INCORPORATION OF POLYOXOMETALATE WITH GELATIN ANDTHEIR FEASIBIITY AS AN ANTI-CANCER DRUG

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INCORPORATION OF POLYOXOMETALATE WITH GELATIN ANDTHEIR FEASIBIITY AS AN ANTI-CANCER DRUG

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Abstract

Polyoxometalates (POMs) are a class of transition-metal oxide clusters with significant applications in material science as well as in biomedical field. Recently, it has been reported that POMs exhibit antitumor activity. POMs containing molybdenum (Mo), tungsten (W), and vanadium (V) with oxygen are three types of POMs with different structural features introduced by our lab as a novel inorganic chemotherapeutic agent for cancer treatment. In this study, Vanadium polyoxometalate (V-POM), which exhibited highest cytotoxicity in 5 different cancer cell lines in our previous *in vitro* experiment, was incorporated with Gelatin type B through polymer modification in hopes of prolonging circulation time and avoiding undesired toxicity. The stability, cellular uptake, and cytotoxicity of the V-POM/gelatin nanoparticles were investigated *in vitro*. The reported study indicates the feasibility of using V-POM/gelatin nanoparticles for anticancer applications.

1. Introduction

Despite the many progresses that were made in medical field last decades, providing effective treatment for cancer still remains as unsolved challenge. As baby boomers are slowly but surely reaching end of their years and as death due to cancer reached 2nd highest in cause of death in United States, right after heart diseases, development of a novel treatment for cancer remains as utmost desired, yet challenging pursuit. Most of the cancer therapeutics that are mainly used and studied is organic compounds. Interestingly enough, cisplatin, currently the biggest selling anti cancer drug, is an inorganic complex.

With that considered, there are merits in studying POMs as a novel inorganic compound for anti-cancer applications.

Polyoxometalates are a transition-metal oxide clusters formed of metal cations connected by oxide anions. Numerous POMs has been synthesized and evaluated, but its potential application in medical field is still rudimentary. Interesting characteristics of POMs are its high antitumoral properties. In the studies, anti-tumor activity of $[Mo_7O_{24}]^{6-}$ was found to be better than that of the most prevalent drug, cisplatin. Polyoxometalate synthesized in our lab, $\{V_6\}$ triesters POMs, also showed better antitumoral activities in inducing tumor growth in *in vivo* model. Despite its significant potency, there has only been one polyoxometalate compound that has been tested on human subject, and due to the high toxicity associated with the compound, its potential application in medicinal field has drastically retracted. The side effects associated with application of POMs is likely due to its instability in water at physiological pH, which results in degradation of polyoxmetalates into smaller inorganic products. Its cellular toxicity may also contribute in deterring its application. However, by combining POMs with the biopolymer, such as gelatin, it is possible to improve its physiological stability as well as its toxicity through the modification of its surface charge, polarity, redox potential, etc.

Gelatin is a well-known natural polymer that has ideal properties in synthesizing Gelatin/POM nanoparticles. With both amino and carboxyl groups on its chain, it is highly biocompatible, since similar proteins are also synthesized in our bodies, and its zwitterionic nature allows simple procedure of polymer modification without large amount of surfactant or harmful organic solvents. Because of the zwitterionic nature of gelatin, the positively charged portion of the gelatin forms hydrophobic complex with

anionic V-POM while the anionic part of the gelatin stabilizes the newly formed particle through electrostatic repulsion.

In this study, V-POMs are dye labeled with Alexa Fluor® 488 Dye (Life Technologies Corp.) for cellular internalization purposes, which results in POM conjugates with 4 negative charges. For Gelatin, bovine, type B with approximately 225 g bloom, about average molecular weight of 50,000 is used. These particles are used to form polymer/POM hybrid nanoparticles through polymer modification where its improvement on stability, cytotoxicity, and internalization will be measured and compared to V-POM alone.

2. Experimental

2.1 Preparation of Gelatin/POM nanoparticles

Three ratios of Gelatin/POM nanoparticles are synthesized: 1:0.5, 1:1, and 1:2. Gelatin solution (60 ml), containing 50 mg of gelatin was prepared and warmed to 70ºC. An aqueous solution of V-POM (2 ml) was prepared by dissolving appropriate amount of POMs (based on the ratio) in the mixture solution of 0.05 ml of DMSO, and 1.95 ml of water. Aqueous V-POM was then added to gelatin solution dropwise at 0.1 ml/hr. During the process, the mixture was continually stirred at 500 rpm and kept at 70 ºC. After 24 hours of stirring, the mixture was put in freezer for next 24 hours. The gelatin/POM hybrid mixture was then put in process of lyophiliazation to remove all the water and obtain gelatin/POM hybrid nanoparticles in solid form.

2.2 Stability

Stability of gelatin/POM nanoparticles and V-POM was measured by a UV spectrometer (UV0810M232, CARY®). Gelatin/POM hybrid nanoparticles and V-POM were diluted

in phosphate buffered solution (1.5ml). Because V-6 POM does not readily dissolve in PBS, minimal amount of DMSO was added to dissolve V-POM. Same amount of DMSO were added to hybrid nanoparticles for control. Each solution was further diluted until its maximum absorbance was between 0.8 to 1. The absorbance was measured every other day for a week. The wavelength was set between 200 nm and 800 nm and PBS was used as blank. All the solutions and control were kept at 32 ºC.

2.3 Cytotoxicity

Crystal violet assay was used to evaluate cytotoxicity of hybrid nanoparticles in comparison to POM alone. SKOV-3 human ovarian carcinoma cell line was used in this *in vitro* study, since V-POM showed highest anti-cancer activity against this cancer cell line. SKOV-3 cell line was maintained in McCoy's 5A medium (Cellgro®, Manassas, VA) supplemented with 10% FBS (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 100 U/mL penicillin and 100 µg/mL streptomycin (cellgro®, Manassas, VA) . The sample size was set as n = 4 and nanoparticles and POMs were diluted to 2, 20, and 200 µM with culture medium. With 12 wells as control, total of 60 wells of well plates were used. For cell seeding, 4,000 cells were seated in 100 µl of McCoy'5A per well. After 24 hours, 100 µl of drug was treated to the treatment group and 100 µl of McCoy was treated to control group. After 72 h-incubation, the plate was washed and fixed with 4% formaldehyde for 10 minutes. After washing with PBS, it was stained with 0.5% crystal violet. After 20-30 minutes of incubation at RT, it was washed with tap water twice. After air drying for a day, the dye is solubilized with Sorenson's buffer and the absorbance was read by a microplate reader (Synergy 2, BioTek®)at 570 nm.

2.4 Internalization

For internalization, an 8-chamber slide (Lab Tek™, Naperville, IL) was used. The 4,000 cells were seated per well. After 24 hours of incubation, each well was treated with Alexa 488 Dye-labeled nanoparticles or Alexa 488 Dye-labeled POM at 0.5 or 1 μM. After 72 h-incubation, the plate was washed and fixed with 4% formaldehyde. After that, the slide was mounted with Prolong Gold® Antifade Reagent with DAPI (Life Technologies Corp,). Visualization of internalization was observed under a fluorescence microscope (IX71, Olympus®).

3. Results and discussion

3.1 Preparation of gelatin/POM nanoparticles

Because gelatin has zwitterionic properties while POM has anionic properties, positive part of gelatin and anionic nature of POM forms hydrophobic complex through electrostatic attraction while the anionic part of the gelatin provides stabilization through electrostatic repulsion. Conceptually, by controlling the surface charge of the POM, proper hybrid nanoparticles should form and cellular toxicity should decrease. Further study would involve characterizing the nanoparticles, including its relative size and pH based on the ratio and visualization through transmission electron microscopy.

3.2 Stability

For the stability, two of the nanoparticles with ratio of 1:1 and 1:2, clearly demonstrated overall improvement in stability while nanoparticle with ratio 1:0.5 and POM alone stability seemed similarly distraught. Nanoparticle with ratio of 1:2 definitely showed highest stability, as the highest peak remained in same wavelength and second peak remained insignificant. For Nanoparticle with ratio of 1:1, the wavelength of highest peak remained constant but the absorbance increased by 20% over the period of 7 days. For

nanoparticles with 1:0.5 ratio, the second highest peak increased by several fold by day 7 where its absorbance surpassed that of the original highest peak. There seemed to be overall trend that with higher the ratio of POM to Gelatin, better the stability. Further study may include synthesis of nanoparticles with ratio of 1:5, 1:10, etc.

3.3 Cytotoxicity

For the cell viability test, overall, all three ratios of nanoparticles performed better than V-POM alone. Similar to the result of stability, nanoparticle with ratio of 1:2 induced greatest cell death in both 1 μM and 10 μM. At the concentration of 10 μM, the nanoparticle with ratio of 1:2 induced cell death by approximately 80% on average, while 75% of the cells in V-POM treatment group died on average. For 1 μM, which was the lowest concentration, 1:2 induced cell death by 75 % on average, while V-POM at the lowest concentration only induced cell death by 50%, which showed at lower concentration, nanoparticles clearly outperformed V-POM alone. Statistically, the p-value is less than 0.05, which means the difference is considered to be statistically significant. Strangely, nanoparticle with 1:2 ratio did not perform so well on highest concentration of 100 μM compared to other nanoparticles and V-POM. (Note for this part of the experiment, equal amount of POMs in nanoparticles were treated to cells, so that equal amount of V-POMs in nanoparticles and POM are treated to cells.) Further study include toxicity test of nanoparticles to normal cell line must be conducted to evaluate the cellular toxicity the POM possess and ability of polymer modification to deduce the toxicity.

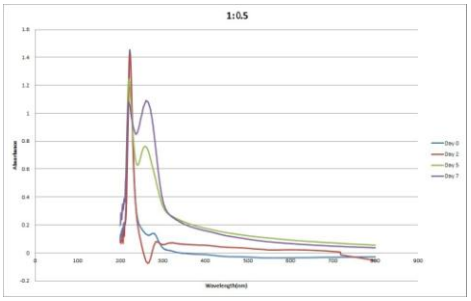
3.4 Internalization

For the internalization, the green images display the nanoparticles inside and outside the cell while blue image display the nucleus of the cell. The superimposed image reveals that the fluorescently dyed nanoparticles and V-POM is in cytoplasm and inside the nucleus. Not surprisingly, nanoparticle with ratio of 1:2 exhibited highest internalization. Compared to images of other nanoparticles and complex at 0.5 μM, the background of the fitc image of 1:2 ratios is dark, while other has green hues. Assuming no leakage has occurred and drug is well spread inside the well, because equal amount of fluorescently dyed POM entered the well, it is safe to assume that most of the drug has entered the cancer cells. Correlation with the other 2 results revealing optimal result in nanoparticle with ratio of 1:2 further supports the need of further need in synthesizing and evaluating the efficacy of nanoparticles with higher ratio in POM.

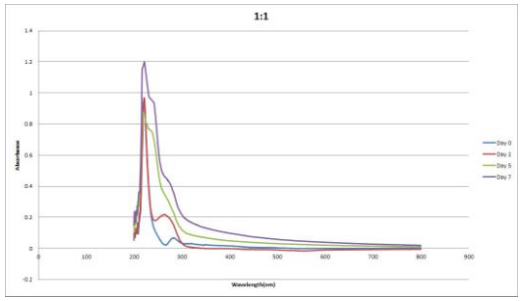
4. Conclusion

As cancer will likely be a rising problem in the medical field, need for early detection method and innovative treatment is needed. Polyoxometalate is a complex that is novel in field of medicine. Although it has failed human trial due to the associated side effects, other studies and this paper show that the associated problems could be severely diminished through polymer incorporation. By utilizing flexible properties of polymers, such as gelatin, the stability could be highly prolonged while increasing anti-cancer effect and internalization. The actual cause of cell death due to POM is still unidentified; therefore, further study must be done on the exact mechanism behind how it induces cell death. Through understanding of its mechanism, it may be possible to have better control over its properties.

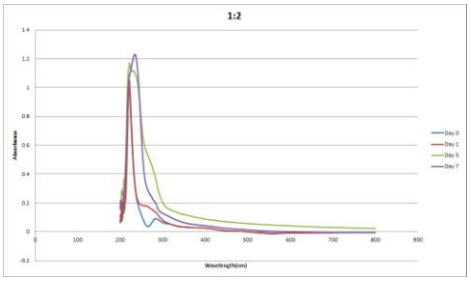
FIGURE I: STABILITY



(a)



(b)



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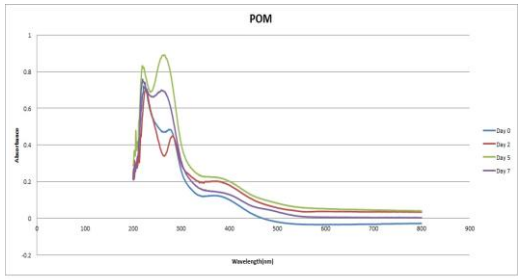


(d)

Figure 1. Stability of (a) 1:.05, (b)1:1, (c) 1:2, (d) POM over wavelength of 200nm to 800nm. First peak on all four nanoparticles were diluted until absorbance was in range of .6 to 1. Nanoparticles 1:2 demonstrated best stability over period of 7 days.

Figure II: Cytotoxicity

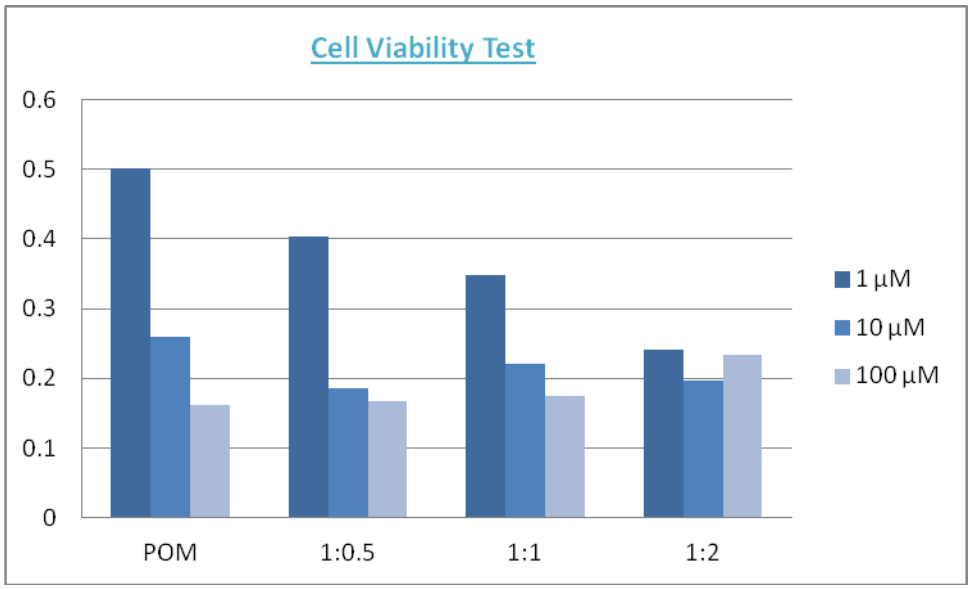
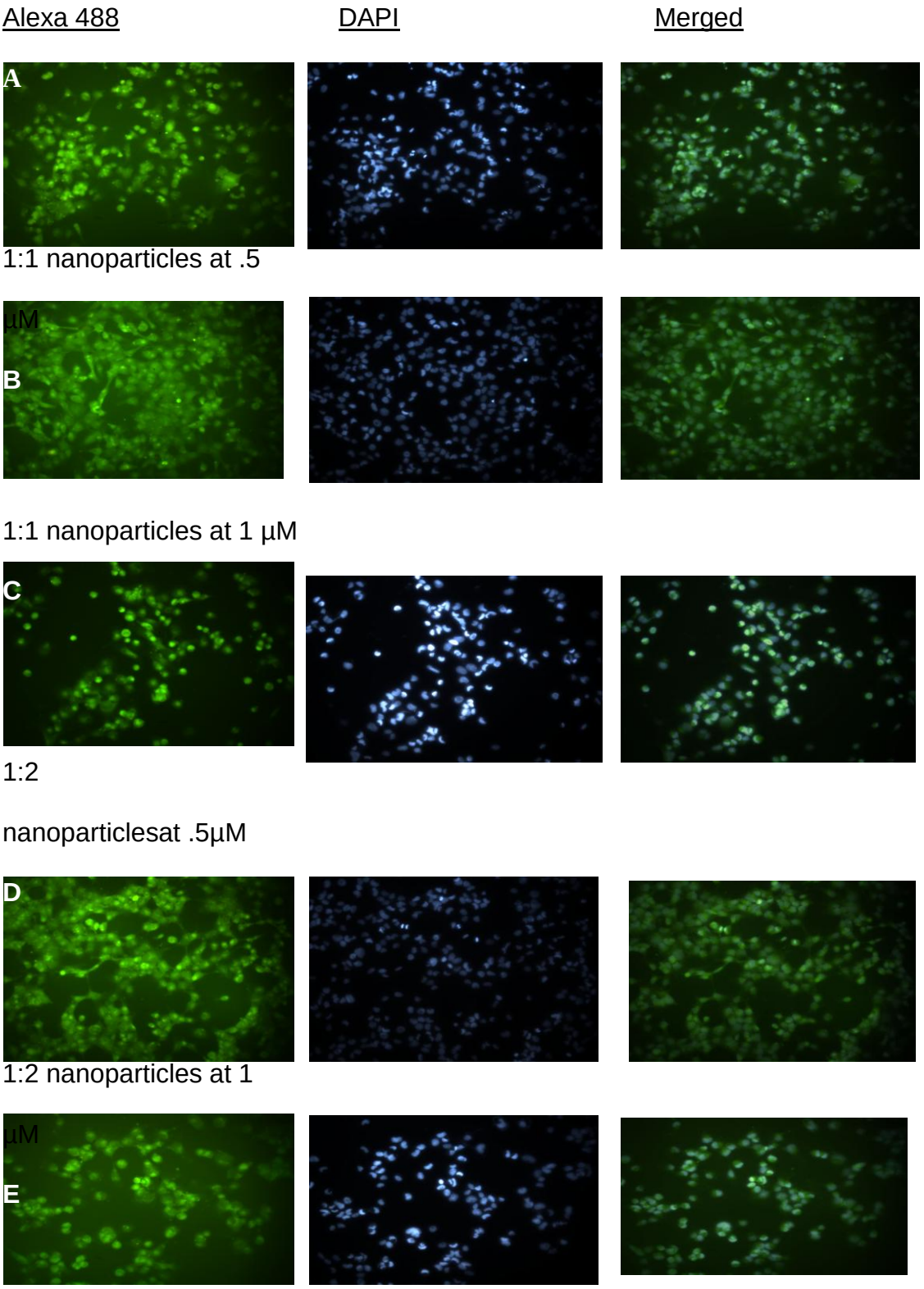


Figure II. Cytotoxicity of POM, 1:.0.5, 1:1, 1:2 nanoparticles against human cancer cell line, SKOV-3. SKOV-3 cell line was sensitive to nanoparticles of 1:2 ratio at lowest concentration.

FIGURE III: INTERNALIZATION



1:.05 nanoparticles at.5 µM

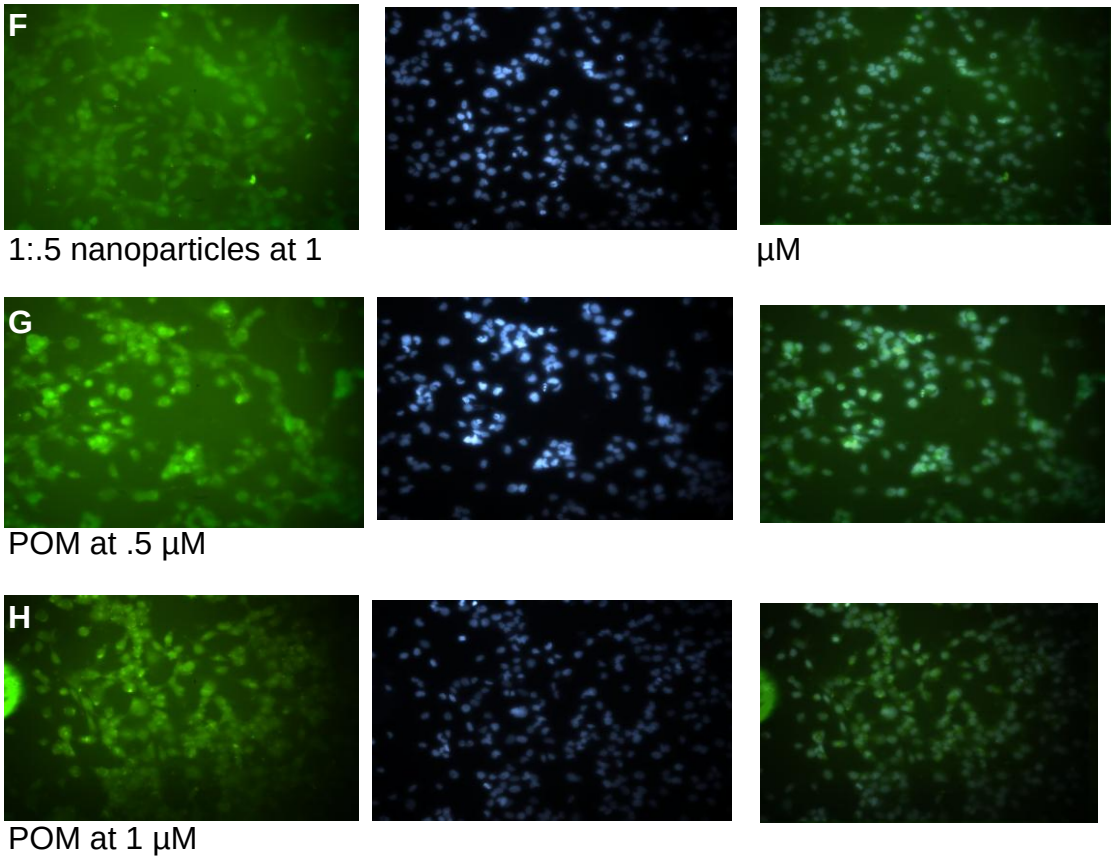


Figure III. Internalization of Alexa 488-labelled V-POM into SKOV-3 cells. SKOV-3 cell's nuclei were counterstained with DAPI.

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