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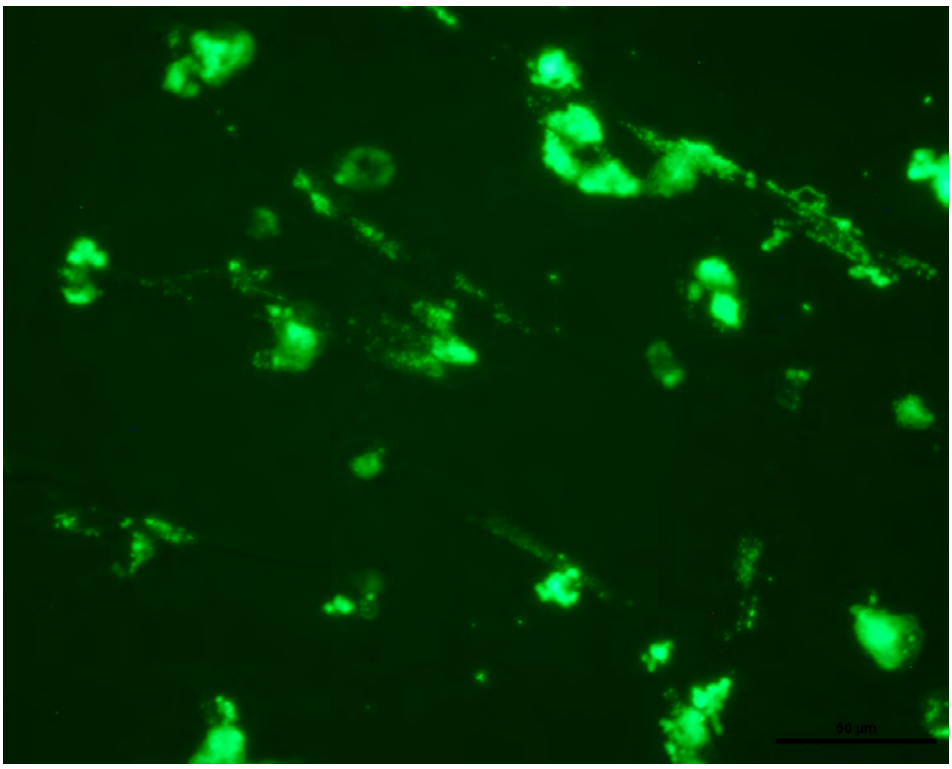
Targeting Oxidative Stress with Functional Polymer



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Contents

Abstract

Oxidative stress occurs when the molecular antioxidant capacity is overwhelmed by elevated reactive oxygen species (ROS). Excess ROS could cause lipid peroxidation which generates a lot of harmful products and acrolein is most reactive among them. Acrolein attacks nucleophiles in biomolecules, like proteins, lipid and even DNA. It is readily incorporated into proteins and forms carbonyl adducts. Oxidative stress, ROS, acrolein and protein adducts are proved by studies to be related to various diseases and acrolein serves as a target to the treatment. The polymer syr 48 is a water soluble polyvinyl alcohol (PVA) with hydrazide side groups. This project was to explore the scavenging effect of the polymer against acrolein. The results showed that syr 48 can scavenge free acrolein and protein adducts in a medium-free environment. When the medium was present, however, the scavenging effect was limited since there was a competition between the polymer and the proteins in the medium. The scavenging effect was enhanced by increasing polymer concentration. We proved that the polymer was interacting with cells by using fluorescent polymer but it was hard to tell whether the polymer was on the membranes or inside the cells due to the limitation of the equipment.

Introduction

Oxidative stress

What is oxidative stress? We have to start from reactive oxygen species (ROS). All aerobic organisms require oxygen to produce cellular energy: adenosine-5-triphosphate (ATP). In this respiration process, oxygen (O_2) is reduced to water (H_2O) along the mitochondrial electron transport chain, where ROS are produced as the by-products of O_2 reduction (Kowaltowski et al., 2009). Major ROS molecules are superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (Toyokuni, 1999). They are highly reactive and will attack biomolecules like protein, lipid and DNA. Among them, superoxide is the primary ROS and hydroxyl radical is most reactive (Papa and Skulachev, 1997). Endogenous ROS are generated during other cellular metabolism as well but mitochondrion is considered as the major source.

Aerobic organisms have intrinsic antioxidant defense system to counter constant ROS attack. This system includes enzymatic and non-enzymatic defense. In enzymatic defense, $O_2^{\cdot -}$ is first dismutated to H_2O_2 by superoxide dismutase (SOD). Then H_2O_2 is reduced by either mitochondrial catalase or glutathione and thioredoxin peroxidase, using glutathione (GSH) and thioredoxin (TrxSH) as hydrogen donors (Kowaltowski et al., 2009). In non-enzymatic defense, vitamin C, vitamin E, GSH and other compounds participate the detoxification process of ROS.

At physiological concentration, endogenous ROS are essential to many signaling pathways and degenerative process (Kowaltowski et al., 2009). When the intrinsic antioxidant defense is overwhelmed by elevated endogenous or exogenous ROS, however, oxidative stress occurs. Excess ROS will disturb normal cell functions even cause cell death if intrinsic antioxidant defenses fail to protect against them. The major effects of ROS are protein and DNA modification, ion homeostasis disruption and lipid peroxidation (Burton and Jauniaux, 2011).

Mainly hydroxyl radical $^{\cdot}OH$ attacks DNA. It can cause DNA breakage; cross-linking with proteins; degradation, deletion and translocation of DNA bases. For protein modification, ROS attack side groups of amino acids and disturb the structures and functions of proteins.

ROS disturb the Ca^{2+} homeostasis in two aspects: on the one hand, ROS release Ca^{2+} from endoplasmic reticulum (ER) and affect the correct folding of the proteins; on the other hand, ROS increase the Ca^{2+} concentration in cytosol, which affects the mitochondrial membrane potential and ATP

synthesis (Burton and Jauniaux, 2011).

In lipid peroxidation progress, the oxidation of polyunsaturated fatty acids (PUFAs) generates many products, ROS, aldehydes, dicarbonyls, hydroxyalkenal, etc (Fig. 1). ROS are transient free radicals while aldehydes have comparatively longer half-life *in vivo*, which could cause more harm to the organisms. Malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), 4-hydroxy-2-hexenal (4-HHE) and acrolein are intensively studied since they react with biomolecules and are, to some extent, related to different diseases (Negre-Salvayre et al. 2008). Among all the unsaturated aldehydes products, acrolein shows highest activity towards nucleophile residues in proteins, lipids and DNAs.

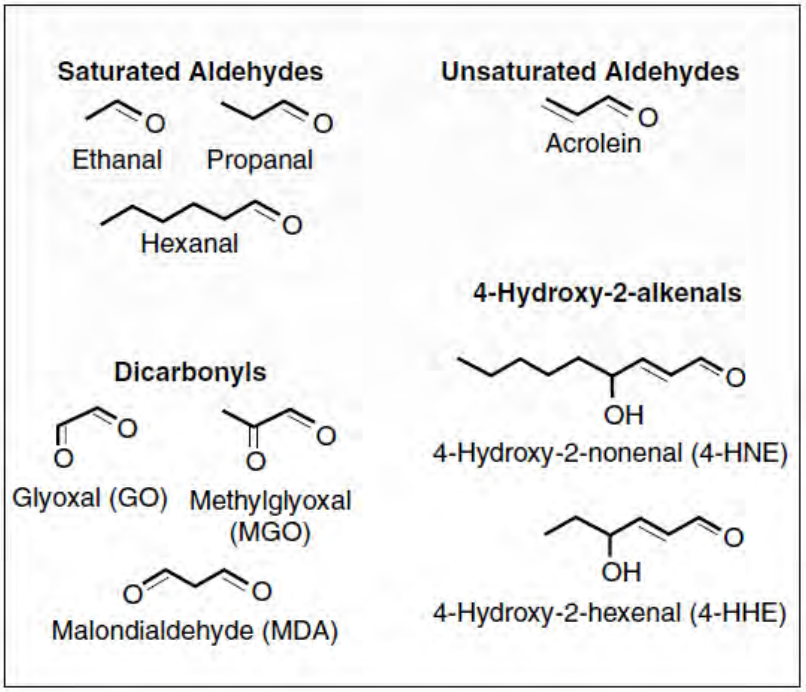


Figure 1 The structures of some lipid peroxidation products, including saturated and unsaturated aldehydes, dicarbonyls and hydroxyllalkenals (Negre-Salvayre et al. 2008).

Acrolein

Acrolein is not only a byproduct of lipid peroxidation in vivo, is also a naturally occurring substance in the environment. It is generated during the combustion of organic matters, like fuel, tobacco, and even cooking oil. Acrolein attacks nucleophiles in cysteine, histidine and lysine, in which way it is readily incorporated into proteins and forms carbonyl adducts. These adducts still process electrophiles therefore they will attack other nucleophiles and form cross-linking between protein-protein or protein-DNA (Burcham et al., 2003;

Burcham and Pyke, 2005). Carbonyl adducts and protein cross-linking impairs the normal cell functions.

Acrolein, as the product of lipid peroxidation, can lead to lipid peroxidation as well. The possible mechanism behind it is that acrolein disturbs membrane integrity by cross-linking membrane bound proteins. Damaged membrane disturbs calcium homeostasis and damages mitochondrial (Hamann and Shi, 2009). As a result, ROS are generated and ROS cause further lipid peroxidation and oxidative stress. What's more, acrolein attacks the thiols group in glutathione (GSH), which is an important endogenous antioxidant (Hamann and Shi, 2009). The depletion of antioxidant further more contributes to the oxidative stress.

The relations with diseases

It has been proved that oxidative stress is involved in the development of many diseases. For instance, high level of oxidative stress decreased cellular GSH and mitochondria dysfunction are found in the patients with Parkinson's disease (Hauser and Hastings, 2013). Mitochondria damage and the resulting ROS generating are directly linked to Alzheimer disease (Bonda et al., 2013). Some studies also point out that oxidative stress is associated with cardiovascular disease (Naranjan et al., 2000), chronic renal failure (Popolo et al., 2013), brain infarction (Yoshida et al., 2010), pregnancy complications (Burton and Jauniaux, 2011), etc.

As one ingredient in cigarette smoke, acrolein is suggested to cause and accelerate pulmonary inflammation. Facchinetti et al. shows that acrolein and 4-HNE stimulates human macrophages to release inflammatory mediators.

Unsaturated aldehydes produced by lipid peroxidation, like 4-HNE, MDA and acrolein, form adducts with proteins. These modified proteins decrease the degradation function of proteasome, which leads to the accumulation of protein adducts in the tissue. Negre-Salvayre et al. pointed out that aging process is closely linked to this accumulation of modified proteins.

In the fact, acrolein-protein adducts are suggested as a bio-marker for oxidative stress (Uchida et al., 1998; Calingasan et al., 1999). Yoshida et al. demonstrates that acrolein-albumin adducts exist in the blood of patients who suffer from brain infarction and the level of adducts is a good marker for the disease. Some researchers even consider albumin as a major antioxidant in human blood to scavenge acrolein and ROS (Medina-Navarro et al., 2010; Yoshida et al., 2010).

Polymer syr 48

Syr 48 is the water soluble polyvinyl alcohol (PVA) with modified side groups (Fig. 2). Degree of substitution is 15%, which is 15% of hydroxyl groups are replaced by hydrazide groups.

Hydrazide groups are characterized by two covalent-bonded nitrogen and at least one of the four substitutes is an acyl group (Fig. 3). Guiotto et al. shows that several hydrazide compounds are capable of scavenging MDA generated by liposome peroxidation. Besides that, some studies demonstrate the cytoprotection function of hydralazine, an anti-hypertension drug with a similar structure to the hydrazide (Burcham et al., 2002; Burcham et al., 2003; Lisa et al., 2004). They point out that hydralazine protects the cells against acrolein in two ways. On the one hand, it reacts with acrolein directly and on the other hand, it inhibits the cross-linking between the carbonyl adducts and other proteins (Burcham and Pyke, 2005).

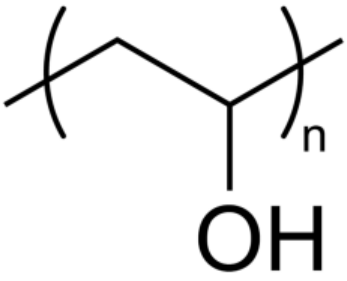


Figure 2 The structure of polyvinyl alcohol

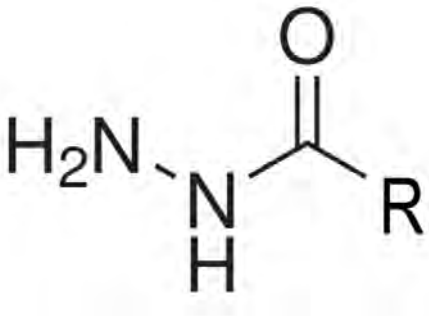


Figure 3 The structure of hydrazide

The aim of this project

Above all, oxidative stress and the resulting ROS, aldehydes are harmful to the organisms and linked to various diseases. Given the short half-life of ROS, acrolein or other aldehydes with a longer half-life serve a better target to the disease treatment. We choose acrolein as the target since it is strongly associated with oxidative stress. Oxidative stress causes lipid peroxidation which generates acrolein and acrolein initiates lipid peroxidation which contributes to additional oxidative stress. Hydrazide groups process electrophiles and the hypothesis is that these electrophiles will attack the nucleophiles in acrolein. The aim of this project is to explore if polymer syr 48, PVA with hydrazide side groups can scavenge acrolein medicated oxidative stress.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM) F-12 HAM, penicillin and streptomycin antibiotics mixture, human serum albumin (HSA), and (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was purchased from Thermo Scientific. Antibiotics mixture of penicillin, streptomycin and L-glutamine was purchased from PAA. Phosphate buffered saline (PBS) was purchased from Biochcrom AG. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific.

Cell culture

Human dermal fibroblast (hDF) cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and L-glutamine. The cells were then incubated at 37 °C, 5%CO_2.

All the assays were conducted with 24 or 6 well polystyrene plates. The seeding day was considered as day 0 and all the assays except fluorescence assay were four days cultures.

Viability test

The viability of the cells was evaluated by using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. MTT powder was dissolved in PBS to reach the concentration 5 mg/mL and then filtered through a 0.2 µm sterile filter. Old medium in the wells were replaced by 500µL fresh medium and then 50 µL MTT solution (10% of the culture volume) was added to each well. After they were incubated for 4 hours, medium was gently aspirated away with a glass pipette and 500µL DMSO was added to each well to dissolve the blue crystal. Absorbance was measured at 570nm/690nm using Tecan infinite M200 microplate reader.

Fluorescent microscopy

The hDF cells were seeded in a 6 well plate with the seeding density 2×10^5 cells/well on day 0. The culture volume was 2 mL and the plate was incubated for 48 hours in the incubator. Different concentration ratios between the

polymer and acrolein, like 1:0, 1:1, 2:1 and 5:1, were incubated in PBS for 24 hours before added to the cells. The cells were then exposed to the incubated polymer for 24 hours on day 2.

On day 3, old medium in each well was removed and the cells were washed with 1 mL PBS twice to remove unattached polymer. Fresh medium was added and the cells were observed under fluorescent microscope.

Then the medium in each well was removed and the cells were washed with 1 mL PBS. The plate was incubated for 5 minutes after 200μL trypsin was added to each well. Then 2 mL fresh medium was added to each well and gently pipetted up and down to detach the cells. The cells in each well were transferred to a 2 mL eppendorf tube and gently centrifuged down, 1000rpm for 5 min. The supernatant was removed and the pellet was re-suspended in a new 6 well plate. The cells were observed under fluorescent microscope again after allowing them to attach for 24 hours.

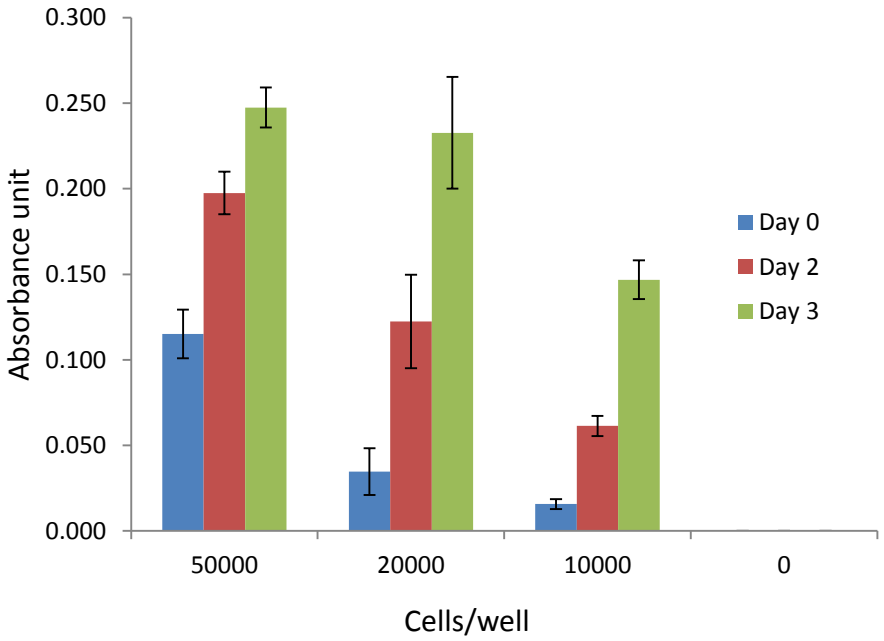
Result

1 Seeding experiment

HDF cells with two different seeding densities, 50,000 cells/well and 20,000 cells/well, were cultured for four days to determine the optimal seeding density for the future assays. Cells' viabilities were measured by MTT assay on day 0, day 2 and day 3. On day 0, the cells were allowed to attach to the plate for 1 h before the viability test was carried out.

A and B in figure 4 display the results in two kind of charts. From fig. 4. B, it can be seen that the cells with 20,000 cells/well seeding density grow faster than the one with 50,000 cells/well seeding density. These two curves almost reach the same absorbance value on day 3. The possible explanation is that the cells with high seeding density reached confluent before day 3 and the cell growth slowed down since the space was limited and culture condition was not optimal. Therefore, 20,000 cells/well was chosen as the seeding density in the later assays.

A



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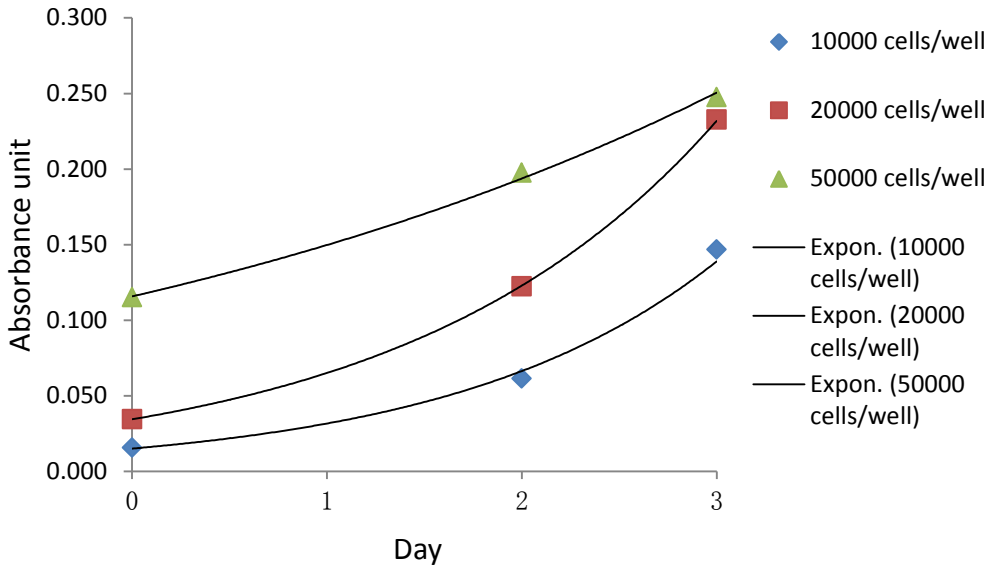

Figure 4 A, The viabilities of hDF cells in a four days culture with two different seeding densities. B, The viabilities of hDF cells in a four days culture with two different seeding densities. Each group represents the mean ± SD of triplicate.

2 The cytotoxicity of acrolein

In order to investigate the cytotoxicity of acrolein, hDF cells were cultured for four days and then exposed to different concentrations of acrolein for 24 hours on day 2. On day 3, MTT assay was carried out to measure the viability.

Figure 5 shows the responses of the cells with two different seeding densities. It can be seen that the viability of 20,000 cells/well group drop significantly under 25μM acrolein while 50,000 cells/well group decreases significantly under 30μM. This figure shows that acrolein is cytotoxic to hDF cell line when it reaches certain concentration.

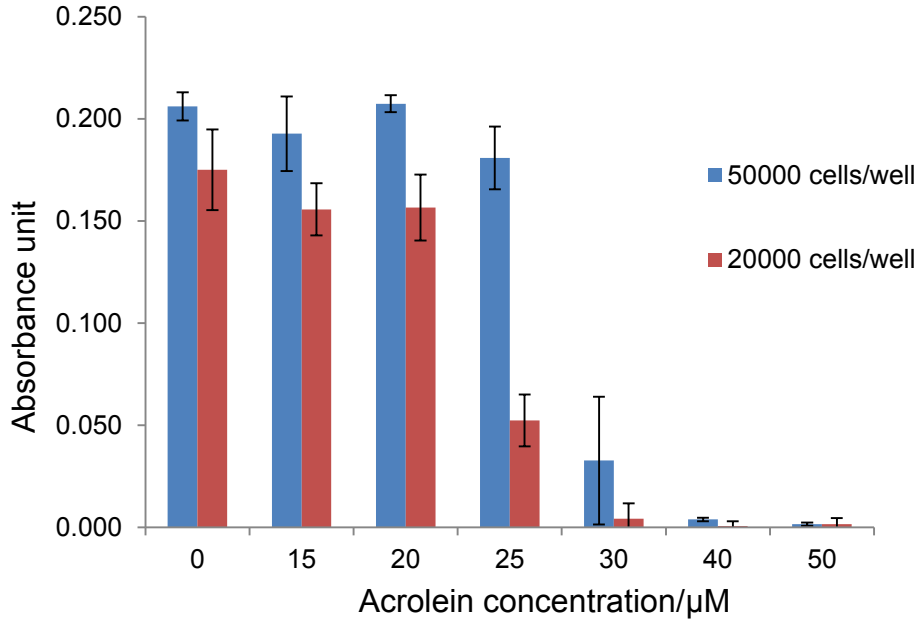

Figure 5 The viabilities of hDF cells under different concentrations of acrolein in a four days culture. Each group represents the mean ± SD of triplicate.

3 The cytotoxicity of incubated acrolein

We mentioned before that acrolein readily incorporates into proteins and forms carbonyl adducts. In the acrolein cytotoxicity experiment, cells died when they were exposed to the mixture of acrolein and medium. The main ingredient of FBS is bovine serum albumin (BSA) and there are a lot of free amino acids in the medium, too. Therefore, the question is: which kills the cells, free acrolein or carbonyl adducts?

Uchida et al. conducted an acrolein titration experiment with BSA and demonstrated that 1 g/L BSA can yield approximate 0.45 mM carbonyl groups within 6 hours. The BSA concentration in the medium we used is about 2.97 g/L and it can react with 1.34 mM acrolein. The highest acrolein concentration in acrolein cytotoxicity assay is 50μM, which means the BSA alone in the medium is excess and enough to neutralize all the acrolein. The thought was that if we incubated acrolein with medium for 24 hours then all the free acrolein would be incorporated into the proteins. We thus conducted a assay in which the cells were exposed to the incubated acrolein and medium for 24 hours, instead of fresh mixture of acrolein and medium. Other procedures of the assay is the same as acrolein cytotoxicity assay and the results are displayed in figure 6.

Figure 6 shows similar pattern as figure 5 and it demonstrates that

acrolein-protein adducts are cytotoxic under certain concentration.

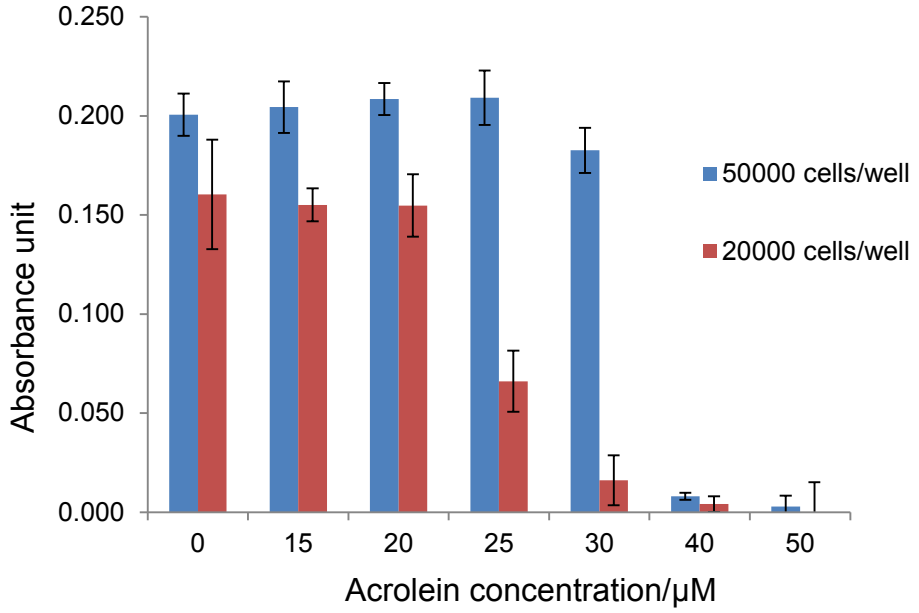

Figure 6 The viabilities of hDF cells after exposed to incubated acrolein and medium. Each group represents the mean ± SD of triplicate.

4 The cytotoxicity of acrolein-HSA adducts

In order to further prove that acrolein-protein adducts are cytotoxic, we incubated human serum albumin (HSA) with acrolein in PBS solution to produce acrolein-HSA adducts. Then hDF cells were exposed to the medium containing HSA adducts for 24 hours. HSA and BSA are homologous proteins. They contain almost the same amount of lysine and histidine and their molecular weights are almost the same. The idea was that using HSA adducts, to some extent, could mimic the BSA adducts when acrolein was incubated with medium.

The responses from the cells under different concentrations are illustrated in figure 7. The viability of high seeding density group drops significantly under 50μM HSA adducts and low seeding density group under 40μM. It seems that HSA adducts are less cytotoxic comparing with the same concentration of incubated acrolein with medium. This may due to the difference between HSA and BSA even though they are homologous proteins. Or HSA adducts tend to cross-link other proteins in the medium and the cytotoxicity decreases.

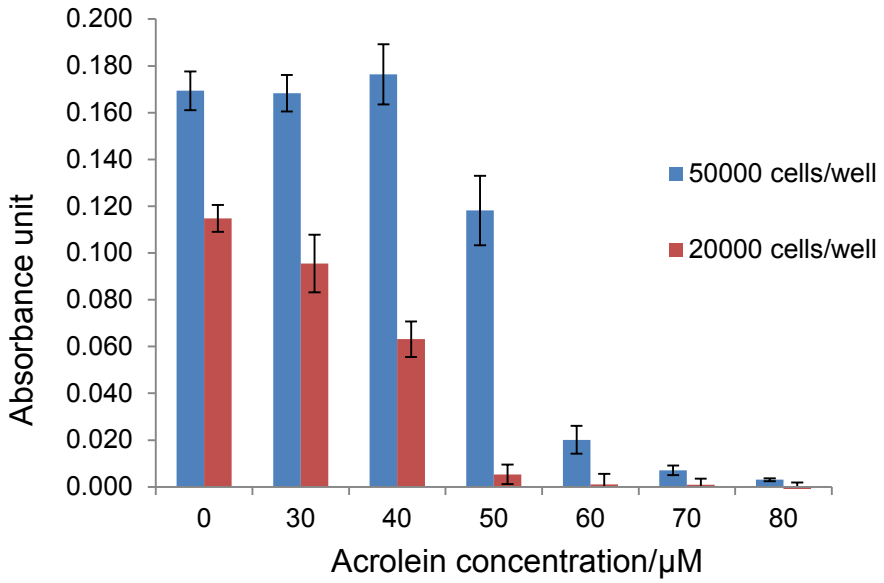

Figure 7 The viabilities of hDF cells after exposed to incubated HSA-acrolein adducts. Each group represents the mean ± SD of triplicate.

5 Pilot polymer experiment

This pilot experiment was to explore if polymer syr 48 could scavenge acrolein as well as if the polymer itself affected hDF cells. The acrolein concentration adopted was 30μM since it could kill most of the cells according to the results we obtained in acrolein cytotoxicity assay. The end concentration of the polymer, which means the concentration of the functional side group, was 30μM. In this four days culture, cells were seeded on day 0; acrolein and the polymer were added in different orders, on day 1 or day 2; the viabilities of the cells were measured on day 3.

The results from different groups are illustrated in figure 8. In ‘polymer acrolein parallel’ group, the cells do not survive when the polymer and acrolein are added at the same time in the medium. In ‘polymer then acrolein’ group, partial viability is surprisingly maintained when the cells are pre-treated with the polymer.

The viability of the cells is slightly affected when the polymer is added on day 1 while it slightly increases when the polymer is added on day 2.

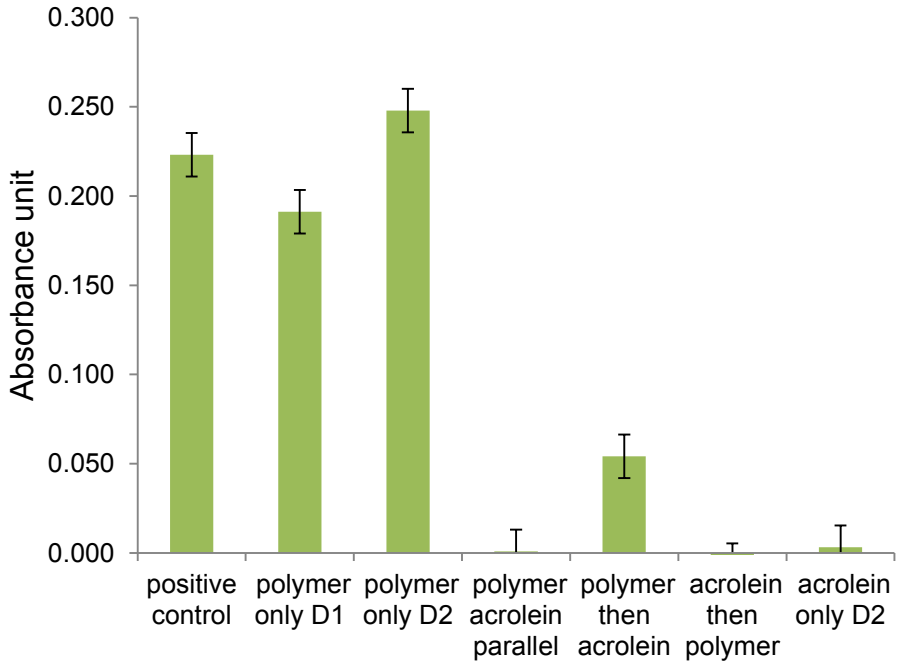

Figure 8 The scavenging effect of the polymer syr 48 against acrolein in a four days culture. The end concentration of the polymer and acrolein was 30μM. For positive control group, no acrolein or polymer was added. For 'polymer acrolein parallel' group, the polymer and acrolein was added on day 2 at the same time. Each group represents the mean ± SD of triplicate.

6 Increasing polymer concentration in pre- and parallel-treatment

From last experiment we can see that the parallel-treatment of 30μM polymer did not scavenge acrolein while the pre-treatment of the polymer saved some cells. Therefore, we tried to increase the polymer concentration in pre- and parallel-treatment to enhance the scavenging effect. In this four days culture, cells were seeded on day 0; the polymer was added on day 1 in pre-treatment group and on day 2 in parallel-treatment group; acrolein was added on day 2; the viabilities of the cells were measured on day 3.

Figure 9 shows the all the results. It can be seen that the cells do not survive in 30μM polymer pre-treatment group, which is contradict with the result we got in experiment 5. There is still no cell viability even when the polymer concentration is increased to 90μM. Probably the positive result for pre-treatment in experiment 5 is wrong.

We can also observe that the polymer, concentration ranging from 30μM to 90μM, has no scavenging effect against acrolein in parallel-treatment. Maybe

even higher polymer concentration is needed.

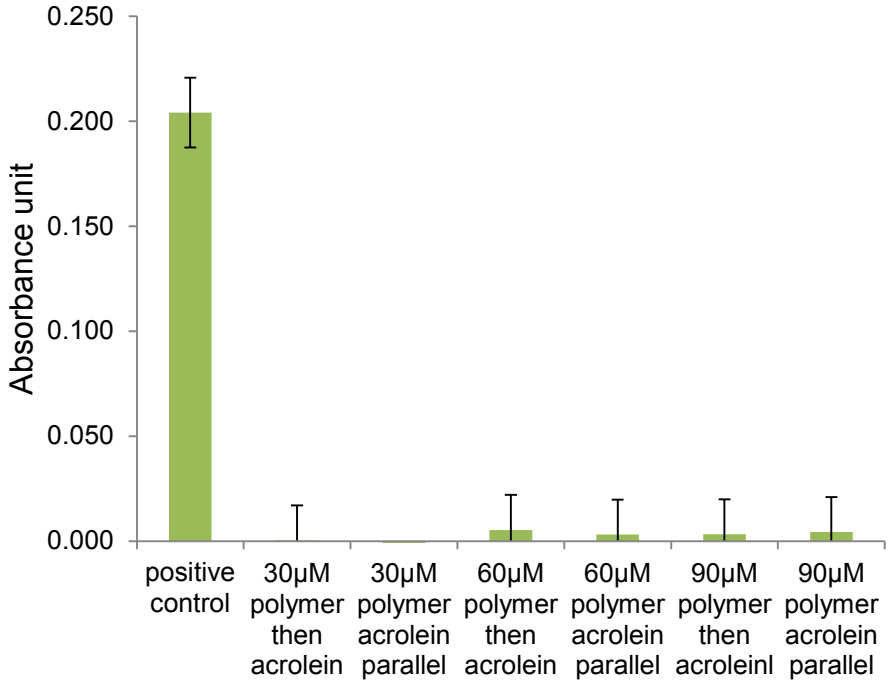

Figure 9 The viabilities of hDF cells with increasing polymer concentration in pre- and parallel-treatment. The end concentration of the acrolein was 30μM and the concentration of the polymer ranges from 30μM to 90μM. For positive control group, no acrolein or polymer was added. Each group represents the mean ± SD of triplicate.

7 Further increasing polymer concentration in parallel-treatment

In experiment 6, the polymer with the concentration ranging from 30μM to 90μM did not scavenge acrolein in the parallel-treatment. In this experiment, we tested the polymer with the concentration ranging from 100μM to 600μM. In this four days culture, cells were seeded on day 0; the polymer and acrolein was added on day 2; the viabilities of the cells were measured on day 3.

The results are illustrated in figure 10. Under 0μM polymer, 'polymer only' group is the positive control with no polymer or acrolein while 'polymer acrolein parallel' group is the group with only acrolein added on day 2.

For 'polymer only' group, it can be seen that the viability of hDF drops with the increasing polymer concentration. For 'polymer acrolein parallel' group, it is obvious that the viability increases with the increasing polymer concentration.

When the concentration reaches 400μM and 600μM, polymer syr 48 displays a significant scavenging effect against acrolein.

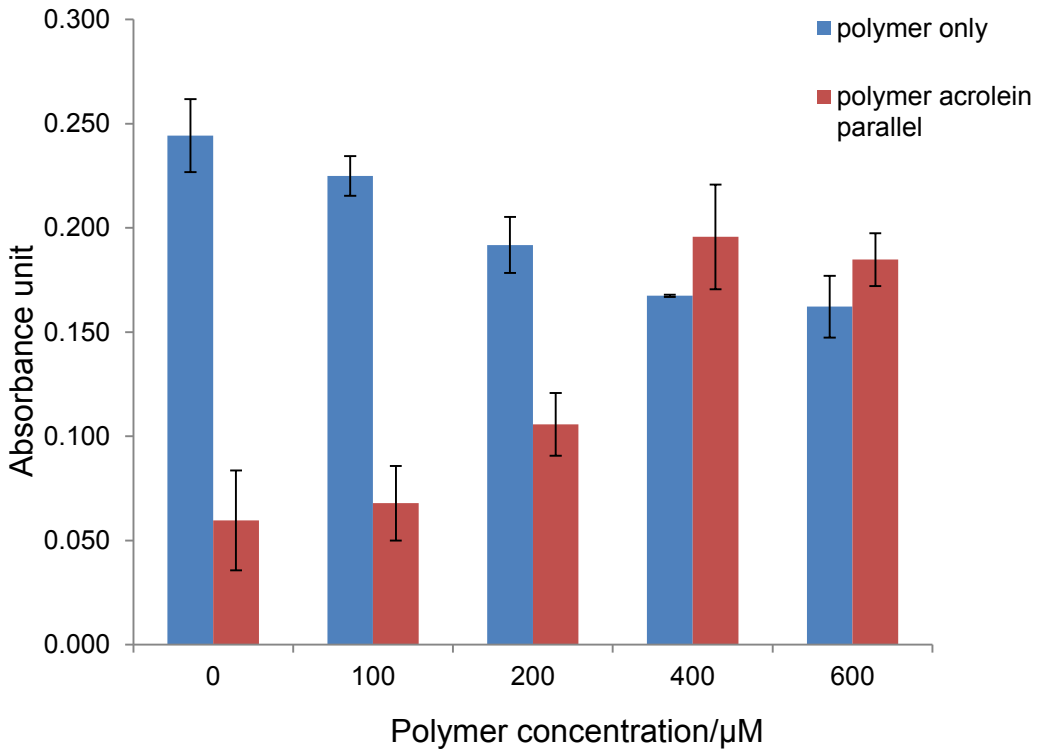

Figure 10 The scavenging effect of the polymer with increasing concentration in parallel-treatment. The end concentration of the acrolein was 30μM and the concentration of the polymer ranges from 100μM to 600μM. For positive control group, no acrolein or polymer was added. For other groups, the polymer and acrolein were added on day 2. Each group represents the mean ± SD of triplicate.

8 Incubating polymer, acrolein with and without medium

This experiment is to explore if the polymer can scavenge acrolein in incubation, with or without the presence of the medium. The polymer and acrolein were incubated for 24 hours, with or without the present of the medium and the end concentration of polymer and acrolein was 30μM. In this four days culture, cells were seeded on day 0; incubated polymer solutions were added to each group on day 2; the viabilities of the cells were measured on day 3.

The results are illustrated in figure 10. When the cells are pre-treated with the polymer, consistent with the result in experiment 6, the viability almost drops to zero. Adding polymer only on day 1 will lead to the slight decrease of the cell viability, which is consistent with the result in experiment 5.

Figure 11 also shows that the cell viability drops when the polymer and

acrolein are added in the medium at the same time (both ‘polymer acrolein parallel’ and ‘incubated polymer acrolein medium’). However, the cells are saved when the polymer and acrolein are incubated for 24h before adding. The reason behind it is probably that acrolein is incorporated into proteins faster than reacting with polymer when the medium is present. The medium or proteins in the medium are competing with the polymer and increasing polymer concentration may enhance the reaction between the polymer and acrolein.

For ‘incubated polymer acrolein medium’ group, partial cell viability was saved when the polymer, acrolein and medium were incubated for 24 hours. This may suggest that the polymer is capable of scavenging protein adducts.

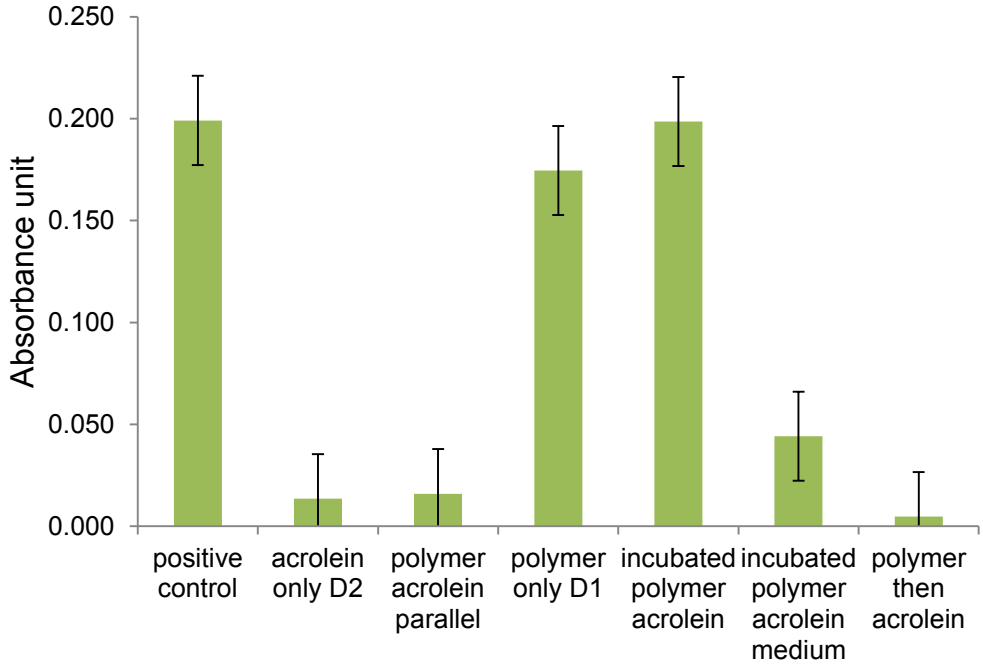

Figure 11 The scavenging effect of the polymer syr 48 against acrolein in incubation, with or without the presence of the medium. The end concentration of polymer and acrolein was 30μM. For positive control group, no acrolein or polymer was added. For ‘polymer acrolein parallel’ group, the polymer and acrolein was added on day 2 at the same time. For ‘incubated polymer acrolein’ and ’incubated polymer acrolein medium’ group, incubated solutions were added on day 2. Each group represents the mean ± SD of triplicate.

9 Increasing polymer concentration in incubation with medium

Given that the polymer can save some cells when it was incubated with acrolein and medium, we suggested that the scavenging effect could be further enhanced if we increased the polymer concentration in the incubation.

Therefore, the polymer syr 48, concentration ranging from 30μM to 200μM, was incubated with acrolein and medium for 24 hours before exposed to the cells. In this four days culture, cells were seeded on day 0; incubated solutions were added to each group on day 2; the viabilities of the cells were measured on day 3.

The results were illustrated in figure 12. This chart demonstrates that increasing polymer concentration in incubation, to some extent, increases the cell viability. The possible reason is that the polymer can scavenge the cytotoxic protein adducts.

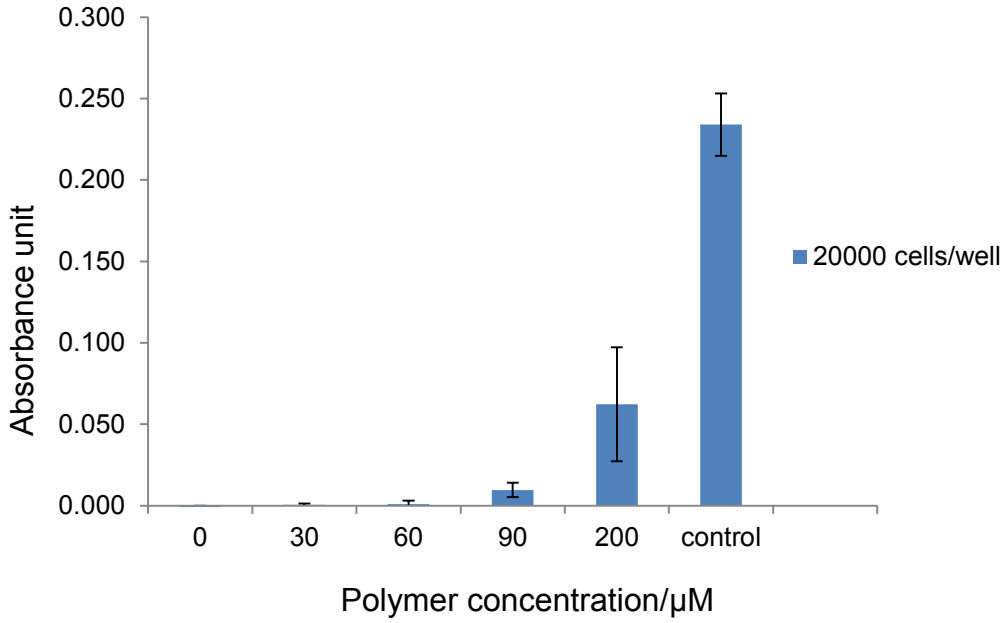

Figure 12 The viabilities of hDF cells with increasing polymer concentration in the incubation of the polymer, acrolein and medium. The end concentration of the acrolein was 30μM and the concentration of the polymer ranges from 30μM to 200μM. For positive control group, no acrolein or polymer was added. Each group represents the mean ± SD of triplicate.

10 Incubating the polymer and HSA adducts

Our thought was that polymer syr 48 may be capable of scavenging protein-acrolein adducts and this experiment is to verify this very idea. HSA was first incubated with acrolein in PBS for 24 hours to generate HSA-acrolein adducts and then HSA adducts was incubated with polymer syr 48 for another 24 hours before exposed to the cells. In this four days culture, cells were seeded on day 0; cells were exposed to HSA adducts and polymer-adduct mixture on day 2, respectively; the viabilities of the cells were measured on

day 3.

Figure 13 displays the results and it demonstrates that the polymer can scavenge HSA adducts in a medium free environment.

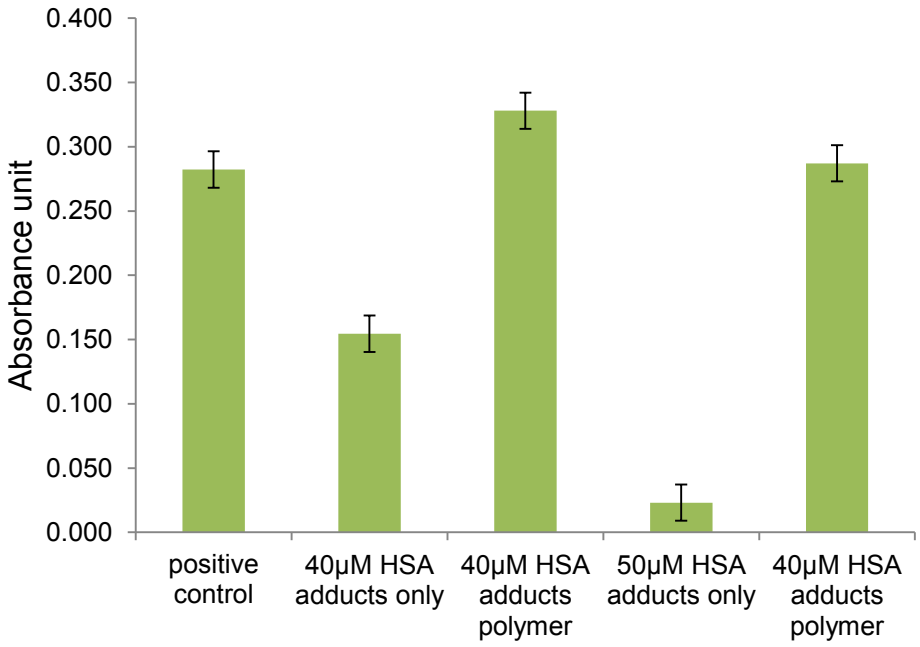

Figure 13 The viabilities of hDF cells when they are exposed to HSA adducts and incubated polymer-adduct mixture. For positive control group, no acrolein or polymer was added. For other groups, HSA adducts and polymer-adduct mixture were added on day 2. Each group represents the mean ± SD of triplicate.

11 Fluorescent polymer experiment

Teramura et al. used fluorescence tagged polymer and studied behaviors of different polymers interacting with cell membranes. They showed that polyvinyl alcohol with alkyl side groups (PVA-alkyl) attached to the membrane through hydrophobic interactions and subsequently took into cytoplasm. In order to understand the interaction between our polymer and hDF cells, we used the polymer syr 48 which was tagged with fluorescein isothiocyanate (FITC). Our polymer was hydrophilic and we thought the polymer may become hydrophobic if the side groups reacted with acrolein. Therefore, we prepared polymer-acrolein mixture by incubating the polymer and acrolein with different ratios, 1:0, 1:1, 2:1 and 5:1.

The cells were seeded on day 0. Different polymer-acrolein mixtures were incubated for 24 hours and then added to the cells on day 2. The end concentration of the polymer was 100μM, the same for each group. On day 3,

the cells were washed with PBS twice to remove un-attached polymer then they were observed under fluorescent microscope (fig. 14).

It can be seen that the groups with only polymer (fig. 14 A) displays significant fluorescence. The fluorescence is much less when the ratio between the polymer and acrolein is one to one (fig. 14 B). Then the fluorescence increases when the ratio increases to 2:1 (fig. 14 C) and 5:1 (fig. 14 D). However, we can not be sure that all the fluorescence comes from the polymer interacting with the cells since there are possibilities that it may attach to the bottom of the plate or get stuck between the cells. Another step is needed.

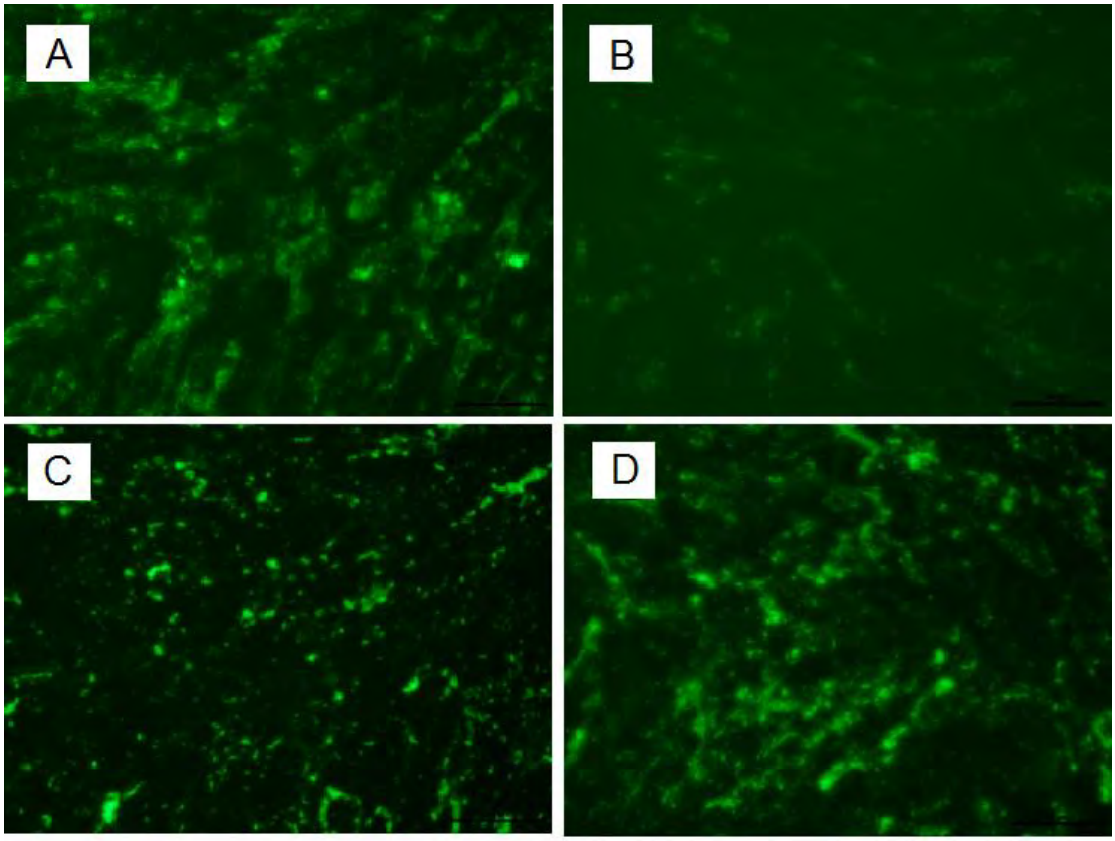

Figure 14 The fluorescent microscopy of hDF cells incubated with the polymer on day 3. The end concentration of the polymer was 100μM. A: polymer: acrolein = 1:0 B: polymer: acrolein = 1:1 C: polymer: acrolein = 2:1 D: polymer: acrolein = 5:1

After the observation, the cells were trypsinized, centrifuged and re-plate to remove the polymer which attached to the bottom or stuck between cells. The cells were allowed to attach for 24 hours then observed again under fluorescent microscope (fig. 15).

For the group with polymer-acrolein ratio 1:1, there is almost no fluorescence left (fig. 15 D). All three other groups still display significant fluorescence, especially those round, shinning objects. The hypothesis is that those round

objects are cells with a different morphology. They may change the morphalogy due to the influence of the polymer but they are still alive since dead cells would be washed away during the washing process.

The fluorescence we can observe now is from the polymer interacting with the cells. They either anchor on the surface of the membranes or enter the cytoplasm of the cells. However, the exact location of the polymer is hard to tell and more sophisticated equipment is needed, for example, confocal laser scanning microscopy.

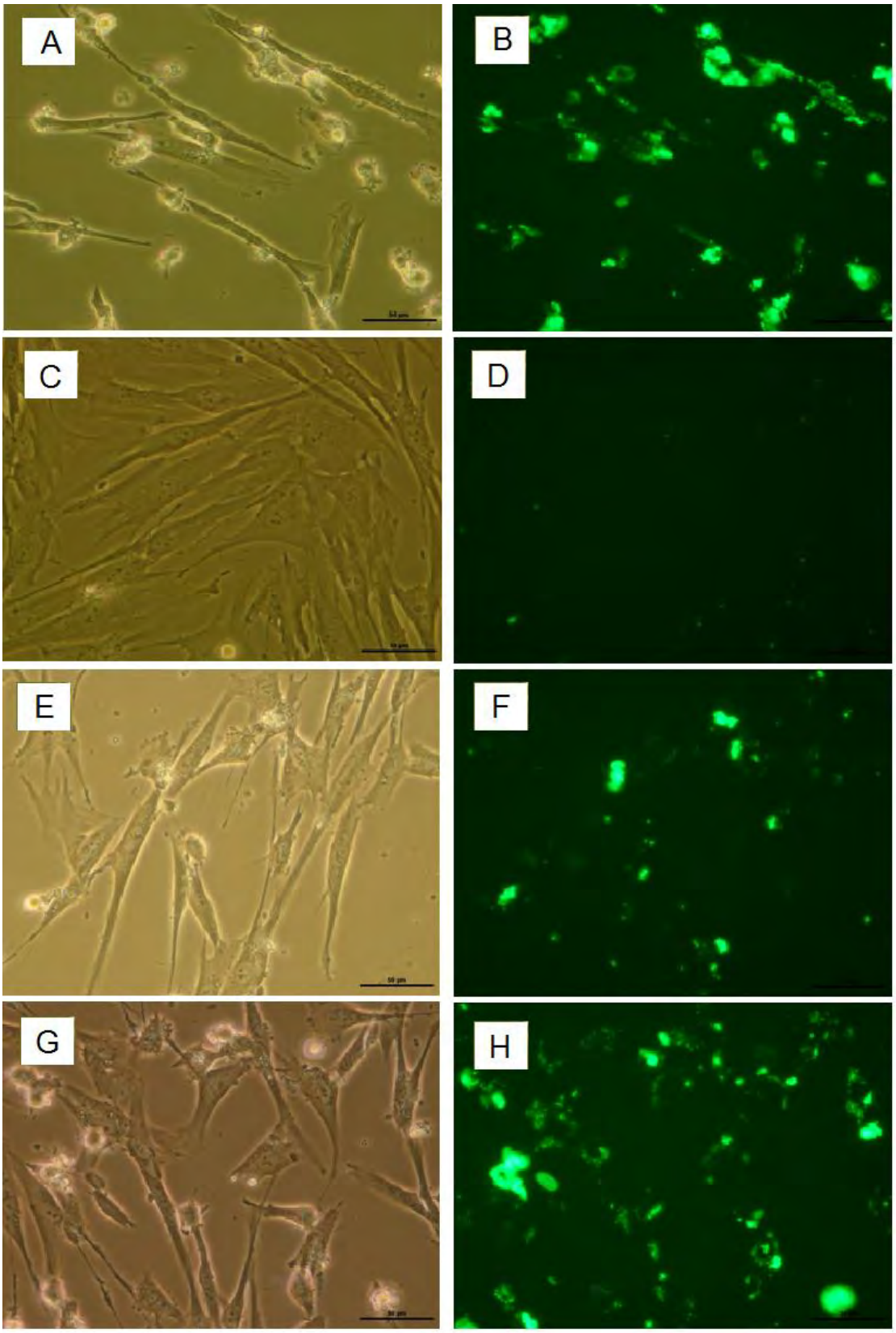

Figure 15 The normal microscopy (left) and fluorescent microscopy (right) of hDF cells incubated with the polymer on day 4. A and B: polymer: acrolein = 1:0 C and D: polymer: acrolein = 1:1 E and F: polymer: acrolein = 2:1 G and H: polymer: acrolein = 5:1

Discussion

The concentration-dependent cytotoxicity

From the experiments 2, 3 and 4, we can see that the cytotoxicity of the acrolein and acrolein adducts is concentration dependent. At low or physiological concentration, the molecular antioxidant defense, like glutathione, can protect the cells against the toxin. When the concentration of acrolein reaches certain level, the antioxidant is depleted and the toxin starts to attack biomolecules, like lipids and proteins. Since the major components of the membrane are lipids and proteins, the integrity of the membrane is compromised and this probably leads to cell death.

The cytotoxicity of protein adducts

Experiment 3 and 4 shows that acrolein-protein adducts are cytotoxic to the cells. Like we discussed before, these adducts are still active and they tend to react with other proteins to form cross-linking. If the cross-linking happens on the proteins floating in the membrane, the integrity of the membrane is disturbed.

The scavenging effect of the polymer

Experiment 8 demonstrates that the polymer can scavenge the acrolein with the same concentration in a medium free environment while experiment 10 shows that the polymer can scavenge the acrolein-HSA adducts in a medium free environment. The principle behind it is probably that hydrazide side groups process nucleophiles which attack the electrophiles in free acrolein and carbonyl adducts. In such a way, the polymer ‘neutralizes’ poisoned acrolein and carbonyl adducts.

However, the scavenging effect of the polymer against acrolein and carbonyl adducts is limited when medium is present. Our hypothesis is that there is a competition between the polymer and proteins existing in the medium when the medium is present. Acrolein reacts with proteins faster than reacting with polymer when the polymer concentration is low. When the polymer concentration is several folds higher, like 400μM or 600μM in experiment 7, more acrolein reacts with the polymer instead of proteins and therefore cells survive.

The effect of the polymer on cells

Figure 16 shows the microscopy of the control group and the group under 600μM polymer in experiment 7. The background of figure 16 B is quite blurry

and there are less cells comparing with the control group. This phenomenon exists in all the groups with high polymer concentration and becomes more and more obvious along the increasing polymer concentration. It is possible that the polymer precipitates on the surface of the plate when the polymer concentration is really high. It detaches the cells from the bottom of the plate and prevents the attachment of the cells. This may explains why the viability of the cells deceases when the polymer concentration increases in the polymer only group of experiment 7.

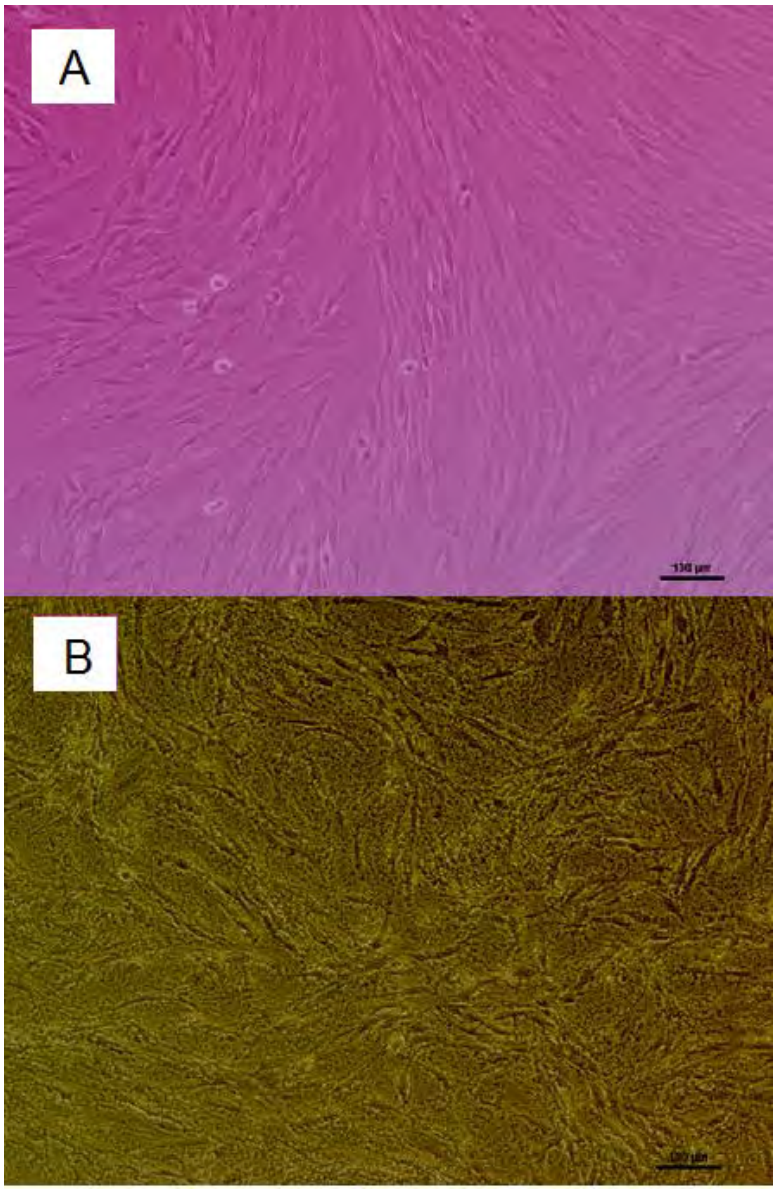

Figure 16 A: The microscopy of control group in experiment 7 on day 3. B: The microscopy of the group with 600μM polymer in experiment 7 on day 3.

The interaction between the polymer and cells

The purpose of using fluorescent polymer is to track the route of the polymer.

Does the polymer interact with the cells or just stays in the medium? Is there any cytoplasmic uptake? Answering all these questions will help us to understand the principles behind the scavenging.

The fluorescent microscopy we employed is just a ‘quick and dirty’ way to know if there is any interaction between the cells and the polymer. The result has showed that there is fluorescence presented on the cells even after multiple washes and changing plate. However, the microscope is not sophisticated enough to tell the exact location of the polymer, on the membrane or inside the cells.

We speculate that there is probably cytoplasmic uptake of the polymer, which enables several more possibilities how the polymer could save the cells. Lipoic acid (LA) is a natural cofactor inside human body and is considered as an antioxidant. It processes two sulphydryl moieties which can scavenge ROS. Jia et al. shows that LA also can protect the cells against the cytotoxicity of acrolein. Some researchers demonstrates that LA can pass the membrane and preform antioxidant functions in several mechanisms, direct ROS scavenging, chelating metals and recycling intrinsic antioxidants (Biewenga et al., 1997; Navari-Izzo et al., 2002; Moini et al., 2002). If the polymer can be taken into the cytoplasm of the cells, there is a possibility that it is capable of preforming these functions. Of course further study is needed to confirm this idea.

The possible application of the polymer

We are collaborating with another group using the same polymer involved animal experiments. They created an air pocket inside the joint of mouse and induced inflammation or oxidative stress by injecting acrolein. Then they injected this polymer to see whether it can reduce inflammation *in vivo*. Maybe this polymer can be applied in human body to reduce inflammation or oxidative stress if the animal trial works.

Future experiments

Like we mentioned before, one further experiment can be done is the confocal laser scanning microscopy. Then the cell membrane, organelles, and nucleus can be dyed with different colors and we can observe whether the polymer can enter the cell membrane. To see if there is cytoplasm uptake or not will help us to better understand the principle behind the scavenging effect.

All the experiments we did before used MTT assay to reflect the cytotoxicity of acrolein and the scavenging effect of the polymer. Maybe we can use other assay to measure the ROS level directly for the future experiments. LeBel et al., introduced a popular method to quantify reactive oxygen species directly

by employing 2',7'-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA can cross cell membranes and be hydrolyzed to 2',7'-dichlorofluorescin (DCFH) by esterases in the cytosol. Then DCFH is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) by ROS in the cell like H_2O_2. In this way, the ROS level can be measured by fluorescence spectrophotometer. However, DCFH has no specificity to ROS. Reactive nitrogen species (RNS) like nitric oxide ($^{\cdot}NO$) can also oxidize DCFH to DCF and whether superoxide ani$O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$) can oxidize DCFH is controversial (Myhre et al., 2003). Some researcher suggested that DCFH assay reflected the overall oxidative stress level rather than ROS level (Wang and Joseph, 1999).

Another experiment can be done is to measure the cellular glutathione (GSH) level since it has been proven that acrolein depletes GSH. Many studies measured GSH concentration and total antioxidant capacity to reflect oxidative stress level (Fernandez et al., 2013; Kim et al., 2013; Shivananjappa et al., 2013). This measurement can be achieved by using commercial GSH assay kits.

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