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作品名稱 **BA-ADA based ROS-responsive nanoparticles for selective drug delivery in cancer cells**

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ABSTRACT

Current medical intervention in cancer therapeutic methods has shown risks and side effects with normal tissues. This includes incomplete cancer eradication. In reference to numerous studies and literature reviews, a stimuli-responsive drug delivery system is selected as an innovative, safe and more assured treatment due to its site-specific release ability. This allows specific intervention upon the given stimulus which response to the presenting disease symptoms. Hence, we designed a ROS(Reactive Oxygen Species)-responsive BA-ADA(4-Hydroxyphenylboronic acid pinacol ester and 1-Adamantanecarboxylic acid bonded molecule) nanoparticle delivery system. In our study, ROS-responsive nanoparticle was designed and prepared based on a synthetic molecule from BA and ADA. A therapeutic payload, Doxorubicin, can be loaded into the nanoparticles and it can be selectively released within cancerous tissues whereby ROS level is over-expressed. This will enhance both therapeutic efficiency and reduce side effects. The stability and ROS-responsiveness of the particle were proven in a series of evidence-based experiments. The results showed a significant difference in cell viability during the experiments with healthy and cancerous cell samples. Further research will be required to extend the experiment in vivo.

KEYWORDS

BA=4-Hydroxyphenylboronic acid pinacol ester
ADA=1-Adamantanecarboxylic acid
BA-ADA particle= particle without drug (Doxorubicin)
ROS=Reactive oxygen species
DOX= Doxorubicin
DAPI=4′,6-diamidino-2-phenylindole
LPS=Lipopolysaccharides
PVA=Polyvinyl alcohol
TEM= Transmission electron microscopy
DLS=Dynamic light scattering
DDS=Drug delivery system

1 INTRODUCTION

1.1 MOTIVATION

Medicine & Health

The cancer rate in children has increased by 13% in the past 20 years (Steliarova-Foucher, et al., 2017). Currently, about 300,000 children worldwide have confirmed cancer every year. We are very saddened by knowing that children who are close to our age or even younger than us are suffering from illness and even death. Our greatest wish is to be able to reduce the pain that patients and their families may suffer in the treatment of cancer. The world of Pharmaceutics has given us hope. We believe that by mastering advanced knowledge in chemistry, biology and pharmaceutics can help us achieve our wish in the near future.

1.2 PROBLEMS

Cancer, the hallmark disease of modern society

A recent research by the World Health Organization (WHO) noted that nearly 1 in 6 deaths was owing to cancer (WHO, 2017). Although medicines for healing cancer might lengthen life to a certain extent, they are not always as effective as expected regarding the survival rates and length improvements (Matsumura & Maeda, 1986). In addition, poor specificity of nearly all of the chemotherapeutic drugs has led to various side-effects and toxicities that have significantly compromised the treatment efficacy and the quality of life of the patients. Thus, researchers have led meticulous studies focused on how to stop this deadly disease in its tracks for years.

1.3 ANALYSIS OF PRESENT SITUATION

Current medical technology has risked damage to normal tissues

Cancer is a very complicated biological phenomenon and can be considered a disease of many diseases. One of the hallmarks of cancer cells is that they divide and multiply rapidly and out of control. Cancer therapies nowadays are finite to surgery, radiation, and chemotherapy. All these three methods risk damage to normal tissues or fragmentary eradication of cancer. Current chemotherapy is mainly aimed at destroying all rapidly dividing cells. The downside of this therapy is that the body's other rapidly proliferating cells, such as in the hair follicles and intestinal epithelium are also killed off, leaving the patient to cope with life-altering side effects (Baudino, 2015).

2 LITERATURE REVIEW

2.1 THE NANOPARTICLE REVOLUTION

Nanoparticles are particles of around several nm to several hundred nm. They have garnered much attention and already been extensively applied in many areas in the last decade. Due to nano-scaled sizes, nanoparticles exhibit various outstanding properties that differ from those of bulk materials (Chan et al., 2012). The properties, such as large superficial area, high surface energy, environmental sensitivity, and biocompatibility, have made nanoparticles widely studied in various fields such as photoelectricity, chemistry, biotechnology. Moreover, they brought us the chance to develop precise, specific, stimuli-responsive release of drug molecules for tackling various diseases.

2.2 REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species (ROS) in the human body are chemically-reactive molecules produced through the complicated biological processes. Typical ROS species include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide ($O^{-2}\cdot$) and hydroxyl radicals (HO•), which may transform from one to another via a cascade of reactions. (D'Autreaux & Toledano, 2007). ROS can be generated endogenously from mitochondrial metabolism or NADPH enzyme-catalyzed reactions as well as exogenously by exposure to UV light or xenobiotic compounds (Nathan, 2003).

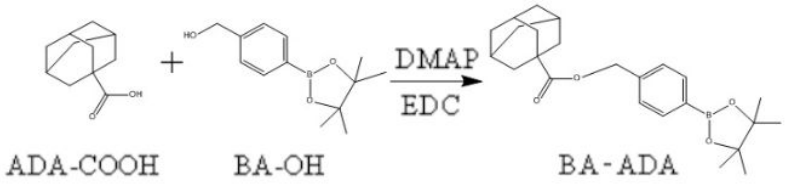
These oxidative species at appropriate concentrations play a crucial part in regulating biological and physiological processes such as cell signaling and apoptosis, native immunity, and so forth.

However, when ROS is overproduced, in the state of oxidative stress, ROS may damage biomolecules causing an array of pathological conditions, including cancer, diabetes, cardiovascular diseases, neurodegenerative diseases, inflammatory disorders and atherosclerosis (Tao & Hu, 2018). Plenty of evidence suggests that many kinds of cancer cells have higher levels of ROS compared with the normal body cells. It was reported that the ROS concentration in cancer cells reaches 100 μM, approximately 100 times higher than that in normal cells. (Pelicano, et al., 2004). As higher oxidative potential was found in cancer cells in tumor tissues, we were inspired to develop cancer-specific killer molecules that can be activated only at the diseased sites with oxidative stress.

3 DESIGN ARCHITECTURE

3.1 DESIGN OUTLINE

The main objective of the present study was to form a type of novel, ROS-responsive nanoparticles. Thus, the design could be summarized into 3 main parts. First and foremost, synthesize biocompatible, ROS-responsive molecules that may be used to prepare nanoparticles. Secondly, fabricate nanoparticles from the prepare ROS-labile molecule, with full materials characterizations (including drug-loading and release). Lastly, ROS responsiveness of the particles as well as in vitro therapeutic efficacy was examined. The design of the study could be depicted in the figure below:



- Figure 1. Design outline of the study.

3.2 HYPOTHESIS

As a drug delivery system(DDS), the ROS-responsive particles (including the payload —— doxorubicin) could be taken by cancer cells via phagocytosis and release a higher amount of drugs in cancer cells than in normal cells due to the property of ROS-responsiveness and the over-expressed ROS inside cancer cells.

3.3 TARGET BENEFICIARIES

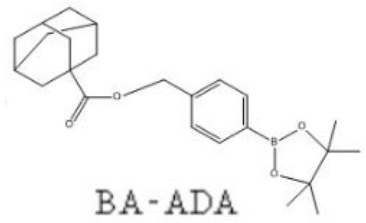
The DDS will be potentially beneficial to patients with cancer in all stages, as this particles can limit the side effects from the drugs, i.e. the particles can deliver drugs into targeted cancer cells and the side effects brought to normal tissues would be reduced.

4 MATERIALS AND METHODS

4.1 MAIN MATERIALS

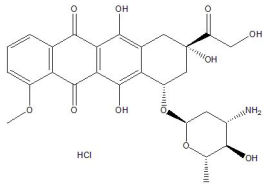
The nanoparticles were synthesized by the Hydroxide of 4-Hydroxyphenylboronic acid pinacol ester (BA) and the carboxylic acid of 1-Adamantanecarboxylic acid (ADA). Doxorubicin(DOX) was used as the drug whereas Polysorbate 80 was like the surface active reactant to stabilize the particles. These main materials would be introduced in the following figure.

❑ Figure 2. Main materials used in the study.

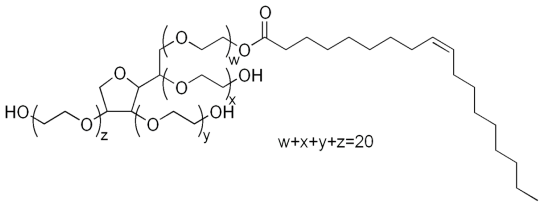


BA(4-Hydroxyphenylboronic acid pinacol ester)
The major material that provides the ROS-responsiveness of the particle.

ADA(1-Adamantanecarboxylic acid)
These two molecules react with each other to make BA-ADA via ester bond (the active from ADA reacts with the OH-from BA).



Drug: DOX(Doxorubicin)
Slows down or stops the growth of cancer cells by blocking an enzyme called topoisomerase II, which is critical for cancer cells to divide and grow.



Surface active reactant: Polysorbate 80
A nonionic surfactant used as a surface active reactant. With several drops dissolved into water, the particles can be stabilized.

4.2 CHOSEN MATERIAL

Besides, the materials mentioned above, during our exploration both by literature reviews and experiments, it was found that some materials did not work out and fulfill the texture we desired for the particle.

■ **Present study** ■ **Future plan** ■ **Materials that we didn't to use**

Drug/ Material	The reason why the particular material was chosen or not chosen in the present study
DOX	DOX can stop the growth of cancer cells by blocking the enzyme called topoisomerase 2 as cancer cells need this enzyme to divide and grow. This drug often induces heart failure, therefore using it precisely can reduce the tragedy. Also, this particular drug is a fluorescent so that we could use it to check the particle's drug releasement.
PTX	Paclitaxel(PTX) is a drug for curing cancer cells. However, it was found that it didn't produce fluorescence light which was considered to be critical in confocal studies. Thus, PTX was not employed in the present project.

Nanoparticle architecture	The reason why the particular material was chosen or not chosen in the present study
Nanoparticle	This is the most stable structure found to create the nano-delivery system after the study and experiments for months.

Formation of the particle		The reason why the particular material was chosen or not chosen in the present study
Materials	**BA-ADA**	BA is the key material for the particle. It can provide the particle with the ability to release drugs selectively. ADA is a material that gives the particle a potential to bond with a variety of materials which can improve the particle's ability in other aspects.
	PEG	It can bond with the particle and form a layer of protection for it, hence the particle wouldn't release drugs within a healthy cell.
Surface active reactant	**Polysorbate 80**	It can stabilize the particle in the early stage formation but it would become useless after the particle is perfectly formed.
	PBS	During our experiments, Phosphate Buffered Saline(PBS) was found to fail in producing its function. Thus, it was not chosen to be used in the final.
Solvent	**DCM**	Dichloromethane(DCM) is found to be able to provide an oily environment required in the project. Hence it can perform the hydrophobic effect.

Table 1. The materials explored in the study.

4.3 EXPERIMENT INSTRUMENTS AND METHODS

Instruments	Usage and methods
Nuclear magnetic resonance spectroscopy	To measure the arrangements of carbons within the product to assure that we have got the BA-ADA molecule.
Mass spectrometry	NH_4^+ and K^+ ions were used to give the sample a positive charge. By subtracting the original mass of the ions by the mass got from the graph of the mass spectrometer, the molecular mass of the compound synthesized could be calculated. To assure the compound we got.
Dynamic Light Scattering	Distributed payload particles into H_2O_2 with different concentrations. Check the diameters of the payload particles at the time intervals of after 1, 4, 7, and 18 hours.
Transmission electron microscopy	To assure the solid interior of the particle.
Ultraviolet-visible spectroscopy	Distributed loaded particles into H_2O_2 with different concentrations. Check the percentage of drug released at the time intervals of after 2, 4,6, and 8 hours.
Plate reader	Distributed particles of different concentrations into RAW and RAW IFN. Check the cell viability by the absorbance after 24 hours.
Confocal microscopy	Dyed the cells by DAPI in order to locate them under the microscopy. thereafter. Distributed particles loaded with Nile red fluorescence into the wells to feed the cells. Study the confocal images after 20 hours.

Table 2. The experiment instruments were used in the study.

5 RESULTS OF THE EXPERIMENTS

The study is divided into three main parts, each of which has different objectives. Therefore, it is separated into three parts: the methods and materials for particle forming, the experimental methods for each part, the materials and equipment used, and the experimental results would be described below in detail in each of the following subsections.

01

THE FORMATION OF PARTICLES

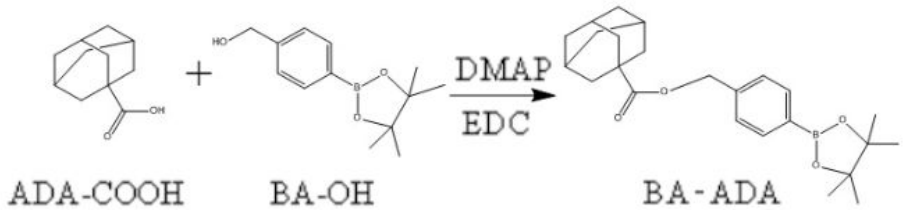
3 main procedures of the formation of the payload particles were as the followings[1]:

1. **Chemical reaction**-the formation of BA-ADA
2. **Add drug**-the formation of payload nanoparticle
3. **Take out the solvent, catalysts, surface active reactant**

----------Further explanation of the formation of the payload particle ----------

1/ Chemical reaction-the formation of BA-ADA

In this reaction, ADA-COOH and BA-OH have been synthesized by using 2 catalysts, namely 4-dimethylaminopyridine(DMAP) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide(EDC). The references suggested that porphyrinogen should not be a good catalyst for this reaction, thus it would not be used as a catalyst in this reaction in the present study. The molecule of ADA was chemically reacted to BA's hydroxide ion with its own carboxylic acid. To confirm the product, Nuclear Magnetic Resonance Spectroscopy(NMR) and mass spectrometer were employed. Thereafter, column chromatography was used to select the product out, from the mixture of unsynthesized materials and catalysts.



❏ Figure 3. Formation structure of the nanoparticle BA-ADA

【1】Experiment 1

NMR spectrum of BA-ADA molecule

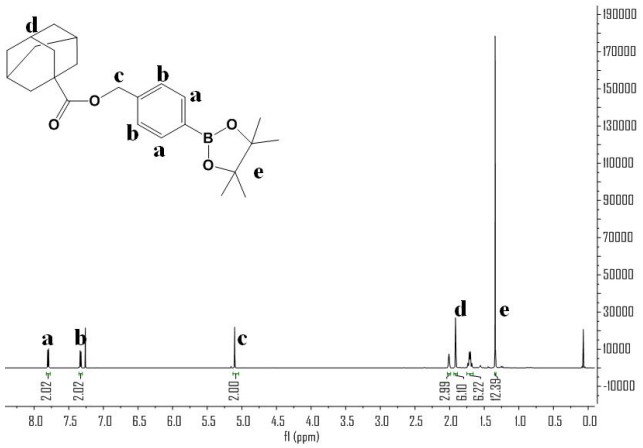
In order to confirm whether the BA-ADA molecule was successfully composed, NMR spectrum was employed. With the different placings of carbons, it could figure out whether the compound required had been reacted or not after a reaction. Also, this is the structure we actually used for nanoparticle preparation.

The figure below presented the NMR spectrum of the compound formed. The structure of the compound, especially the position of carbon shown in the figure.

The reaction had been completed and the raw materials used in the study had been synthesized into the desired BA-ADA molecules.

[1] The detail of the procedures in forming the nanoparticle was presented in Appendix 1. It was adapted by the procedures suggested by Qiu et al.(2015), who had successful composed an oxidation-responsive polymer.

1H NMR (600 MHz, CDCl3, δ): 7.73 (d, 2H), 7.32 (d, 2H), 5.05 (s, 2H), 1.1-2 (m, 15H), 1.26 (d, 12H).



❏ Figure 4. NMR spectrum of BA-ADA molecule.

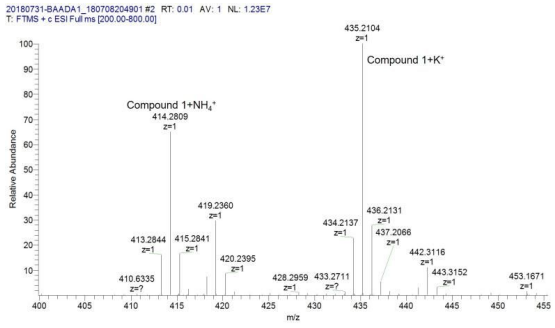
【2】Experiment 2

Results from mass spectrometry of BA-ADA molecule

In order to further confirm that the synthesized product was the desired BA-ADA molecule, this study used a mass spectrometer to provide a more complete understanding of the product. Mass spectrometry is one of the most powerful tools for identifying compounds, including to determine the elemental composition of a sample, the masses of molecules and to elucidate the chemical structures of molecules.

NH_4^+ and K^+ ions were used to give the sample a positive charge, as only the molecular mass of a sample with a positive charge was able to be measured during mass spectrometry. By subtracting the original mass of the ions by the mass got from the graph of the mass spectrometer, the molecular mass of the compound synthesized could be calculated.

After calculation, the mass of the sample should be approximately 396.1124 g/mol and it was the same as the result got from the mass spectrometer. The result supported that the BA-ADA molecule had been successfully synthesized. The figure below showed the result provided by the mass spectrometer.



❏ Figure 5. Results from mass spectrometry of the BA-ADA molecule.

2/ Add drug ——conjugate the nanoparticle with payload

BA-ADA and the therapeutic payload (DOX) were dissolved in the solvent DCM while adding a few drops of surface active reactant (polysorbate 80) into the water. Since both BA-ADA molecules and DOX drugs were oily, they both are soluble in organic solvents such as organic solvent and polysorbate 80 was used as a stabilizer. This simple

emulsion would be held inside an ultrasonic cell crusher noise isolation chamber for 80 minutes until the emulsification completed.

After the particles were stabilized, the synthetic solution would be concentrated in a centrifuge for several times to remove DCM and unused polysorbate 80. Then the concentrated sediments of the payload nanoparticles could be obtained.

02

CHECK PARTICLE'S STABILITY

In order to have a stable particle, the followings needed to be achieved:

1. **A consistent particle width**
2. **A spherical shape**

And so far the particle formed have achieved all of that which made it stable.

----------Further explanation of the formation of the payload particle ----------

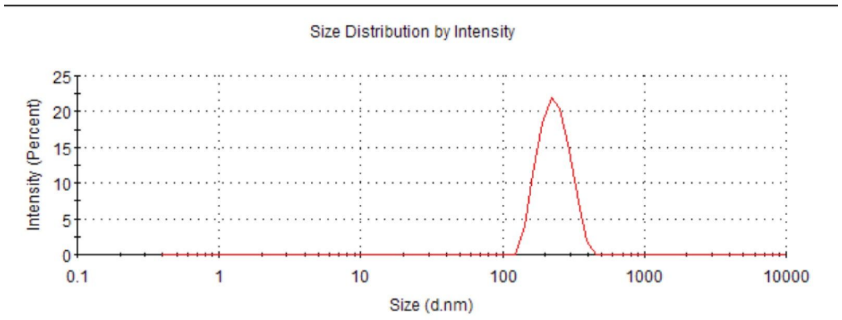
1/ A consistent particle width

【1】Experiment 3
DLS result of the BA-ADA nanoparticles

The results of the particle' average width could be displayed by Dynamic Light Scattering (DLS) to understand the particle size and particle size distribution of the nanoparticles produced in this study.

As shown in the figure below, the average diameter of the nanoparticles was 244.6 nm and the PDI was 0.155, indicating that the particles met the requirements of this study.

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 244.6	Peak 1:	235.8	100.0	57.45
PdI: 0.155	Peak 2:	0.000	0.0	0.000
Intercept: 0.804	Peak 3:	0.000	0.0	0.000
Result quality : Good				



❏ Figure 6. DLS result of the BA-ADA nanoparticles.

2/ A spherical shape——particle was fully solid at the interior

In order to assure the particles were fully solid at the interior and it has a spherical shape, Transmission Electron Microscope (TEM) was employed.

【2】Experiment 4
BA-ADA nanoparticle in a TEM image

The drug (DOX) could only be stored within the particle which was in a solid state. Thus, it was essential to assure the interior of the particles were entirely solid and the TEM was used.

By the TEM image showed below, it was clear that the particle was solid in the interior.

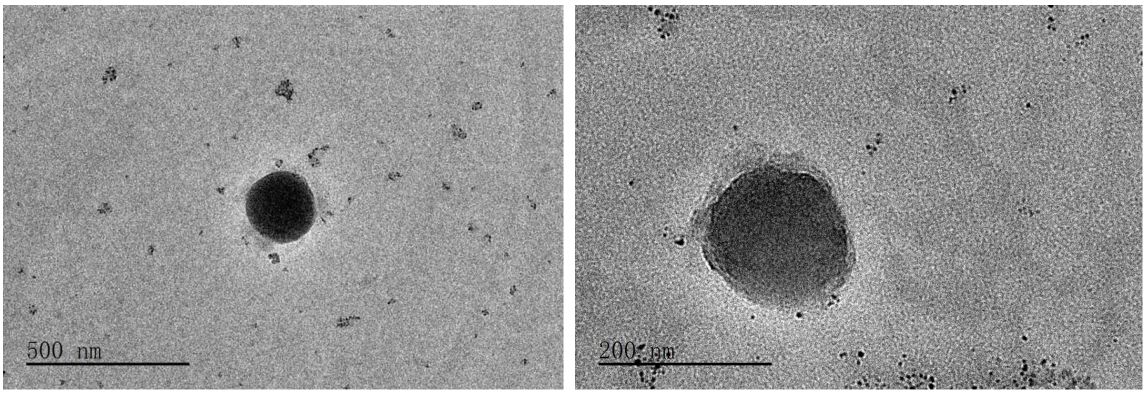


Figure 7. TEM result of BA-ADA nanoparticles enlarged into a scale of 500 and 200 nm.

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03 CHECK PARTICLE'S ROS RESPONSIVENESS

Hydrogen peroxide (H_2O_2) is a key member of the class of ROS. All cells will have some amount of H_2O_2 but in cancer cells, its level is much higher. In the following experiments, H_2O_2 would be employed as an agent to represent the ROS within cells for assessing the property of ROS responsiveness of the particles.

------Further explanation of the check of the particle's ROS responsiveness-------

【1】Experiment 5
Nanoparticles in the presence of various concentrations of H_2O_2

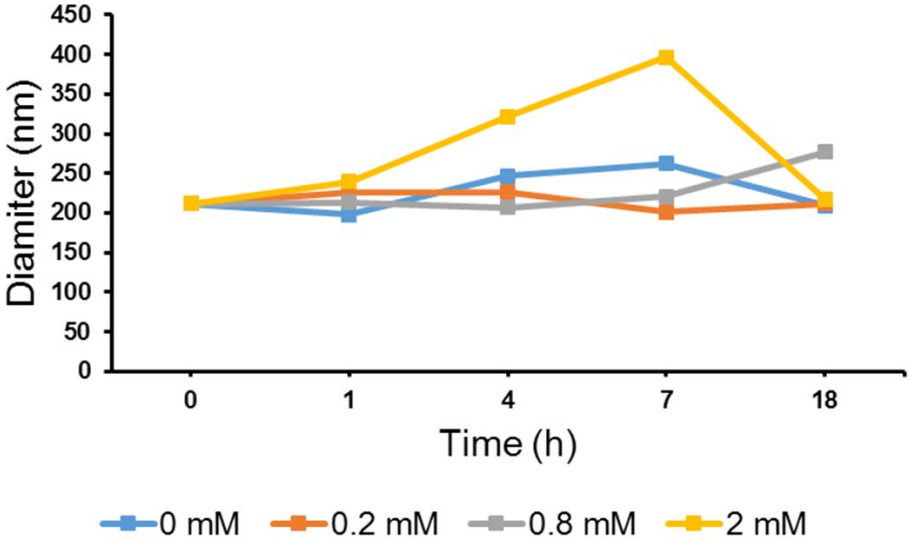
Our nanoparticle DLS was in a design that the particles should break off and release the drug loaded in cells with a high level of ROS. Thus, in the present experiment, payload particles would be distributed into plates with different concentrations of H_2O_2, ranging from 0 to 2 mM. Pure water with zero H_2O_2 was held as a control group.

The concentration of 0.2 mM H_2O_2 simulated the H_2O_2 level of normal cells. The concentration of 0.8mM and 2 mM H_2O_2 could be considered as the medium and high level of concentration in cells respectively.

As before breaking off, the particles would swell up. Thus, zetasizer would be employed again to check the size of the payload particles in different concentrations of H_2O_2 at different time intervals.

Results could be presented in Figure 8. According to the DLS, the particles size of the payload particles in H_2O_2 of different concentrations varied in different time intervals. Diameters of particles in higher concentrated H_2O_2 became larger after hours.

All of the results supported that the particles were with ROS responsiveness.



❑ Figure 8. The diameters of nanoparticles in different concentrated H_2O_2 over time.

【2】Experiment 6
DOX release profile of BA-ADA nanoparticles measured by UV–Vis method in the presence of various concentrated H2O2

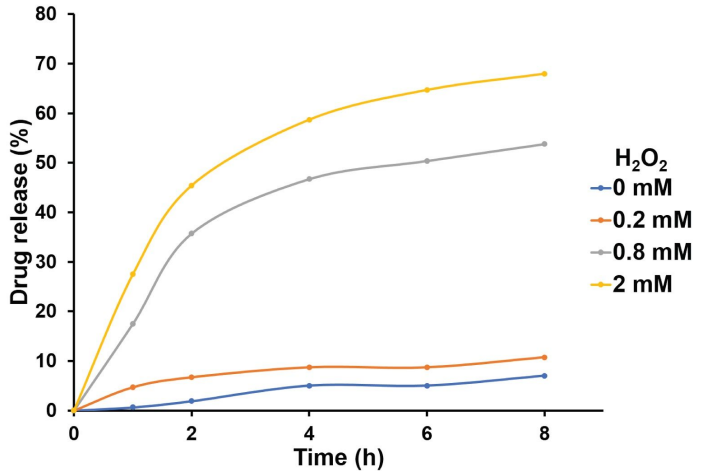
As the drug (DOX) was able to absorb UV- lights, this experiment was designed with this characterization to have a deeper understanding of the drug release mechanism of the nanoparticles. In the present experiment, payload particles would again be distributed into plates with different concentrated H_2O_2, ranging from 0 to 2 mM, the intensity of UV- lights would be assessed for checking the percentage of drug released by the payload particles in different concentrations of H_2O_2.

UV-Vis spectrometer would be utilized to check the percentage of drug released in different concentration of H_2O_2 at different time intervals. By using UV-Vis method, the percentage of drug released of the payload particles in H_2O_2 of different concentrations over time could be presented in figure 9. The results can be summarized into one key point:

Percentage of drug released in higher concentrated H_2O_2 were higher after hours.

Obviously, these results supported the hypothesis that the BA-ADA payload particles synthesized in the present study would release the payload-drug(DOX) with high H_2O_2 or ROS level. The longer the time, the more the amount was released.

With the results drawn by Experiment 6, it could be known that the responsiveness of the BA-ADA payload particles was sensitive as the particles would be decomposed faster and trigger more drug releasement when they were in higher level of ROS.



❑ Figure 9. DOX release profile of BA-ADA nanoparticles measured by UV–Vis method in the presence of various concentrations of H_2O_2.

【3】Experiment 7
Analysis of cell viability (MTT) of normal and inflamed RAW with BA-ADA nanoparticles

In order to test out whether the nanoparticles themselves (without payload) were with poison or not, a group of cell experiments with normal and inflamed RAW cells with a broad range of particles in different concentrations was made.

The cell type RAW 264.7 was used in the present study.In this experiment, interferon (IFN) was employed to stimulate the RAW and make them inflamed.

Before the experiment was launched, normal and inflamed RAW were settled separately into a 96-well plate. Then, nanoparticles were dispersed into pure water and distributed into the plate, with the concentrations of particles from 0 to 200 μg/ml. O μg group was served as the control group in which there was no particle. After 24 hours, we used live cells to reduce 3-(4,5-dimethylthiazole-2)-2,5-diphenyltetrazolium bromide (MTT solution) into blue-violet insoluble crystalline formamidine. Then, Dimethyl sulfoxide (DMSO) was employed to dissolve the blue-violet crystalline formazan. Finally, the absorbance at 490 nm was measured with a Microplate Reader, and the amount of formazan was checked. Thus, The survival rate of normal and inflamed RAW in different nanoparticle solutions could be compared. The figure below presented the results of the experiment drawn from the microplate reader.The results can be summarized into two key points:

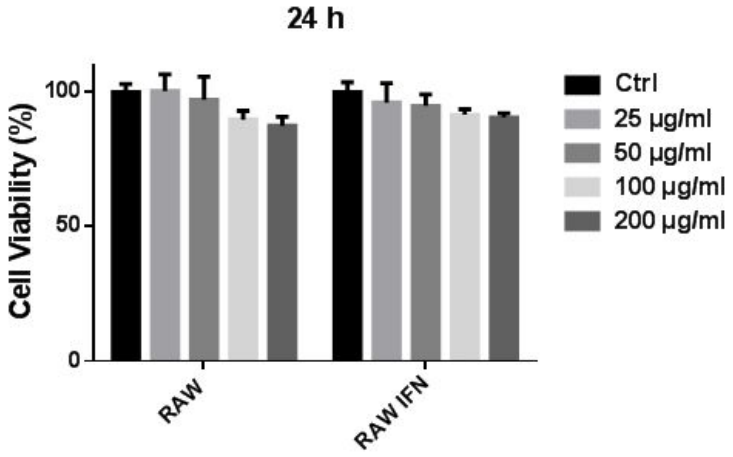
1. The cell viability rates of RAW and RAW IFN was with no significant difference among all groups with different particle concentrations.

2. The cell viability of RAW was similar to RAW IFN cells in all particles concentration groups.

The cell viabilities of RAW and RAW IFN did not vary in accordance with the particle concentrations. Even the result from Group E (RAW with the highest particle concentration) did not show an apparent difference with the one of Group A which was with no particles. Also for RAW IFNs, the same results were got.

The results showed that the cell viabilities of both cells with different concentrations of particles were similar after 24 hours. Also, they were similar to

both of the control groups. Thus, the conclusion could be made that the nanoparticles themselves were not poisonous and they with biocompatibility.



❑ Figure 10. Biocompatibility of BA-ADA nanoparticles for inflamed and RAW cells.

【4】Experiment 8

Confocal study by using Nile red and DAPI for RAW and inflamed RAW

Confocal microscopy was employed in this experiment to verify whether the nanoparticles would be phagocytized by cells and release the drug in response to ROS.

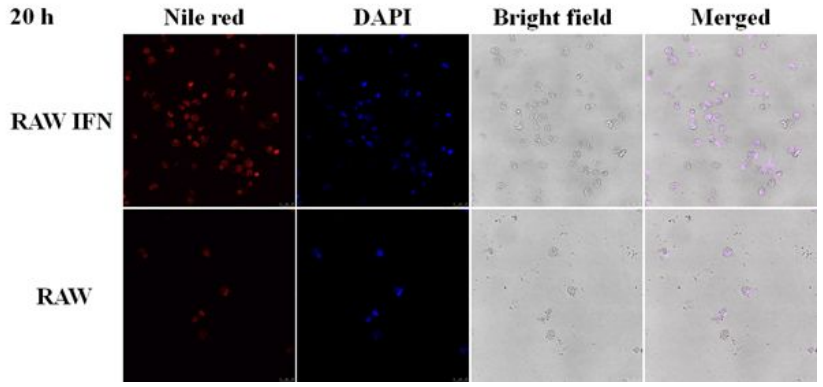
In the present experiment, RAW 264.7 were used as materials. IFN was employed to stimulate RAW and make them inflamed.

The confocal study was separated into two parts, the first part was to set up a foundation for the study, where 4',6-diamidino-2-phenylindole(DAPI)was used to dye up the cell's nucleus so that the cells could be located.

In the second part, Nile red was employed as a fluorescent material which was loaded into the particles for modifying drug release. As the materials were with fluorescent colors, they were able to be seen through the study of confocal when they were released.

The confocal images were shown in the figure below. The final results wrapped up the conclusion as it made the conclusion literally visible. After 20 hours, by the confocal image of RAW IFN, red fluorescence produced by the Nile red were inside the RAW IFN obviously, which indicates that the nanoparticles would release the payloads as the RAW IFN were with high ROS. However, the payloads were not triggered to be released in RAW.

Therefore, the confocal study could conclude that the BA-ADA particles were DDS with ROS responsiveness.



❑ Figure 11.Confocal images of normal and inflamed RAW cells dyed by DAPI with the particles loaded with Nile red

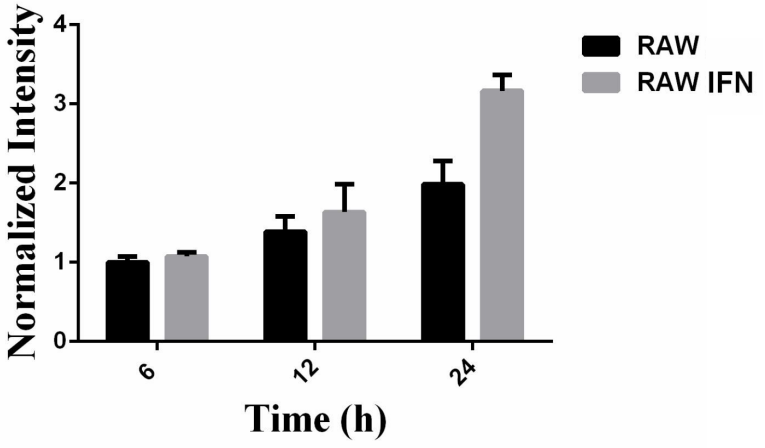
【5】Experiment 9
Intensity of fluorescence detected in different time intervals in normal and Inflamed RAW

The experiment that corresponded the confocal study was the one about the intensity of fluorescence released into the cells that were detected by a flow cytometer. Likewise, Nile red was employed as a fluorescent material loaded with the particles for modifying drug release. The intensity of fluorescence was checked at the intervals of after 6, 12 and 24 hours. The results were shown in figure 12 and they could be summarized into one main point:

The amount of fluorescence released in RAW IFN was more than the one in the RAW.

The apparent difference could be found between the normalized intensity of fluorescence released in RAW IFN and RAW after 24 hours. Due to the selective ability of payload release of the nanoparticles, the discrepancy between the amount of fluorescence released in RAW IFN and RAW became larger over time.

The result of this experiment confirmed that the BA-ADA nanoparticle was an effective DDS with ROS responsiveness. The particles would selectively trigger the payloads to be released in RAW IFN, causing a relatively higher amount of fluorescence leaked, i.e. high ROS cells. The ability of ROS responsiveness was supported.



- Figure 12. The amount of fluorescence intensity released from Nile red loaded BA-ADA nanoparticle of the normal and inflamed RAW cell checked by a flow cytometer.

【6】Experiment 10
Analysis of Cell viabilities (MTT) of RAW with BA-ADA payload nanoparticles and free DOX

The first cell discussion was done upon a group of normal RAW (RAW) and inflamed RAW (RAW IFN). In order to make the results more convincing, nanoparticles loaded with different concentrations of DOX were employed with a free DOX set of groups and a control group. Free DOX set was consisted of groups with different concentrated DOX but not protected by nanoparticles. Control groups were held with neither particle nor drug.

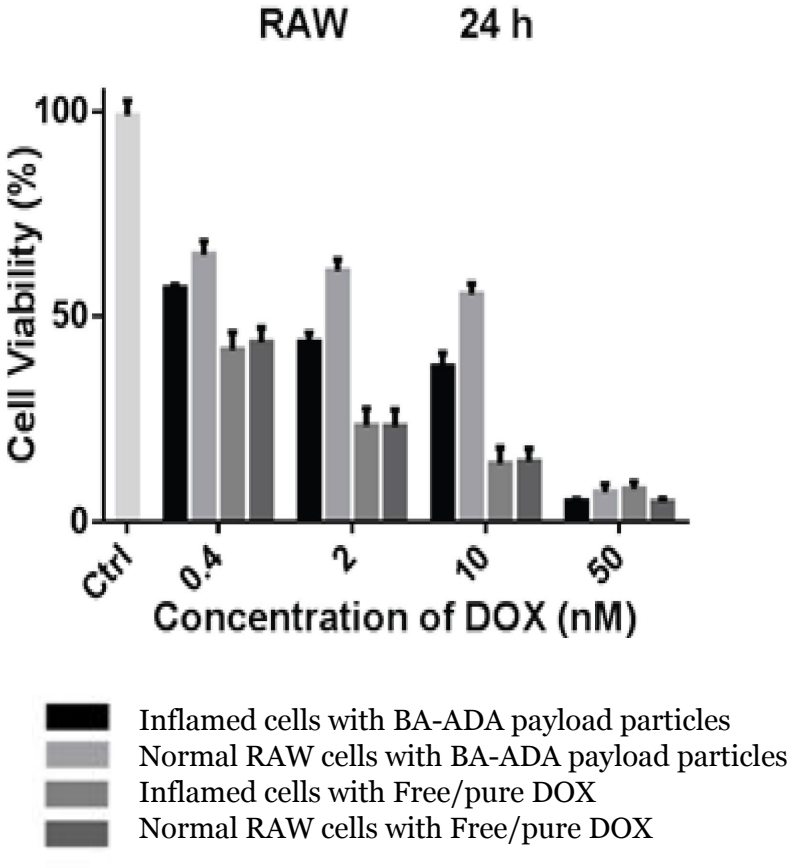
In this experiment, RAW 264.7 was used as the materials. Two different matters, lipopolysaccharide (LPS) and Interferon (IFN) were employed to stimulate RAW and make them inflamed.

After 24 hours, the results could be shown by the microplate reader and the summarizations were as the followings:

1. In all groups, cell viabilities of normal RAW with particles and DOX were higher than the one of inflamed RAW with particles and DOX.

2. The cell viability of the control group, i.e. normal and inflamed RAW in pure water with neither particles nor drug, was 100%.

It was as expected, since DOX was poisonous, when the normal RAW were fed with free DOX, their cell viability became as low as the inflamed RAW. However, when the normal RAW was fed with payload particles, their viabilities were higher than the one which were fed with free DOX. Apparently, the results proved that the nanoparticles could serve as protectors for the normal RAW from the aggressive effects of DOX. More essentially, the viability of normal RAW wasn't affected much by the payload particles comparing to the group with free DOX. Thus, it's pretty clear that the BA-ADA particle could be served as a selective drug release system of both of the cells. The results were presented in figure 16.



❑ Figure 13. Cell viability of DOX and payload BA-ADA Nanoparticles for RAW.

【7】Experiment 11
Analysis of cell viabilities (MTT) of Bel, MCF-7 and L02 with BA-ADA payload nanoparticles and free DOX

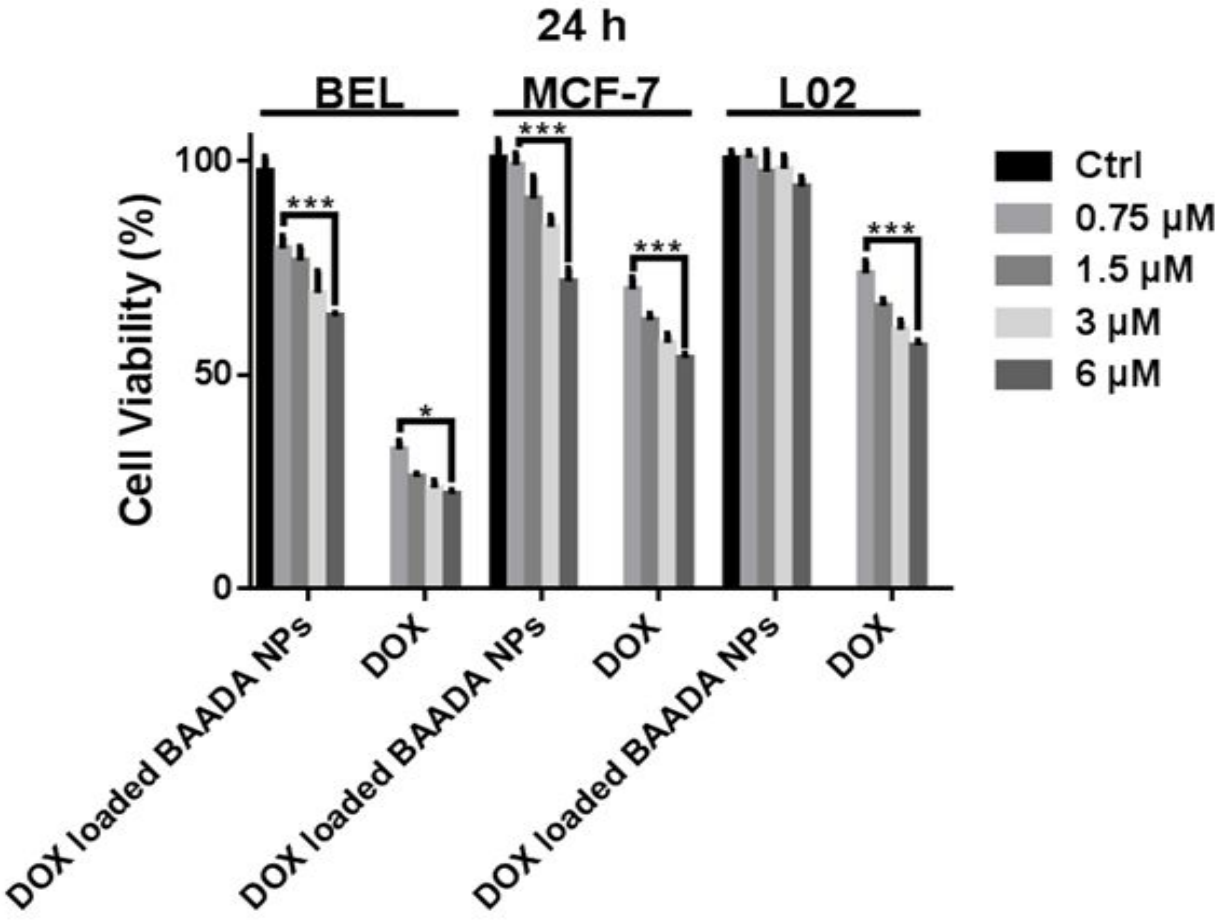
To have a more advanced exploration of the effects of payload nanoparticles on cells, we moved on to using two groups of cancer cells (human hepatocarcinoma cell BEL and human breast carcinoma Cell MCF-7) and normal cells (human hepatocyte L02)in the present experiment. Their cell viabilities would be assessed with payload particles and free DOX.

After 24 hours, the results could be shown by the microplate reader and they could be summarized as below:

1. For the group of normal cells, the viabilities were higher when they were fed with payload particles than the ones with free DOX. The discrepancy enlarged when the concentration level of DOX increased.

2. In the case of phagocytizing payload particles, viabilities of the normal cells were higher than the ones of the two cancer cell groups. With the DOX concentrations increased, the discrepancy became larger.

The results of this experiment were further evidence that the nanoparticles could serve as normal cell protectors from the aggressive effects of the drug and they could be considered as a drug release system with the effective ability of selectivity. causing a significant drop in viability of cancer cells. The results of this experiment were shown in the following bar charts of the following figure.



❑ Figure 14. Cell viability of Bel, MCF-7, L02 with payload BA-ADA nanoparticles and DOX.

6 DISCUSSION

OUR PROJECT IS STILL IN ITS EARLY STAGES

Although literature have proved that the design of DDSs demonstrated a promising way to effectively deliver therapeutic agents to disease sites and it would become more and more popular in the biomedical field (Tao & He, 2018), it is a long way for our project to go before clinical trials in human patients.

Thus, more experiments in both vitro and vivo have been planned and we have our mission to pay our efforts in improving our odds in the fight against cancers.

During our journey of this study, the main challenge we had faced was the selection of materials. Plenty of times had been consumed by reviewing literature as well as conducting experiments for forming our particles and choosing therapeutic drugs. It had been our plan of employing vesicles to form our DDS and numerous trials had been used to test their stability. However, the vesicles were found to be with insufficient stability during some experiments and we had to select the alternatives. At last, the choice of nanoparticles was confirmed. However, plenty of time and efforts had been consumed to refine the procedures in order to produce the particles with perfect purity. Besides, our sweat and blood had been used in selecting therapeutic drugs. Only the drug would be utilized with the conditions that it had been proved clinically to be effective in conquering broad type of cancers and it must be sensitive in the experiment process, such as with the property to produce fluorescence light which was critical in confocal studies. We were blessed that DOX had come to us finally. Last but not least, we trusted all of the efforts we had devoted were worthy.

7 FUTURE PLAN

Surface characteristics of the nanoparticles can also determine their lifespan during circulation in the blood stream. In order to ensure the particles can travel through the circulation into the cancer cells, we are planning to bond the particles with PEG in the coming extended study. PEG are hydrophilic polymer molecules, which can hinder the binding of plasma proteins (opsonization), and thus preventing substantial loss of the given dose. PEGylated nanoparticles are often referred as “stealth” nanoparticles, because without opsonization, they remain undetected by the Mononuclear phagocyte system (Rizvi & Saleh, 2018) and resist serum protein adsorption (Zhao, 2012). Thus, it is trusted that PEG can form “another layer” of protection for increasing the stability of the particles and at the same time, reducing the risk of them to release drugs within healthy tissues.

Moreover, the pH-responsiveness and GSH-responsiveness of the BA-ADA particles would be addressed in the extended study. It has been reported that a pH gradient exists between intracellular and extracellular compartments in tumors, which results from the rapid proliferation and growth of tumor cells exceeding blood transportation, causing an inadequate supply of nutrients and oxygen, and thus generating lactic acid due to glycolysis (Karimi et at., 2016). Regarding Glutathione (GSH), the intracellular GSH level inside cancerous tissues are in the range of 0.5–10 × 10− 3 M, which is four times higher than the GSH levels in normal tissues (Wang et al., 2017) and this makes the neoplastic tissues more resistant to chemotherapy (Estrela et al., 2016). However, such drastic differences in the GSH level could be utilized as a promising platform to synthesize nanoparticles to selectively release therapeutic drugs in intracellular reductive environments in a triggered fashion (Uthaman et al., 2018).

However, given the acidic tumor microenvironment, pH-responsive nanoparticles would collapse and release their payload into the cytosol, promoting drug concentrations to exceed the capacity of the efflux transporters which support sufficient drug release within the cancerous tissue (Kanamala et al., 2016).

In our coming study, whether BA-ADA nanoparticle can be employed as a multiple-stimuli responsive nanoscale DDS would be examined. Multistimulirase Responsive DDSs that are sensitive to several stimuli can further enhance controlled drug release sites where multiple stimuli coexist. It is believed that the system would be responsive for high drug loading, more precise targeting without premature leakage, rapid drug unloading as well as higher accumulation at lesions. Considering the complexity of the tumor microenvironment and sophisticated physiological barriers in the human body. However, we have the confidence to do all our best to make a contribution in combating cancer and protecting human health.

8 CONCLUSION

Delivery of therapeutic drugs to the disease sites without harming healthy cells in the surrounding environment has attracted increasing attention as it may result in both enhanced treatment outcome and reduced side effects. Thus, stimuli-response drug delivery systems are of crucial importance due to their site-specific release ability in the existence of a stimulus that has relation with certain disease symptom. One of the most popular biological stimuli is ROS because their overproduction have been happening in several types of diseases and the emerging ROS-sensitive materials have immense potential in the aspect of biomedicine development.

The present study succeeded in synthesizing a type of nanoparticles (BA-ADA) which could be "loaded" with drugs (DOX) which were effective in preventing growth or recurrence of cancer cells. BA-ADA nanoparticles were considered as excellent DDS as in their construct, BA was ROS responsive and ADA was pH-responsive as well as GSH-responsive which would make multiple drug release within the target areas become possible. In this stage of the study, the ROS responsiveness was mainly addressed through a set of precise experiments.

The followings have been proved by the experiments conducted:

1. The nanoparticles by themselves were not poisonous to cells.
2. The nanoparticles could release payloads in the area of high concentration of H_2O_2 which was considered as an important signaling molecule related to ROS.
3. The longer the time, the higher the intensity of the payload released.
4. The drug (DOX) of the payload particles could selectively be released inside inflamed RAW, i.e.the payload particles were with a selective DDS.
5. The selectivity of drug release of the payload particles was effective to cancer cells--a significant higher amount of drugs were released to the cancer cells and decreased cell viability was led.

9 CONTRIBUTION

The present project was undergone by the supervision and full support by Professor Wang Rui Bing, Mr Chen Sun and Miss Kou. Prof Wang and Miss Kou provided us with the guidelines in Biochemistry and we equipped ourselves with the knowledge in Biology, Organic Chemistry and Pharmaceutics by self-study, literature review and the academic talks we were invited to attend every week in the University of Macau. Not only these three mentors, we were very thankful to all of the university students who taught, discussed and inspired us throughout our precious learning process.

The concept of the construct of the payload nanoparticles are nurtured by Prof. We were entrusted with the responsibility to try and to synthesize the materials and we were more than happy that we managed to form the particles after the efforts for months. Prof and Mr Chen also designed the framework of the experiments with us in order to verify the nanoparticles. During the process, all of the experiments, cell culturing, material and equipment managements were conducted by us with supervision by Mr Chen.

Lastly, we declare that both the Chinese and English version of the present report, novelty reports, progress reports and powerpoints were prepared by our own efforts.

10 MEMBERS' REFLECTION

Zelia

I am very pleased to be one of the members of our team as we all do our best in the project with our passion and devotion. Moreover, I am very impressed that we have worked as an effective team, all of us can develop our potentials and exert our talents. I would like to thanks professor, teachers, and tutor who taught me a lot when I have difficulties in doing experiments. In addition, I appreciate to my caring and thoughtful teammates that I neither speak Mandarin well nor really know a thing at first as I was in arrears for few months in the early stage, but they taught me patiently and explain the whole experimental procedure to me. No matter how our performance is and what result we will get, I trust we will have won ourselves and we will get the trophy of honor for ourselves.

Rocky

The most fruitful lesson I have learnt from the project is about problem analyzing and solving. In the project, the most remarkable lesson is actually getting my hands on cell culturing. I have also had a great experience in working with peers as a team. I finished the project with all of my heart and soul and I enjoy this wonderful process very much. After all, the journey along writing this report is filled with excitements.

Chris

I have great experience in exploring my possibility in pharmaceutical and biochemistry which I found I was really interested in. I would like to thanks Professor, teachers and my teammates, Rocky and Zelia. It is because they had helped me find my interest in pharmaceutical and biochemistry. More essentially, I found that I have got more self-confidence in assessing and solving problems in the lab. I trust it would be the most valuable experience in this stage of my learning life.

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- Prof. Wang Rui Bing: our professor
- Miss Kou Weng Si and Mr. Sun Chen: our mentors for assisting us
- Our families: for their understanding and mental support

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APPENDICES

APPX.1 FORMATION OF THE NANOPARTICLES

1-Adamantanecarboxylic acid (1.98 g, 11.0 mM) was dissolved in 100 mL of anhydrous DCM

Then, DMAP (1.34 g, 11.0 mM), EDC (4.22 g, 22.0mM) and BA (2.34 g, 10.0 mM) were added to the above solution. After 15 min at 0 °C, the yellowish solution was stirred at room temperature for another 2 h. Rotary evaporation was then performed. Transforming the yellowish solution into a white powder(BA-ADA molecule) The crude product was purified by chromatography on a silica column using a mixed eluent of petroleum ether and ethyl acetate (v/v = 2/1), affording the compound required (the particle, 4.10g, 92%) as a purified solution, with which rotary evaporation was performed again to collect the pure BA-ADA particle.

Adding the BA-ADA molecule into 99% DCM(200mL)and a few drops of 1% Tween 80, gives us a colourless solution. With the help of the ultrasonic cell crusher, the molecules are bonded together to create a particle. To follow up we created a vacuum chamber to remove DCM. To remove tween 80, we put the solution into a centrifuge(4000 rpm, duration: 3 minutes) to take out the big useless particles. After that, we continuously used a centrifuge(14,000 rpm, duration: 3 minutes) to take out the supernatant liquid. Finally, we got our sediments(particles) and we dissolved it into 500 microlitres of water. For further experiments, we create our own concentration of particles.

APPX.2 TEAM ORGANIZATION

Getting to know about us

All of the members are committed to our mission-be professional and be united. Although we have different experimental, biochemical specialties and interests, all of us have been working together energetically to pursuit for perfection.

Besides enhancing our knowledge and skills, we are able to identify our potentials and strengths. We enjoy our learning process as well as the friendship and team spirit developed during the days and nights in which we condense all of the drops of our sweat and the bits of our heart blood. Last but not the least, if any success in our team achieves in the contest, all of the honor should be dedicated to our most respectable and amiable, Professor Wang, Miss Kou Weng Si and Dr. Sun Chen, and our supportable and affectionate parents.

Team profile

Aurora Zelia Hui	Rocky Lao Sap Tou	Chris Fong Hong U
Grade 10	Grade 9	Grade 9

【評語】090022

They designed a ROS-responsive BA-ADA nanoparticle delivery systems. A chemotherpeautic drug can be loaded into the nanoparticle to kill the ROS-high cancer cell.

1. They need to demonstrate the cancer cells have a higher ROS.
2. They need to further demonstrate the underlying mechanisms.