities while also functionalizing the surface with useful carboxyl groups that can help in binding drugs [21,45-46]. The first step was to determine the feasibilities of low-cost purification of NDs, with the utilization of two strong oxidative acids, impurities surrounding the surface of the NDs were cleaved off while also allowing for the enrichment of useful binding groups [21], utilizing this method can allow for basic equipped labs to perform purification at a low cost. Our characterization studies using the Raman Spectrometry revealed that acid washed purified NDs still had some impurities as compared to the

commercially purified NDs, shown by Figure 8 and 4, respectively, but in comparison to the unpurified state that they had previously occupied figure 6 there has been a significant reduction in impurities. Compared to out pre-bought purified NDs figure 4we saw that the NDs were on track to becoming purer, but further acid washes may be required in order to see. The next step was to see if NDs can induced cytotoxicity in cells given high concentrations. Several studies have reported using 100 ug/ml [26-28] had little to no toxicity, therefore we wanted to determine how many cells would be able to uptake NDs before cell death occurred. Given figure 9 revealed cell viability and growth was not impeded at any of the concentration (0-400 ug/ml), an explanation to this is that the ND passed through the cell without interfering heavily in its functions.

Functionalization is the process of surface modifications on a NP and in our case, NDs. For our functionalization studies we choose to focus on CD73, given its role in tumor microenvironment management [36-40] and h-WNT-1, with its involvement in uncontrollable proliferation of the cancer cells [41-44], which has increased their interest in lung cancer. We also utilized the PEG-8001 as this has been known to be a safe agent for binding via a polymer link on the surface of NDs [30,35]. In addition, we sought to create a specialized gene carrier that is able to seek out a particular cell type, able to bind to it and not release it until function has concluded.

Taking into consideration NDs surface properties, PEI800 has been attached and has been shown to be an anchor in which NDs were able to stick to the cell [29-32], by attaching Anti-CD73, which would serve as a targeting system by seeking out the high concentration produced by the A549 cells. Attaching the h-WNT-1, as our therapeutic agent, has been shown to be an effective oncogene agent [33] and therefore can help slow the growth progression of our cancer

cell model, A549. After utilizing the process of MTT, figure10 revealed that the proposed vehicle did inhibit cell growth, however, further testing is required to prove its effectiveness.

Future Prospects

In the future, we plan to conduct more test utilizing NDs as a novel vehicle in future *in-vitro* experiments, expanding our observations in vivo studies utilization of animal models such as mice to test out the capabilities of the NDs as a vehicle within a closed living environment. We also hope to further optimize this model in the efforts that we can further improve its function.

References

[1] Hulla J, Sahu S, Hayes A. Nanotechnology: History and future. Human & Experimental Toxicology. December 2015:1318-1321. doi:10.1177/0960327115603588 Google Scholar

[2] Ibrahim Khan, Khalid Saeed, Idrees Khan. Nanoparticles: Properties, applications, and toxicities. Arabian Journal of chemistry. March 2017: 908-931. https://doi.org/10.1016/j.arabjc.2017.05.011. Google Scholar

[3] Mochalin, V., Shenderova, O., Ho, D. et al. The properties and applications of nanodiamonds. Nature Nanotech 7, 11–23 (2012). https://doi.org/10.1038/nnano.2011.209 Google Scholar

[4] Danilenko, V.V. On the history of the discovery of nanodiamond synthesis. Phys. Solid State 46, 595–599 (2004). Google Scholar

[5] Wirth, Thomas, Nigel Parker, and Seppo Ylä-Herttuala. "History of gene therapy." Gene 525.2 (2013): 162-169. Google Scholar

[6] Griffith, F. (1928). The significance of pneumococcal types. Epidemiology & Infection, 27(2), 113-159. Google Scholar

[7] Becker, P. S. (2002). Hematopoietic stem cell gene therapy for inherited bone marrow disorders: past accomplishments and continued challenges. Journal of Cellular Biochemistry, 85(S38), 55-64. Google Scholar

[8] Fiandaca, M. S., & Bankiewicz, K. S. (2010). Gene therapy for Parkinson's disease: from non-human primates to humans. Current opinion in molecular therapeutics, 12(5), 519-529. Google Scholar

[9] Thomas, C. E., Ehrhardt, A., & Kay, M. A. (2003). Progress and problems with the use of viral vectors for gene therapy. Nature Reviews Genetics, 4(5), 346-358. Google Scholar

[10] Witschi, H. (2001). A short history of lung cancer. Toxicological sciences, 64(1), 4-6. Google Scholar

[11] Proctor, R. N. (2004). The global smoking epidemic: a history and status report. Clinical lung cancer, 5(6), 371-376. Google Scholar

[12] Daga, A., Ansari, A., Patel, S., Mirza, S., Rawal, R., & Umrania, V. (2015). Current drugs and drug targets in non-small cell lung cancer: limitations and opportunities. Asian Pacific Journal of Cancer Prevention, 16(10), 4147-4156. Google Scholar

[13] Zimmermann, S., Dziadziuszko, R., & Peters, S. (2014). Indications and limitations of chemotherapy and targeted agents in non-small cell lung cancer brain metastases. Cancer treatment reviews, 40(6), 716-722.Google Scholar.

[14] Tomanin, Rosella, and Maurizio Scarpa. "Why do we need new gene therapy viral vectors? Characteristics, limitations and future perspectives of viral vector transduction." Current gene therapy 4.4 (2004): 357-372. Google Scholar

[15] Bondon, N., Raehm, L., Charnay, C., Boukherroub, R., & Durand, J. O. (2020). Nanodiamonds for bioapplications, recent developments. Journal of Materials Chemistry B, 8(48), 10878-10896. Google Scholar

[16] Terranova, M. L., Orlanducci, S., Rossi, M., & Tamburri, E. (2015). Nanodiamonds for field emission: state of the art. Nanoscale, 7(12), 5094-5114. Google Scholar

[16] Khabashesku, V. N., Margrave, J. L., & Barrera, E. V. (2005). Functionalized carbon nanotubes and nanodiamonds for engineering and biomedical applications. Diamond and Related Materials, 14(3-7), 859-866. Google Scholar

[17] Qin, J. X., Yang, X. G., Lv, C. F., Li, Y. Z., Liu, K. K., Zang, J. H., ... & Shan, C. X. (2021). Nanodiamonds: Synthesis, properties, and applications in nanomedicine. Materials & Design, 210, 110091. Google Scholar

[19] World Health organization (WHO), Cancer, accessed on 10/23, updated on: 02/03/22, Web Linked

[20] V. Mochalin, O. Shenderova, D. Ho and Y. Gogotsi, The properties and applications of nanodiamonds, Nano-Enabled Medical Applications 2020 Pages 313-350 Google Scholar

[21] Pichot, V., Comet, M., Fousson, E., Baras, C., Senger, A., Le Normand, F., & Spitzer, D. (2008). An efficient purification method for detonation nanodiamonds. Diamond and Related Materials, 17(1), 13-22. Google Scholar

[22] Gao, Z. W., Liu, C., Yang, L., Chen, H. C., Yang, L. F., Zhang, H. Z., & Dong, K. (2021). CD73 severed as a potential prognostic marker and promote lung cancer cells migration via enhancing EMT progression. Frontiers in Genetics, 12, 728200. Google scholar

[23] Zhang, H., Cao, Y., Tang, J., & Wang, R. (2022). CD73 (NT5E) Promotes the Proliferation and Metastasis of Lung Adenocarcinoma through the EGFR/AKT/mTOR Pathway. BioMed Research International, 2022. Google Scholar

[24] Schrand, A. M., Hens, S. A. C., & Shenderova, O. A. (2009). Nanodiamond particles: properties and perspectives for Bioapplications. Critical reviews in solid state and materials sciences, 34(1-2), 18-74. Google Scholar

[25] Mochalin, V. N., Shenderova, O., Ho, D., & Gogotsi, Y. (2012). The properties and applications of nanodiamonds. Nature nanotechnology, 7(1), 11-23. Google Scholar

[26] Schrand, A. M., Huang, H., Carlson, C., Schlager, J. J., Ōsawa, E., Hussain, S. M., & Dai, L. (2007). Are diamond nanoparticles cytotoxic? The journal of physical chemistry B, 111(1), 2-7. Google Scholar

[27] Liu, K. K., Cheng, C. L., Chang, C. C., & Chao, J. I. (2007). Biocompatible and detectable carboxylate nanodiamond on human cell. Nanotechnology, 18(32), 325102. Google Scholar

[28] Solarska-Ściuk, K., Gajewska, A., Glińska, S., Michlewska, S., Balcerzak, Ł., Jamrozik, A., ... & Bartosz, G. (2014). Effect of functionalized and non-functionalized nanodiamond on the morphology and activities of antioxidant enzymes of lung epithelial cells (A549). Chemico-biological interactions, 222, 135-147. Google Scholar

[29] Zhang, X. Q., Chen, M., Lam, R., Xu, X., Osawa, E., & Ho, D. (2009). Polymer-functionalized nanodiamond platforms as vehicles for gene delivery. ACS nano, 3(9), 2609-2616. Google Scholar

[30] Kim, H., Man, H. B., Saha, B., Kopacz, A. M., Lee, O. S., Schatz, G. C., ... & Liu, W. K. (2012). Multiscale simulation as a framework for the enhanced design of nanodiamond-polyethylenimine-based gene delivery. The journal of physical chemistry letters, 3(24), 3791-3797. Google Scholar

[31] Neuhöferová, E., Křivohlavá, R., Jakobsen, K. Q., & Benson, V. (2022). Interaction of non-targeted nanodiamond carriers with specialized rodent cells: preliminary ex vivo and in vivo study. Journal of BioScience and Biotechnology, 11(1), 23-29. Google Scholar

[32] Chipaux, M., van der Laan, K. J., Hemelaar, S. R., Hasani, M., Zheng, T., & Schirhagl, R. (2018). Nanodiamonds and their applications in cells. Small, 14(24), 1704263. Google Scholar

[33] Polakis, P. (2012). Wnt signaling in cancer. Cold Spring Harbor perspectives in biology, 4(5), a008052. Google Scholar

[34] Polaris, P. (2012). Wnt signaling in cancer. Cold Spring Harbor perspectives in biology, 4(5), a008052. Google Scholar

[35] Křivohlavá, R., Neuhöferová, E., Jakobsen, K. Q., & Benson, V. (2019). Knockdown of microRNA-135b in mammary carcinoma by targeted nanodiamonds: potentials and pitfalls of in vivo applications. Nanomaterials, 9(6), 866. Google Scholar

[36] Kowash, R. R., & Akbay, E. A. (2023). Tumor intrinsic and extrinsic functions of CD73 and the adenosine pathway in lung cancer. Frontiers in immunology, 14, 1130358. Google Scholar

[37] Han, Y., Lee, T., He, Y., Raman, R., Irizarry, A., Martin, M. L., & Giaccone, G. (2022). The regulation of CD73 in non-small cell lung cancer. European Journal of Cancer, 170, 91-102. Google Scholar

[38] Bertolini, G., Compagno, M., Belisario, D. C., Bracci, C., Genova, T., Mussano, F., ... & Roato, I. (2022). CD73/adenosine pathway involvement in the interaction of non-small cell lung cancer stem cells and bone cells in the pre-metastatic niche. International Journal of Molecular Sciences, 23(9), 5126. Google Scholar

[39] Giatromanolaki, A., Kouroupi, M., Pouliliou, S., Mitrakas, A., Hasan, F., Pappa, A., & Koukourakis, M. I. (2020). Ectonucleotidase CD73 and CD39 expression in non-small cell lung cancer relates to hypoxia and immunosuppressive pathways. Life sciences, 259, 118389. Google Scholar

[40] Xia, C., Yin, S., To, K. K., & Fu, L. (2023). CD39/CD73/A2AR pathway and cancer immunotherapy. Molecular Cancer, 22(1), 1-17. Google Scholar

[41] Kansara, M., Tsang, M., Kodjabachian, L., Sims, N. A., Trivett, M. K., Ehrich, M., ... & Thomas, D. M. (2009). Wnt inhibitory factor 1 is epigenetically silenced in human osteosarcoma, and targeted disruption accelerates osteosarcoma genesis in mice. The Journal of clinical investigation, 119(4), 837-851. Google Scholar

[42] Luo, X., Ye, S., Jiang, Q., Gong, Y., Yuan, Y., Hu, X., ... & Zhu, W. (2018). Wnt inhibitory factor-1-mediated autophagy inhibits Wnt/β-catenin signaling by downregulating dishevelled-2 expression in non-small cell lung cancer cells. International Journal of Oncology, 53(2), 904-914. Google Scholar

[43] Mazieres, J., He, B., You, L., Xu, Z., Lee, A. Y., Mikami, I., ... & Jablons, D. M. (2004). Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. Cancer research, 64(14), 4717-4720. Google Scholar

[44] Rubin, E. M., Guo, Y., Tu, K., Xie, J., Zi, X., & Hoang, B. H. (2010). Wnt Inhibitory Factor 1 Decreases Tumorigenesis and Metastasis in OsteosarcomaWIF-1 Modulates Tumor Progression in Osteosarcoma. Molecular cancer therapeutics, 9(3), 731-741. Google Scholar.

[45] Rouhani, P., Govindaraju, N., Iyer, J. K., Kaul, R., Kaul, A., & Singh, R. N. (2016) Purification and functionalization of nanodiamond to serve as a platform for amoxicillin delivery. Materials Science and Engineering: C, 63, 323-332. Google Scholar.

[46] Sushchev, V. G., Dolmatov, V. Y., Marchukov, V. A., & Veretennikova, M. V. (2008). Fundamentals of chemical purification of detonation nanodiamond soot using nitric acid. Journal of Superhard Materials, 30(5), 297-304. Google Scholar.