ENGINEERING AGGREGATES WITH CHEMICAL LINKERS FOR TISSUE ENGINEERING APPLICATION

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NATIONAL UNIVERSITY OF SINGAPORE

2006

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A THESIS SUBMITTED FOR THE DEGREE OF

MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOENGINEERING

NATIONAL UNIVERSITY OF SINGAPORE

2006

ACKNOWLEDGEMENT

This research began one and half years ago when I settled in A/P Hanry Yu's lab, when I started my second lab rotation. The first person I really would like to thank is my direct supervisor Hanry Yu. He has been impressing on me for his enthusiasm in research and his mission for high quality work. I am very grateful to him for showing me the way of research as well as the consistent help and advice he has been providing me as close as a relative and a good friend.

I am also deeply indebted to my co-supervisor Dr. Tan Choon Hong, who has been keeping an eye on my research and was always there whenever I need his advice during all the time of research and writing of this thesis.

I am especially obliged to Ong Siew Min, Tee Yee Han, Nguyen Thi Thuy Linh and Zhao Deqiang who are all my colleagues of the project, giving me the feeling of being at home at work. My former colleague, Dr Tang Guping, although he left Singapore one year ago, I still want to extend my gratitude to him, without whom I could never explore out the way in this absolutely new research field

Needless to say, that I need to thank all of my colleagues in Prof. Hanry Yu's lab, who provided me a lot of constructive ideas and advices during my research and discussions of my thesis, especially Dr. Chia Ser Mien, Susanne, Khong Yuet Mei,

Toh Yi Chin. I also want to thank Toh Yi Er for her techinical support on microscopy.

I feel a deep sense of gratitude for my father and mother who formed part of my vision and taught me the things that really matter in life. The encouragement of my father still provides a persistent inspiration for my journey in this life.

Finally I want to extend my appreciation to all of the friends who has been caring for me and helping me during the past two years.

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SUMMARY

This thesis explored a novel way to engineer artificial multicellular structures combining principles from tissue engineering, cell surface engineering and chemistry. Instead of using classical tissue engineering approach, which involves seeding cells into polymer scaffold or hydrogels, we tried to work on cell aggregates as building blocks for tissue engineering. The one-native cell-surface ketone epitopes produced by cell surface modification provides a stable molecular handle for the attachment of other molecules to cells. By combining the function of reacting with non-native groups on cell surface, and that of linking different cells, we synthesized five different kinds of chemicals shown to construct aggregates efficiently. The chemicals within defined concentrations have low cellular toxicity. In addition, the size distribution of the aggregates can be controlled by concentration and nature of the linkers, such as molecular weight. After comparing the aggregation efficiency and the viability of the aggregates, PEI-2000-hy was chosen as the model chemical linker for further study.

During the further study of aggregates by PEI-2000-hy, we discovered that this method provided a simple and efficient way to build multi-cellular structures, such as cell aggregates. Bi-functional chemicals, with the combined functions of biotin hydrazide and avidin, were used. Using this one-step linking system, we were able to achieve cellular aggregates rapidly and efficiently. In order to find out the important factors for the linkers to be an efficient cell glue, neutral tetra-hydrazide was

synthesized and found to be non reactive to the modified cells, which distinguished the positive charge possessed by PEI as an important factor for PEI-hydrazide to be an efficient linker. Besides studying the aggregating ability of this system, we also studied its cytotoxicity. Inconsistent with published data, we found that sodium periodate oxidation is the most cytotoxic step in this chemical-linking system. However, by taking the advantage of charge interaction between the positive linker and negative cell surface as well as the specific covalent interaction between ketone sialic acids and hydrazide, we were able to form multi-cellular structures using relatively low concentration of chemical linker and kept the overall viability of cells. In order to further prove the feasibility of this new system, the cell aggregates were cultured in suspension, and showed increased viability up to seven days. Fluorescent linkers were synthesized and applied in this aggregating system. The ability to directly observe the presence of fluorescent linkers on cell surfaces enabled us to track the fate of linker. Disappearance of fluorescent linkers from cell surfaces during suspension culture hinted us the existence of natural cell-adhesion molecules which took over the role of gluing the cells together compactly.

In the initial process of engineering aggregates, we could only control the size distribution of aggregates by changing chemical concentrations but not the shape of the aggregates. However, in the final stage of the study, we managed to control the shape of the aggregates by micropatterning and micromanipulation, which demonstrated the possible usage of this system in tissue engineering.

LIST OF FIGURES AND TABLES

LIST OF SYMBOLS

PDMS	Poly (dimethylsiloxane)
PEI	Poly (ethylenimine)
PAMAM	Polyamides and amines
2-IT	2-iminothiolane
HAS	Human serum albumin
FITC	Fluorescein 5'-isothiocyanate
EMCH	E- maleimidocaproic acid hydrazide. HCl
DAB-Am-4	Polypropylenimine tetramine dentrimer, Generation 1.0
DAB-Am-8	Polypropylenimine octaamine Dendrimer, Generation 2.0
DAB-Am-16	Polypropylenimine hexadecaamine Dendrimer, Generation 3.0
PI	Propidium iodide
PBS	Phosphate buffered saline
DMEM	Dulbecco’s modified Eagle’s medium
MTS	Mitochondrial reduction of tetrazolium salts into soluble dye
FBS	Fetal bovine serum
DMSO	Dimethyl Sulfoxide
MWCO	Molecular weight cut-off
EDTA	Ethylene diamine tetra-acetic acid
CTG	CellTracker™Green
CTB	CellTracker™Blue

Chapter 1 Introduction

1.1 Tissue engineering

1.1.1 Overview of tissue engineering

In the field of tissue engineering, principles of engineering and life sciences are integrated to develop biological substitutes that can restore or improve tissue functions [1, 2]. Isolated cells or cell substitutes, tissue-inducing substances, and cells placed on or in matrices, have been the most general strategies for creating new tissues [2]. Engineered tissues can be used to improve burn treatment, dental implants, bone, and cartilage transplants, as well as to replace the function of organs such as liver and kidney [3]. There are several challenges before these types of treatment are fully realized, including finding reliable sources of compatible cells, utilizing the stem cells efficiently and differentiating them properly into functional tissue, and optimizing the design and fabrication of scaffold.

Tissue engineering usually starts with cells derived from the patient or from a donor. According to the specific application, different cell types are needed from different sources. For example, articular, auricular, and costal chondrocytes are able to produce cartilaginous matrix that forms mechanically bonds with native cartilage, which makes them applicable in cartilage tissue engineering [4]. Primary hepatocytes are most commonly used in current liver engineering therapies although highly functional

hepatocyte cell lines are being developed [5-7]. Besides these mature cell types, immature cells in the stem cell stage can also be used [8]. Bone marrow stem cells are popular for bone and cartilage tissue engineering nowadays. Recently, people found that human embryonic stem cells can rescue injured hear in a clinical trial [9]. In addition to cell sources, some kind of 3-D scaffold is required to provide physical support for cells to grow outside of the human body. The design and fabrication of scaffolds has attracted much attention recently [10, 11]. In order to form hierarchical structures, which are similar to native tissues, chemical and mechanical signals are also needed at appropriate times and places to induce cellular growth. People immobilized galactose, which is specifically targeted to asiaglycoprotein receptors (ASGPR), on hepatocytes membrane, on poly (D, L-lactic-co-glycolic acid) (PLGA) surface to promote specific cell adhesion [12]. It was also found that the hepatocyte functional fate could be engineered in vitro by variable mechanochemical properties of the extracellular microenvironment [13], as well as the uses of growth factors [14].

Applications of tissue engineering can be broadly classified into two types. One is its therapeutic application in which the tissue is either grown in a patient or outside the patient before it is transplanted [15-20]. The other application is diagnostic applications, in which the tissue is made *in vitro* and used for testing drug metabolism, uptake, toxicity, pathogenicity, etc. [21-24]. In both applications, how to cause biological tissues to regenerate *in vitro* is the key problem. Development of this field is stimulated by that in gene therapy, polymer science, and cell biology [25]. With fast

development of these areas, it is possible that laboratory-grown tissue replacements will become a common medical therapy during the early decades of the 21st century [26]. However, different from simple cell culture, in which cells reproduce their own structure with essential nutrients provided in a proper environment, high level of structures must be produced before functional tissue can be constructed [27]. What determines cell organization and differentiation in tissues? Is it possible to permit the fine control of tissue architecture for the engineered tissues to become clinically useful? All of these questions require solving.

1.1.2 The strategy of Scaffolds and their limitation

There are many different ways to engineer tissues. The majority one relies on forming homogeneous, porous scaffolds that are then seeded with cells [1, 28-32]. These scaffolds are traditionally made from polymers, hydrogels, or organic/inorganic composites. They play the function of providing the required mechanical support for the cells and a frame for growth and differentiation [2, 30]. The overall tissue size and shape can be molded by biodegradable scaffolds. Flexibility of scaffold makes it possible to optimize the microgeometry for cell recruitment. In addition, the synthetic polymer can be controlled to degrade as the tissue forms [33-37]. It is now well known that viability and function of surface-attached cells depend on the properties of the surface. In fact, synthetic surfaces can be chemically modified to replicate the chemical [38, 39] and physical [40-42] features of tissues, rendering materials active for specific types of cell populations. Beside proper surface properties, mechanical

strength of three-dimensional scaffolds, in most tissue engineering is also required for implantation and interconnected channels are essential prerequisites for cell growth and nutrients to permeate the entire scaffolding [43-46].

The design of scaffolds for tissue engineering contains several levels, which include macroscopic level (on a scale of millimeters to centimeters); an intermediate level (hundreds of microns), involving the topography of pores and channels; and the molecular level, involving surface texture and chemistry (tens of microns) [10]. Current research and development in biomaterials are trying to solve problems across these spectrums. Studies of basic biological and biophysical processes at the molecular and cellular level, are required so that we understand what processes the cells need help with and what events they can accomplish by themselves [47-49]. Studies at this end of the spectrum have led to the development of new tools for biologists to use in fundamental studies of cell behavior, which in turn lead to better bioactive biomaterials. At the other end of the spectrum, scaffolds are needed to direct the macroscopic process of tissue formation [50-53]. There are two challenges existing. Firstly, the first generation of degradable polymers widely used in tissue engineering, was adapted from other surgical uses and has some deficiencies in terms of mechanical and degradative properties. New classes of degradable materials are being developed [54-56]. The second challenge is how to fabricate these relatively delicate polymers into scaffolds that have defined shapes and a complex, porous, internal architecture that can direct tissue growth [57-59]. A variety of new

approaches are being developed under the classical engineering constraints of cost, reliability, government regulation, and societal acceptance. Micropattern and computer-based printing techniques, which are among the emerging new strategies, will be reviewed in 1.1.3 and 1.1.4 separately.

Despite development of scaffolds for tissue engineering application, there exist several problems with this method. The first one is that penetration and seeding of cells is not effective enough. Uniformity of cells throughout the scaffold, without proper external guiding signals, is also a problem. Although significant progress has been made in designing scaffolds that enable effective seeding and cell migration [60], it is still far from optimal. The second problem is that natural organs usually contain many cell types, and it is a challenging technical problem to place different cell types in defined positions [61, 62]. The third problem is that different types of scaffold are required for engineering tissues which differ in properties. Besides the above problems, the absence of vascularization is the key problem for solid scaffold larger than 200 *u*m. Currently, many scientists are trying to use different ways to construct the vascularization tissue [52, 63-66].

1.1.3 Micropattern in tissue engineering

Function of tissue is modulated by the spatial organization of cells on a micrometer scale. So it is quite important to engineer tissue to replicate natural cellular structures so that we can understand, simulate and measure their *in vivo* functions. However,

selective attachment of cells on surface has always been a technological challenge. People tried severaldifferent ways such as scratched extracellular matrix pattern [67] to guide attachment, spreading and migration of cells. Recently the silicon microfabrication techniques and development in surface chemistry made it possible to design the biochemical composition of substrate [68, 69], the matrix surrounding a cell [70, 71] and the cell type contacting each other [61, 62]. Normally a template to which cells attach preferentially is microfabricated before the selective cell attachment is achieved. The template can be made of metals [72], self-assembled monolayers [73], polymers [74], extracellular matrix proteins [75] or cell adhesive peptides [76].

An alternative to this template-based pattern is to deliver cell suspension onto specific regions of a substrate by microfluidic channels [77, 78]. However, this method can only be applied to a few cell types with slow metabolism. Another alternative way is to use a stencil, which is a thin sheet containing holes of specialized shapes and sizes. Metallic stencils were micromachined to generate cellular micropatterns as early as 1967 [79]. However, the difficulty of metallic to seal against the substrate and the challenge involved in fabrication of metallic stencils with diameters around 10-15um, the size of single cell prevents the further application of metallic stencils [80]. More recently, people have successfully made cellular patterns of many adherent cell types through the fabrication of Poly (dimethylsiloxane) (PDMS) stencil [81]. The stencil can be applied to cell culture substrate before cell seeding and peeled off manually

after seeding. The stencils can be replicated many times from the same master since the replication process does not damage the mold, which make precise repeatability possible over large surface areas.

A common drawback of all these methods mentioned above is that they are topologically constrained to two-dimension. In order to reproduce tissue structure functional in three-dimension, people tried to fabricate three-dimensional microfluidic structures by stacking membranes in PDMS using proto-typing [82-84]. Although fabrication 3D PDMS mold is much more complex than fabricating simpler structures, this is a versatile technology to pattern multiple types of cells or proteins in complex continuous surface. Since tissues of mammalian organisms always exhibit complicated micro-architecture related with different cell types, the ability to pattern different cell types in 3D defined structures paves the way to study the relationship between function and structure of tissue in single cell resolution.

1.1.4 Organ printing – a novel approach in tissue engineering

Besides micropattern to control the shape of engineered tissue *in vitro*, methods to print patterns and structures of scaffold are worked out recently as novel ways to replace traditional techniques in tissue engineering [85]. Computer designs are utilized in some approaches to fabricate complex 2-D and 3-D structures directly from the basic elements. Several different printing technologies have shown the ability to create porous polymer scaffolds with both macroscopic and microscopic structures

[86]. However, seeding cells in these scaffolds only leaves a homogeneous mass of cells which does not resemble the heterogeneous structure of tissue. There are some more advanced methods of cell seeding which possibly could place different types of cells and biomaterials into the scaffold in organized patterns. They could thereby create heterogeneous constructs [87, 88]. One possible way to accomplish this seeding approach would be using a tool to print cells into single layers of scaffolds, then the entire tissue-like constructs can be built by using a layer-by-layer approach [88].

Based on the concept of printing cells, several researches have been done during the past few years. Previous experiments have been done to demonstrate that embryonic chick spinal cord cells could be printed to a substrate using a laser guidance machine [89, 90]. In addition, both prokaryotic and eukaryotic cells were shown to remain viable after printing cell patterns with a modified laser transfer technique [91]. A recently modified ink jet machine was used to print patterns of bovine aortal endothelial cells [92-94]. All of these above techniques have the ability to enhance the traditional cell-seeding process in tissue engineering by placing single or multiple cell types into scaffolds precisely controlled by computer.

Until recently, this technology of printing cells was limited to the printing of 2D tissue constructs. A new opportunity for extending the printing technology to three dimensions is created by the emerging use of thermo-reversible gels [95]. The gels, which are nontoxic, biodegradable, thermo-reversible, can be used as a sort of “paper”

and the cells are used as the “ink.” 3D constructs could be generated by dropping one layer of gel onto another layer of gel, which has already been printed with cells. This technology termed “organ printing” [92, 94] enables complex 3D organs with exact placing of different cell types to be printed in a few minutes. Previous work also showed that cell aggregates which are placed closely in a 3D gel can fuse into structure defined by initial location of the aggregates [96]. This proves the feasibility of this method in the area of tissue engineering.

1.2 Cell Aggregates

1.2.1 Reaggregate approach in tissue engineering

Regeneration of simple animals and whole vertebrate tissues was achieved in reaggregation experiments several decades ago [97]. It is attempted to regenerate more or less complete tissues or organs from dispersed cells of a particular origin under specifically controlled culture conditions. The technique includes dissociation of tissue enzymatically or mechanically, reaggregating of the dispersed cells into multi-cellular spheres by rotation in suspension, and culture of spheres in regular culture dishes, spinner flasks, or in conical tubes within roller drums [98, 99]. Suspension cultures of 3D spheres allow tissue growth in all three dimensions [100]. It was also found that compared with cells in monolayer cultures, the cells in 3D-spheres have higher proliferation rates and their differentiation more closely resembles that *in situ* [101-103].

1.2.2 Previous way to get aggregates

One of the oldest ways that 3D spheroids of cells can be obtained is by spontaneous cell aggregation, which can generate somehow spherical cellular structures or by culturing cells on artificial substrates that induce cellular differentiation and maintain cellular function. Malignant cells are able to adhere to each other to form homotypic aggregation [104] or adhere to other cells resulting in heterotypic aggregation [105]. However, because of mass transportation limit, accumulation of metabolic waste and lack of nutrient becoming progressively serious deep within the spheroid, most of the proliferating cells were present on or near the surface [106].

For cells in suspension to grow as 3D aggregates or spheroids, it is required that the adhesive forces between the cells are greater than that between cells and the substrate the cells are cultured on. The simplest way to prevent adherence of cells to substratum is to use liquid overlay technique, which prevents deposition of matrix [107]. Using this method, spheroids are formed following a biphasic process. In the first phase, cells migrate towards each other on the substratum and aggregate into spheroids, whereas in the second phase, cell growth results in the increase of spheroid size [108, 109]. In order for spheroid to form in this way, different substratums are required for different types of cells. For example, primary hepatocytes spheroid can be formed by culturing cells on positively charged surfaces or dishes coated with an extracellular matrix protein such as proteoglycan [110], poly-(2-hydroxyethyl methacrylate) [101,

111], or poly-N-isopropyl acrylamide [112]. Breast cancer cells are grown over an agar base or reconstituted basement membrane [107].

Liquid overlay cultures in static environment are useful in studying individual spheroids, whereas spinner flasks are used to provide dynamic suspension when greater numbers of spheroids are cultivated. Spinner flasks are stirred tank bioreactors, in which mixing of impeller keeps the cells from settling down. The movement of fluid theoretically plays the role of assisting mass transportation of nutrients and wastes into and out of the spheroids separately [113]. Although the most widely used method for culturing large numbers of multicellular spheroids was spinner flask culture [114], roller tubes and gyratory shakers were also used somehow successfully. People found that 80% of hepatocytes can form spheroids within 6hrs of spinner culturing, which is much faster than previous methods, which normally take 24hrs to 96hrs [115].

Rotary Cell Culture System was developed by NASA and it introduced a revolutionary concept [116, 117]. In this system, cells are maintained in a dynamic suspension in liquid media mixed by small hydrodynamic forces. Fluid turbulence and shear forces are minimized in this system, in which the vessel is completely filled with media and there is a semi-permeable membrane to eliminate bubbles. This system successfully integrates co-localization of cells, 3D cell-cell interactions, cell-matrix interaction and minimal shear forces, which provides a mild environment

for 3D spheroid cell culture with adequate mixing for mass transportation. This is a great advantage as higher fluid turbulence in the spinner flask was shown to damage fragile animal cells and affect the integrity of membrane as well as normal metabolism [118].

Other methods used by previous people to get cell aggregates include scaffold-based culture and hanging drop method. Hepatocyte-like spheroid structures could form in three-dimensional peptide scaffolds from putative liver progenitor cells [119]. Hydrogel-coated textile scaffolds was also found to be a good candidate in liver tissue engineering as they permit favorable hepatocytes attachment, spheroid formation and thus the maintenance of function [120]. The recent emergency of hanging drop method provides a mild, straightforward way to produce spheroids of homogeneous size, which are applicable to many anchorage-dependent cell types [121, 122].

1.2.3 Previous application of cell aggregates

Previous application of reaggregates experiments can be divided into two types according to time scale, short-term experiments, which last from minutes to a few hours and long-term ones which last from several hours to a few days. Short-term reaggregation has been used widely to analyse cell–cell interactions, cell surface properties, and to characterize cell adhesion molecules [123-125]. The reaggregate approach in longer time scale allows study of the formation of tissue-like cell arrangements. However, reaggregate approaches do not have a cellular pattern from

which the tissue originates [126]. Thus, a primary goal of the reaggregate approach is not to simulate normal tissue formation but to reveal basic mechanisms involved in this process. Take the aggregates formed in monolayer cultures for example, the reaggregate approach enables us to follow the process of tissue formation from single cells to organized spheres in a controlled environment so that we can understand better the inherent principles of tissue formation [127].

1.3 Cell surface engineering

1.3.1 Introduction to cell surface

The cell membrane of mammalian cells contains several different components, including lipids, proteins and carbohydrates. These components are constructed to generate the sophisticated functions of the membrane, such as uptake of molecules selectively into the cell, specific communication between cells as well as that between cell and extracellular matrix [128]. Besides its complexity, the cell membrane is also a dynamic structure that changes its chemical constituents and its overall composition from time to time according to the change of its environment. One example is that during tissue development it is by changing carbohydrate and protein handles on the outer plasma membrane that the individual cell influences tissue morphogenesis. Because of the heterogeneity of cell membranes, they become a challenging environment in which non-native chemical species are introduced. In addition to this, chemical modification on the cell surface should be insured not to induce undesirable changes in cell behaviors, which further complicates the area of cell surface

engineering. Despite the difficulties, there has been rapid progress in this area, in which scientists have explored several types of chemical strategies to engineer cells surface, such as insertion of molecules into cell membrane, reactions using exogenous enzymes, inhibition of metabolic pathway, metabolic engineering and covalent ligation to cell surface chemical groups.

1.3.2 Chemical strategies to engineer cell surfaces

The first type of chemical strategy to engineer cell surface is insertion of molecules into cell membranes. A number of groups have successfully displayed both naturally occurring and synthetic bioactive molecules on the cell surface by employing the lipophilic nature of mamamliam cell membrane. To achieve this goal, a fatty moiety is attached to the biomolecule of interest and it incorporates into the membrane when the reconstructed molecule is applied to the cell, leaving the biomolecule exposed on the cell surface. Two main classes of fatty compound have been used for this application, which are named GPI-anchored proteins [129, 130] and cholesterol-tethered compounds [131]**.** Protein transfer using these fatty anchors has great potential for pathogenic study of disorders and diseases where cell surface molecules are aberrant.

Enzymes are widely used in the process of glycosidic bonds formation in carbohydrate chemistry, which renders the use of enzyme-catalyzed chemical transformations as a feasible alternative approach to traditional ones. Applying

exogenous glycosyltransferase, such as fucosyltransferases [132] and sialyltransferases [133] on the existing surface glycoforms and with an appropriate activated sugar donor can be performed on the cell surface. However, the exogenous application of tolerant glycosyltransferases has been replaced to a certain extent by the utilization of endogenous metabolic machinery for cell surface engineering.

Because the glycosylation of proteins and lipids is an important factor influencing the molecular complexity and functionality of the cell surface, inhibition of carbohydrate metabolism presents an alternative chemical strategy for engineering cell surface. Diverse complements of enzymes are required for a monosaccharide to be converted into an active sugar donor [134, 135]. This enables the inhibition of specific enzymes, which thereby makes it possible to subtly change the surface glycosylation. The development of inhibitors for selective glycosylation will be useful for a number of therapeutic applications, including treating cancers and autoimmune diseaseas. Natural products have become the resources of some inhibitory molecules, such as carbohydrate mimetic alkaloids from plants and microorganisms [136]. Specifically designed synthetic drugs are important additions to those natural occurring inhibitors. Most inhibitors exert their effects by competing with the natural enzyme substrates, which can be sugar donor or acceptor species, and acting as transition state analogues of the enzyme–substrate complex.

Some enzymes involved in the biosynthesis of cell surface molecules are tolerant to their substrates structural variability. It makes metabolic engineering an alternative strategy for altering the chemical functionality of cell surfaces. Technically, unnatural precursors of cell surface moieties can be used to incubate cells followed by taken up and metabolized by the cells. Then unnatural structure will be incorporated on the cell surface. One example of this strategy is the incorporation of unnatural sialic acid into cell surface [137-139]. This approach has been used on different cell types to alter the structure of sialic acids on cell surface, which has potential therapeutic applications because changing the structure of the sialic acids will possibly affect cell recognition and adhesion events.

Besides the four techniques mentioned above, there is another type of strategy for modifying the chemical structure of cell surfaces, which is utilizing direct covalent reaction. There are two different ways to achieve this. The first one is reactive molecules can be ligated directly to cell surface natural generating functional groups, such as amines or thiols. The other approach involves generation of non-native functional groups on the cell surface, which can react specifically with the molecule of interest.

Although it was found that it is not easy to alter the behaviour of living cells by labeling cells with reactive molecules on cell surface functional groups, previous work suggested that this might be a viable technique in the area of tissue engineering.

It was found that by encapsulating the implanted cells with a biocompatible polymer, such as poly (ethylene glycol) (PEG), the chance for the implants to be recognized and destructed by host immune cells was reduced [140, 141]. Since functional group on PEG can reactive with amines exposing on cell surface, cells can be completely surrounded by PEG molecules and viability and normal functions of the cells are not be affected compared with untreated cells. However, the major drawback of this approach is that there is no specificity between cell surface functional groups and reactive molecules. To solve the problem above, another approach can be used for engineering the molecular on cell surface, which is to generate unnatural functional groups at specific sites of the cell surface molecules. These reactive groups don't normally appear on the cells surface, so they can be used to chemoselectively ligate suitably functionalized molecules. Two different types of chemical groups, reactive carbonyls, which are usually in the form of aldehydes and ketones, and azides are the focus of current research. Aldehyde and ketone groups on the cell surface can selectively reacted with hydrazide, aminooxy or thiosemicarbazide functionalities [142]. Three different ways have been used to incorporate these functional groups into cell surface, including application of exogenous enzymes, direct chemical reaction and metabolic engineering.

The first method used to introduce aldehyde groups at specific sites in cells surface is the application of exogenous galactose oxidase, which oxidizes terminal galactosyl and *N*-acetylgalactosaminyl residues. Compared with this exogenous enzyme method,

there is a simpler one for the introduction of aldehydes, which is direct oxidation of sialic acids with sodium periodate [143]. This method is very rapid and it was found to be concentration dependent, which is selective for the sialic acid when mild conditions are employed [144, 145]. Although it is a bit crude technique, it has been demonstrated that aldehyde groups can be incorporated into the cell surface by mild sodium periodate oxidation, which did not affect the viability, or morphology of the cells [146]. Besides these two methods, the delivery of modified sialic acid to cell surface by metabolic pathway is also a powerful technique for cell surface engineering. Ketone and azide groups could be delivered to cell surfaces in this way by employing functionalized mannosamine derivatives, such as *N*-levulinoylmannosamine (ManLev) and *N*-azidoacetylmannosamine (ManNAz) [147, 148]. Although ketones and azides could be effectively metabolized into sialic acids on cell surface in numerous cell types without adverse effects on the viability of cells, the level of ketone expression on cell surface depends on the species of cells. It is possibly because in different cell types, the tolerances of the enzymes in the sialic acid metabolic pathway to structural variations are also different.

1.3.3 Applications of surface engineered mammalian cells

Cell surface interactions are of fundamental importance to the functions of cells and tissues both *in vivo* and *in vitro*, whereas cell surface modification may probably affect cellular functions. This fact makes the various available strategies of engineering mammalian cells surface have a wide range of applications, especially in

the research area of pharmaceutics and biomedical engineering. They can be divided into two categories, drug delivery, tissue engineering and cell based strategies.

1.3.3.1 Drug delivery

One of the key issues to be solved in drug delivery is how to deliver drugs to a particular cell type and organ without specific receptors on cells or transport mechanisms for the drug. Chemically engineering cell surfaces provides a way to facilitate the specific interactions between cell surface and drugs or drug delivery systems. For example, synthetic adenovirus receptor was incorporated into cell surface by metabolic engineering to potentially enable gene therapy [149]. Recently, synthetic receptors for exogenous proteins were specifically introduced into cell surfaces to specify the permeability of cell membrane to large drug molecules [150]. In addition, tumor cells could also be tagged by incorporation of unnatural sugar residue into the sialic acid molecules on the cell surface, which provided a strategy for selectively killing tumor cells [151].

1.3.3.2 Tissue engineering and cell-based therapies

Cell-cell interaction and cell-matrix interaction are quite important factors for the development or repair of tissue. Since both of them are controlled by cell surface properties, cell surface engineering is potentially useful in the field of tissue engineering. The major problem with transplantation of a tissue or organ from a donor to a patient is the immune rejection of the tissue or organ by the host. Cell surface engineering provides a way to prevent foreign cell or tissue being recognized by

immune system. For example, pancreatic islets have been encapsulated in poly (ethylene glycol) (PEG) to block the binding of immune cells to the foreign tissue [140]. Cell surface engineering was also found to assist nerve regeneration, especially spinal cord. Büttner *et al.* have shown that the length of neuritis, the surface of which were metabolically engineered, can be more than twice compared to control cells [152]. Besides the application above, it was also found that cellular aggregation could be induced by cell surface engineering [146]. Since three-dimensional reconstruction of tissues is the ultimate goal for tissue engineering, this finding provides an important approach in this area.

1.4 Application of Poly (ethylenimine) and dentrimers in bioengineering

The beauty of chemistry is that we can design and synthesize chemicals with required properties in various applications, such as scaffolds in tissue engineering, vectors for gene delivery, carriers for drug delivery etc. In the following section, the primary chemical, poly (ethylenimine) and dentrimers used in this project will be introduced to illustrate their chemical properties and biological applications.

1.4.1 Chemistry of Poly (ethylenimine) and dentrimers

Poly (ethylenimine) (PEI) has been used for many years in common processes including paper production, shampoo manufacturing and water purification. There are two forms of the polymer: linear and branch [153]. Both of them are produced by

cationic polymerization from two different kinds of monomers, aziridine monomers for branched one and 2-substituted 2-oxazoline monomer for linear one (as shown in Fig 1) [154]. The standard form of PEI for gene transfection is branched PEI, which shows significantly higher transfection efficiency.

The basic unit of PEI has one nitrogen atom following every two carbons. In branched PEI, there are primary, secondary and tertiary amino groups, both of which can be protonated, rendering PEI as the organic macromolecule with the highest cationic-charge-density potential [155]. PEI has an effective buffering capacity at a broad range of pH value, which is closely related to its high efficiency in gene transfection [155].

The word “dendrimer” came from the Greek *dendron* and *meros* which mean ‘tree’ or ‘branch’ and ‘part’ separately. ‘Arboroles’ or ‘cascade polymers’ are also used to name “dentrimer” [156]. Dendrimers are well-defined chemicals, with a low polydispersity compared with traditional polymers. The dendritic branching results in semi-globular to globular structures, mostly with a high density of functionalities on the surface together with a small molecular ‘volume’.

The dendrimer design can be based on a large variety of linkages, such as polyamines (PPI dendrimers) [158], a mix of polyamides and amines (PAMAM dendrimers) [159] and more recent designs based on carbohydrate [160] or containing ‘third period’

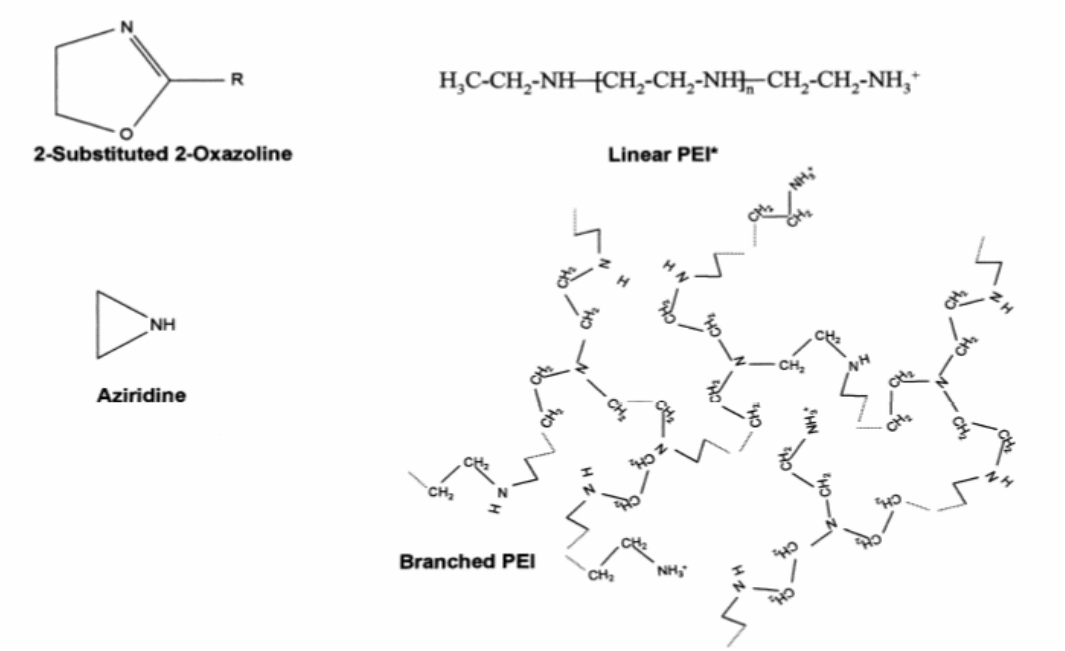
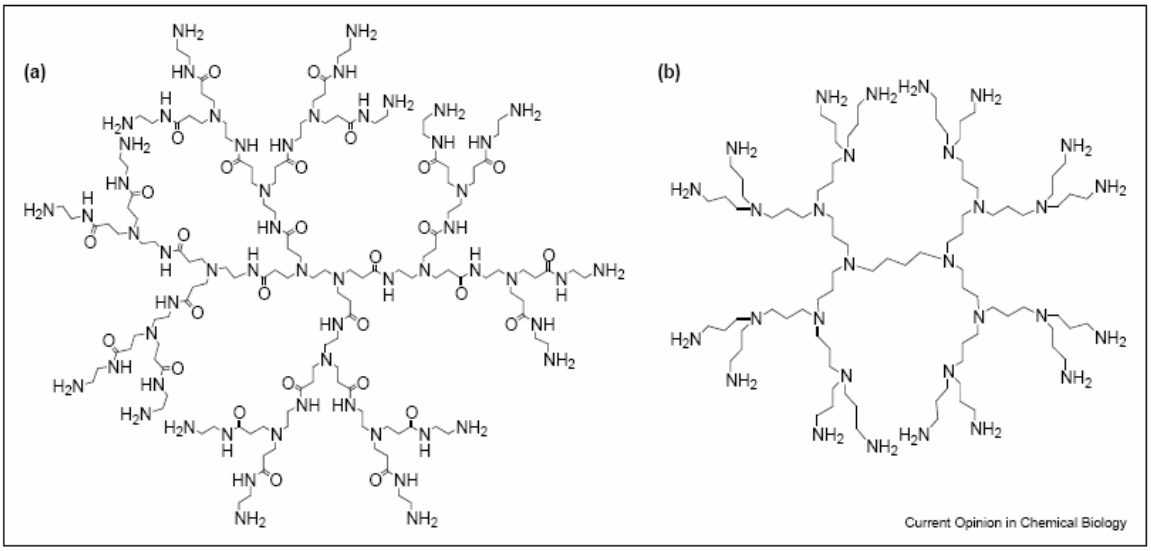


Fig 1.1 Stuctures of PEI percursors and end products. Adapted from [154].



Two commercially available frameworks. **(a)** A second-generation PAMAM dendrimer. **(b)** A third-generation poly(propylene imine) dendrimer.

Fig 1.2 Structures of two frameworks of Dentrimers. Adapted from [157].

elements like silicon or phosphorus [161]. Dendritic structures are chemically synthesized by two different approaches, either divergent or convergent. In the divergent approach the dendrimer is synthesized from the core and built up generation by generation [162]. The alternative convergent approach starts from the surface and ends up at the core, where the dendrimer segments are coupled together [163].

The structure of dentrimers can be divided into three parts: the multivalent surface, with a high number of potential reactive sites, the ‘outer shell’ just beneath the surface having a well-defined microenvironment protected from the outside by the dendrimer surface, and the core, which is protected from the surroundings in higher generation dendrimers, creating a microenvironment surrounded by the dendritic branches [164]. The three parts of the dendrimer can be tailored specifically for the desired purposes. For example, the multivalent surfaces on the dendrimer can contain a large number of functional groups, making the dendritic surfaces suited to multivalent interactions which are important in biological systems [165].

1.4.2 Biological application of PEI and dentrimers.

Since transfection was first introduced as a technique in mammalian cells in 1966 [166], both viral (adenovirus and retrovirus) and nonviral carrier systems have been used to treat several genetic diseases, such as cystic fibrosis [167-170] and several kinds of cancer [171, 172]. Although viruses have been the most popular vectors for gene delivery, there are several problems when viral vectors are used in clinical

treatment, for example, the transfection efficiency *in vivo* is restricted due to the inflammatory properties of viruses; inappropriate tropisms prevent them to target tissues [173]. Non-viral vectors with low immunogenicity have been investigated as alternatives for viral vectors. Cationic polymers with large diversity of structures and molecular design can be recruited to produce vectors with different properties [174]. These designed polymers, such as poly (L-lysine) are able to condense DNA into discrete particles through electrostatic interaction and stabilize the polyplexes by enclosing it with hydrophilic coating. Positively charged polyplexes interact strongly with cell membrane which is negatively charged followed by taken up by the cells through endocytic pathways [175].

However, the efficiency of non-viral vectors to transfect cells has yet to be improved which is mainly because a large fraction of the polyplexes from cationic polymers and DNA are delivered into lysosome and degraded there finally. Fortunately, some kinds of polymers, such as Poly (ethylenimine) (PEI) [153, 155] and dentrimers [176-178], can mediate gene transfection with relatively high efficiency. Abundance of secondary and tertiary amino groups in these chemicals prevents the lowering of pH in endosomes and lysosomes, preventing degradation of polyplexes. These polymers also induce osmotic swelling of the endosome and lead to the rupture of endosome, followed by releasing DNA into cytoplasm [179, 180].

Besides the potential application of dentrimers in gene transfection, significant advances have been made in the synthesis and study of glycodendrimers and peptide dendrimers in the past few years. Application of these dendrimers has facilitated the understanding of the study of carbohydrate–protein and protein–protein interactions. For example, glycodendrimers with surface carbohydrate units have been used to study the protein–carbohydrate interactions that are implicated in many intercellular recognition events [181, 182]. Dendrimers with surface peptides or amino acids incorporated into the framework as branching or core have potential applications as protein mimics, antiviral and anticancer agents, vaccines and drug delivery systems [183-185].

1.4.3 Cytotoxicty of PEI and Dentrimers

Both PEI and dentrimers were reported to be toxic to cells. It was found that PEI was involved in causing lysosomal disruption in rat hepatocyte when the concentration of PEI amines is 0.001 M. However, the stability of lysosomes were not affected when the concentration of PEI is at or below 0.0002 M_{Amine} [186]. Fusogenic effects of both linear and branched PEI on liposomes have been reported, which showed that branched PEI disrupting liposomal membranes made from phosphatidyl serine, whereas the effect was not great when the liposomes were constructed from phosphatidylcholine / phosphatidyl serine [187]. These results collectively suggested that low concentration of PEI will not harm plasma membranes. After PEI polyplexes was systemically delivered *in vivo,* PEI could induce multiple cellular responses such

as apoptosis [188]. Systemic application of linear PEI polyplexes in mice led to liver necrosis, activation of lung endothelium, adhesion of aggregated platelets and shock after injection of elevated doses [189].

Generally speaking, amino-terminated dentrimers are cytotoxic [190]. Studies on rodent muscles showed that amino-terminated PAMAM dentrimers was more myotoxic than cationic liposomes and proteins [191]. In addition, both amino-terminated PAMAM and PPI dentrimers show a molecular size dependent increase haemolytic effect on a solution of rat blood cells [190]. Recent comparative studies of anionic and cationic PAMAM dentrimers conclude that carboxyl functionlized PAMAM dentrimers are less toxic than amino-terminated one [192]. From the few systematic studies on the *in vivo* toxicity of dentrimers, injection of PAMAM dentrimers with 10 mG/kg concentration do not appear to be toxic, independent on the dentrimer surface properties [193]

The reason for toxicity of PEI and dentrimers to cells is probably because of the favored interactions between negatively charged cell membranes and the positively charged polymer surfaces, which enables the polymers to adhere and damage the cell membrane and cause cell lysis.

1.5 Project outline

Classical tissue engineering involves seeding cells into polymer scaffold or hydrogels,

culturing the cells-scaffold composite for a period of time followed by transplanting the tissue into recipient body. However, there are several problems with this method, including ineffective cell seeding, difficulty involved in placing different types of cells in scaffold and absence of vascularization. An alternative way is to use cell aggregate instead of individual cells as building blocks for tissue engineering. Traditional way to get cell aggregates was to use cell adhesion molecule, which was uncontrollable and usually takes several days. An alternative way is to use biofunctional molecules [194, 195]. However, those molecules always target cell surface proteins, which are important in intracellular signal transduction. Modification of oligosaccharides, which are not directly correlated to cellular functions, provides an alternative approach for aggregation formation. Sialic acid on the top end of glycoprotein was oxidized and made to react with biotin hydrazide. Subsequently, avidin was added to achieve multicellular structure. However, it takes a relatively long time for the aggregates to be formed by this 2-step reaction. Since the reactions all took place in non-physiological environment, it is imperative to shorten the length of time for aggregation formation so as to keep the viability of cells in aggregates.

In order to reduce the time required for cell aggregation and to promote general usage of this technique, we would like to design special chemical linkers using conjugation method. Instead of treating the modified cells by two-step reaction, we synthesize bi-functional chemical linker with at least two hydrazide groups, which shortens the time for multi-cellular structures to be formed. We try to synthesize different types of

chemical linker in the first part of this work and one of them will be selected out as the model for further study of this aggregating system. Cytotoxicity test, which shows the toxicity of the chemical to cells, aggregation efficiency analysis, as well as live and dead assay, which characterizes the overall effect of this linker on viability of cells in aggregates, will be used as the parameters to choose the best chemical for further study. In the second part of the work, the factors affecting the aggregating ability of the chemicals are to be studied to explore the advantages and mechanism of this system. In order to prove the feasibility of this system as an alternative approach for generating tissues *in vitro*, the aggregates will be cultured in suspension up to one week. The viability and functionality changes will be tracked. Fluorescence tag is also conjugated to the chemical linker and used to track the fate of chemical linkers in aggregates, which may stimulate further study on understanding the aggregate formation and changes during culture. In order to find out whether this aggregating approach can be performed in a more controllable way, we try to combine this technique with micropatterning and micromanipulation to control the size and shape of the multi-cellular structures, which may further prove the possible profound use of this system in regenerating 3D tissues *in vitro*.

Chapter 2 Preliminary study of chemical linkers for aggregates formation

2.1 Cell surface modification detected with streptavidin - FITC

As mentioned in the work done by Oetke and his group members [146], sialic acids on myoblast cell surfaces could be oxidized into non-native aldehyde groups. The modification could be detected by biotin hydrazide and streptavidin – FITC either by confocal laser scanning microscopy or by flow cytometry. They also assessed the persistence of cell surface modifications on myoblast cells after 24 hours of culture and found that cells retained the aldehyde groups at a reduced level corresponding to 27.6 ± 2.2% of the initial aldehyde group number. Similar results were obtained when this was repeated with biotinylated cells. In our experiment, we used confocal laser scanning microscopy to detect the surface modification as well as tracking the persistence of non-native aldehyde groups on HepG2 cells. After modification, we cultured the cells up to 3 days and tracked the presence of aldehyde groups on the cell surfaces. Inconsistent with published data, we found that the aldehyde groups only stayed on cell surface for one day after modification (Fig 2.1, B,C,D), whereas the biotinylated ones persisted for 3 days (Fig 2.1, E,F,G). It is possible that the free aldehyde groups on cell surface are more easily to be endocytosed, whereas the biotinylation prevent the aldehyde groups from endocytosis.

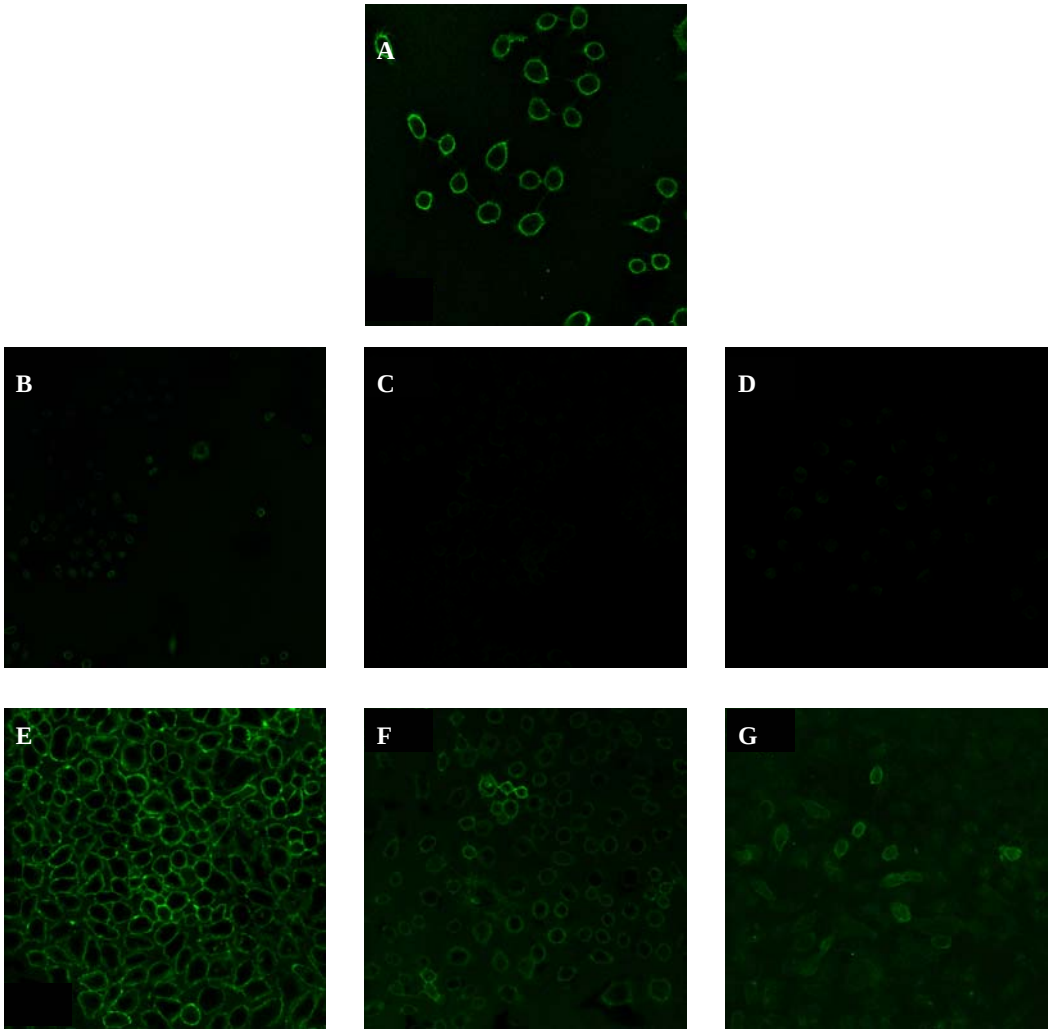


Fig 2.1 Distribution of non-native aldehyde groups on HepG2 cells after modification observed by Olympus Fluoview 300. The excitation wavelength is 488 nm. The cells were modified with 1 mM $NaIO_4$ for 15 minutes, followed by biotinylation and stained with Streptavidin-FITC (green). The cells were imaged using confocal laser microscopy. Cells were biotinylated and stained immediately after modification (A) or cultured for 1(B), 2(C), 3(D) days separately after modification and before biotinylated and staining or cultured for 1(E), 2(F), 3(G) days after modification and biotinylation before staining.

2.2 Synthesis of various types of chemical linker

Biotin hydrazide and avidin were used to aggregate cells by Debank, P.A et.al [146].When this system was tried at the early stage of the work, it was found that this way of aggregating cells involve so many steps that we could not obtain satisfactory cell aggregates after several months of optimization In addition, cells were placed in non-physiological conditions for several hours. Due to these disadvantages, we designed several types of chemicals which can aggregate the cells rapidly.

Polyethylenemines [155] and dentrimers [176] have been used widely in the area of gene delivery for several years, taking advantage of their cationic properties. They were chosen as the backbones for the hydrazide chemicals because of their abundance of amine groups, which can be easily modified into other functional groups. Reaction is performed in aqueous environment and involves two steps (Fig 2.2). In the first step, PEI and dendrimers, which bear many primary amino groups reacted with 2-iminothiolane [196] resulting in the product with sulfhydryl groups. 2-iminothiolane (2-IT) is normally used to incorporate thiol groups into proteins by reactions of amino groups (Fig 2.2) [197]. EMCH in the second step reaction contains both maleimide groups and hydrazide groups [198]. The maleimide group reacts specifically with sulfhydryl groups at neutral pH to from a stable and non-reversible thioether linkage. Maleimide reacts with sulfhydryls 1,000 fold faster than with amines at neutral pH, the reaction favors primary amines at pH > 8.5. Since all the original chemicals used here has far more than 1 primary amino group in the molecule, it is possible to

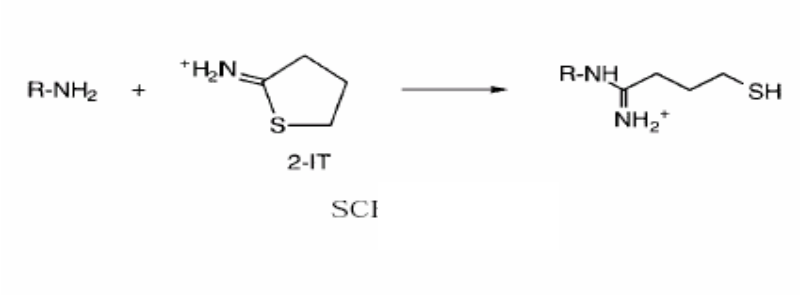


Fig 2.2 Reaction scheme of 2-iminothiolane and amino group. Adapted from [197].

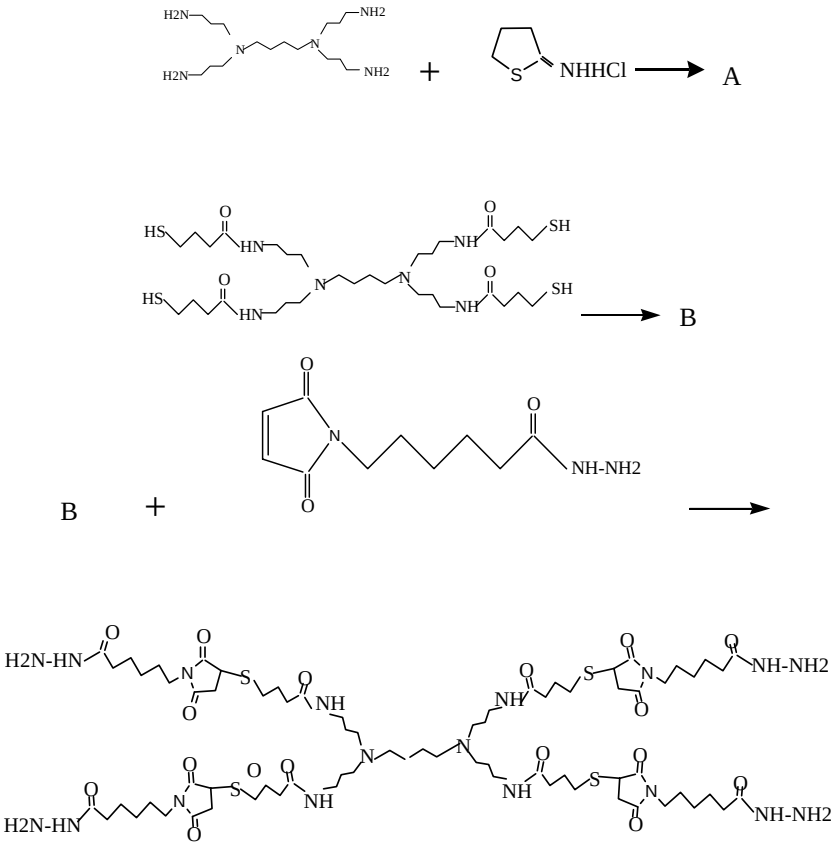


Fig 2.3 Route of synthesis of chemical linkers.

conjuage one or more sulfhydryl groups, which will be replaced into hydrazide groups by EMCH , resulting in the molecules with at least two hydrazide groups. 3 types of Dentrimers with 4, 8 and 16 arms separately and 2 types of PEI with molecular weight of 600, 2000 were used as the initial chemicals for synthesizing the linkers. We named the two series of hydrazide linkers as DAB-AM-4/8/16 iminothiolane-hydrazide and PEI-600/1200 iminothiolane-hydrazide separately. Using this protocol, we were able to get product with reproducible NMR spectrum (not shown here), from which we can see the distinct differences between the final products and the initial reagents. Due to the time limit, NMR data was not analyzed elaborately. So we are not sure how many hydrazide groups are on each molecule, which requires further characterization.

2.3 Cytotoxicity of chemical linkers

The effect of PEI and dentrimers on mammalian cells have been discussed by many previous papers [186, 187, 199, 200]. However, cytotoxicity of the hydrazide chemicals, which were seldom synthesized and characterized, was not tested before. In order to assess the cytotocity of the chemicals, we used MTS assay to test and compare the effects of the different types of chemical linkers. Cells were incubated in different concentrations of chemical solution in serum free DMEM followed by incubation with MTS for 4 hours. By correlating the number of live cells with the absorption of the resultant solution, we calculated the percentage of live cells under different conditions.

From results in Fig 2.4 we found that for the same series of chemicals, take DAB-iminothiolane-hydrazide as an example, the cytotoxicity of the chemical increases as the molecular weight increases. It is possibly due to the increased number of amino groups on the molecule. This is also in accordance with the general finding that increasing molecular size of polymers may result in increased cytotoxicity [201]. When all of the five types of hydrazide chemicals are compared, DAB-AM-16-iminothiolane-hydrazide shows the highest toxicity to HepG2 cells in MTS test. It is probably because this chemical bears most primary amino groups, which increases the chance for the cationic chemical to adhere to cellular surface and cause cell lysis. Although we are not sure how many hydrazide groups are on each molecule for these five types of chemicals, from NMR data, we can estimate that only a small part of the amino groups on the original multi-amino chemicals were converted into hydrazide groups. So most likely, the factor that decides the cytotoxicity of the chemical is still the structures of the original chemicals.

2.4 Ability to aggregate cells

All of the chemicals mentioned above act successfully as chemical linkers, which are shown in Fig 2.5. For the DAB-AM-iminothiolane-hydrazide and PEI-iminothiolane-hydrazide synthesis, both of these two categories of linkers function well, whereas all of the original chemicals cannot aggregate cells using the same condition. It seemed to us that if we can really synthesize chemical linker with all of the molecules fully

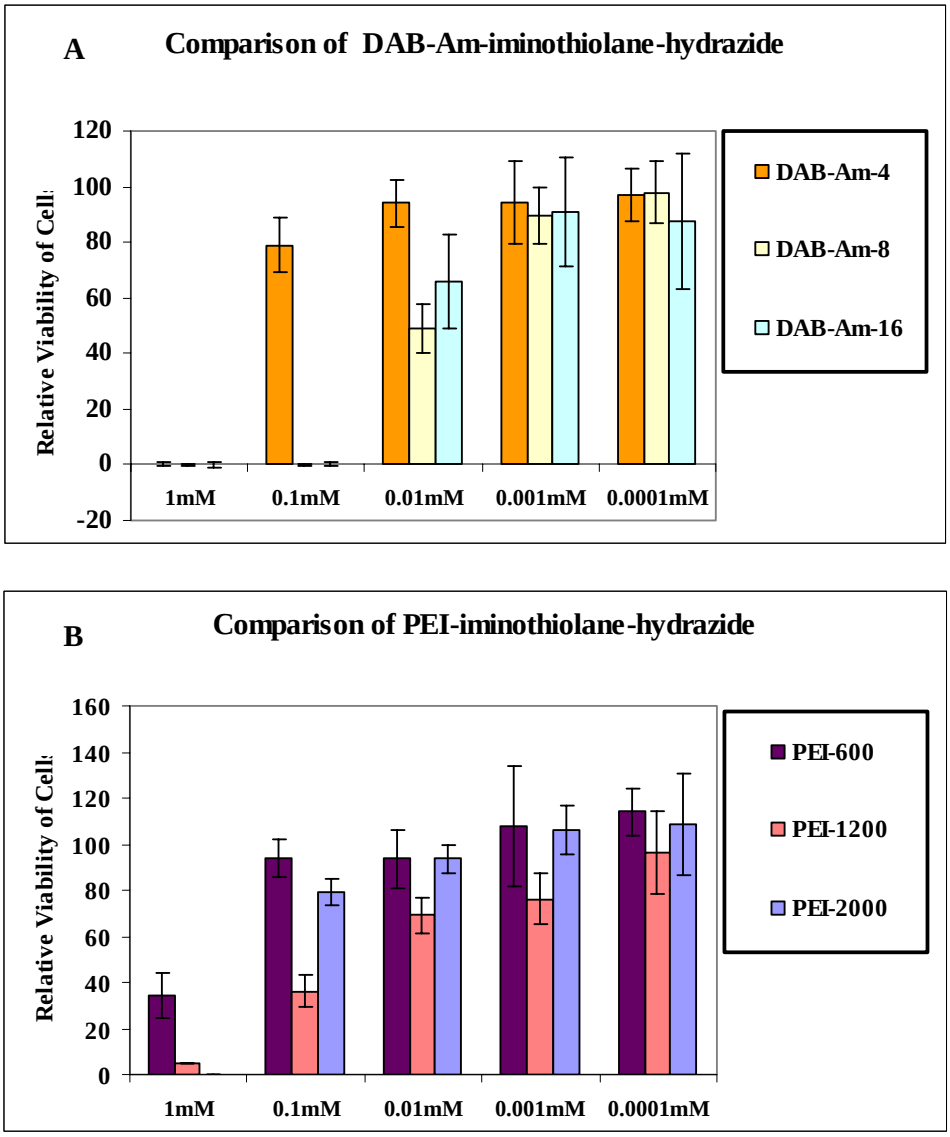


Fig 2.4 The cytotoxicity of chemicals tested by MTS assay. Five different concentrations of each chemical were added into 96-well plate. The viability of the cells was calculated based on the absorption of the resultant solution. A. cytotoxicity test of the four kinds of DAB-AM-iminothiolane-hydrazide. B. cytotoxicity of the two kinds of PEI derived hydrazide.

conjugated, the concentration of the chemical for aggregation could be lowered further down to micro scale. From all of images taken, we can also conclude that it is hard to control the shape of the aggregates, which always possess randomly

branch-like structures. In addition, the size of the aggregates in the same sample is also not uniform (Fig 2.5).

When all of the chemicals were applied to aggregate cells, it showed that all five types of chemicals synthesized from dentrimers/PEI, 2-iminothioalne and EMCH can aggregate cells rapidly, whereas the original chemicals without hydrazide groups cannot. So the key player for the formation of cellular aggregates by chemical linkers should be the conjugated hydrazide group, which can react specifically with ketone groups on the modified cell surface. Since all of the five chemicals have several amino groups on each molecule, even only a part of them can transverse into hydrazide groups, it is still possible that some of the molecules after reaction will bear more than one hydrazide groups, enabling them to act as linkers.

2.5 Size distribution of aggregates

Fig 2.5 shows that cell aggregates can be formed by all of the five kinds of synthesized chemicals. However, our aim of this project is not only to get cell aggregates but also to control the size and shape of the aggregates. Can we achieve this by using this rotating system? In order to answer this question, we tried to quantify the size of aggregates formed by chemical linkers in rotating tubes. We used three different concentrations for each type of chemical. At the end of experiment,

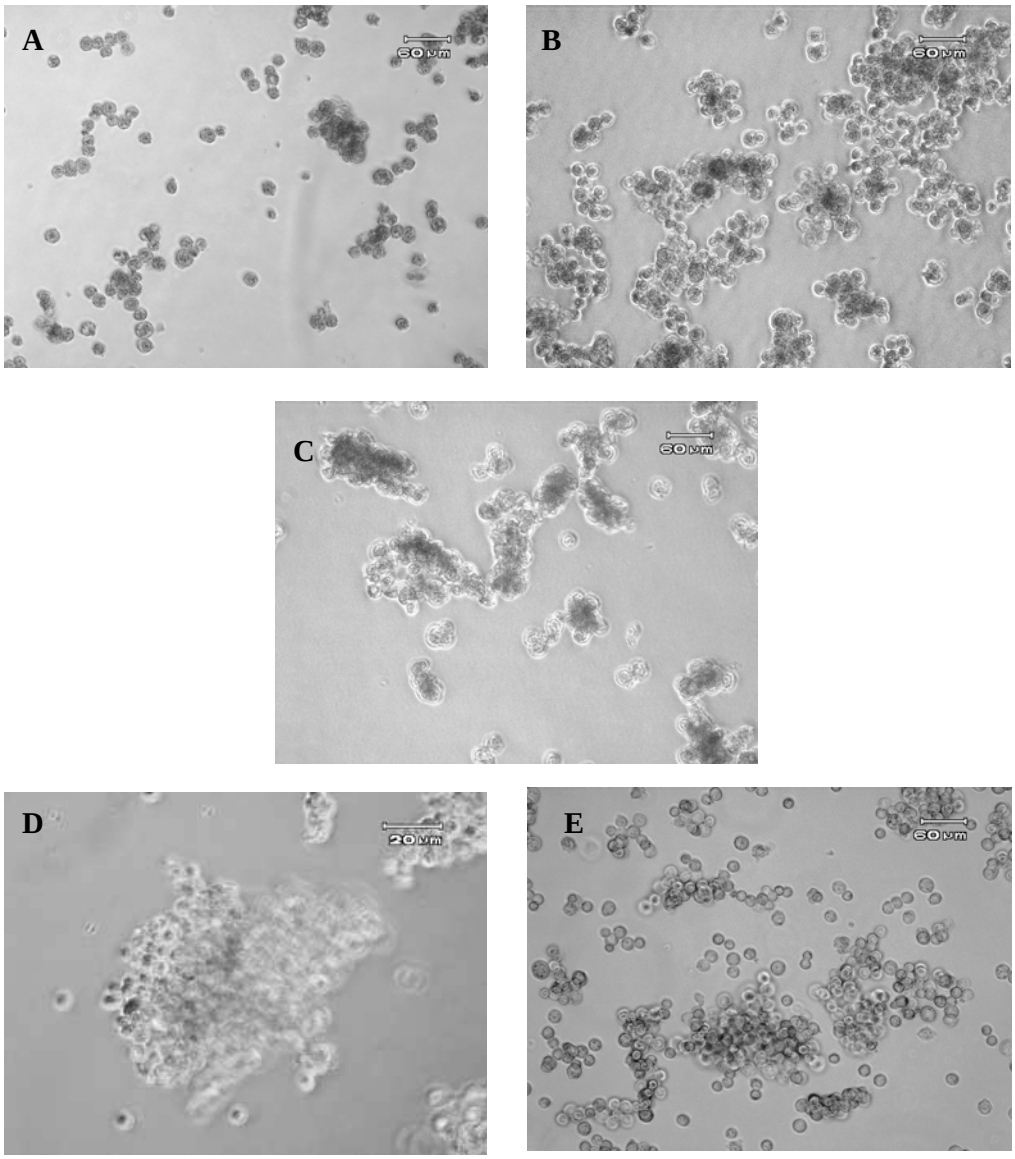
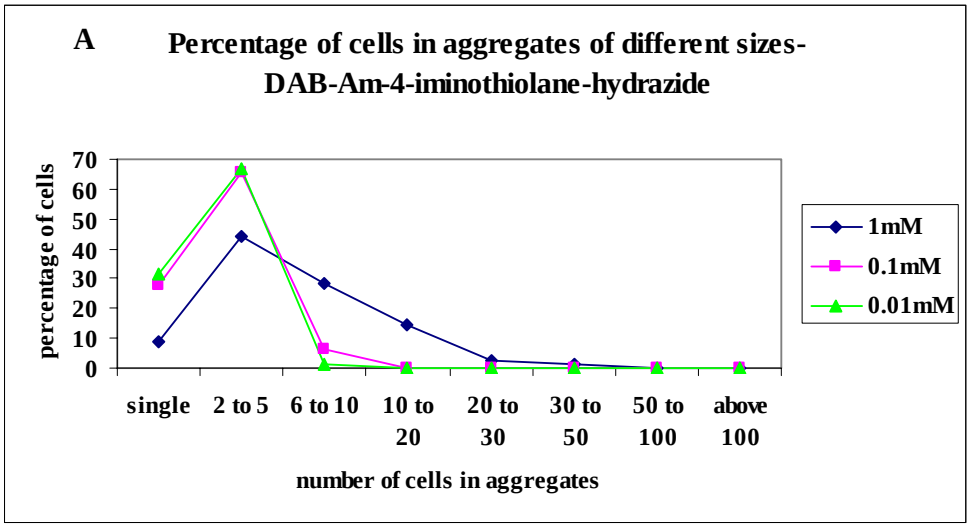
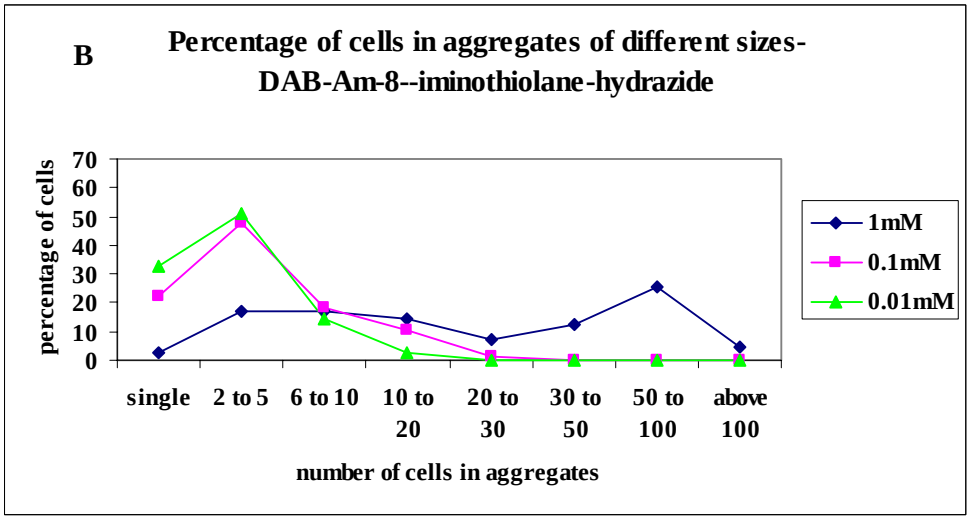


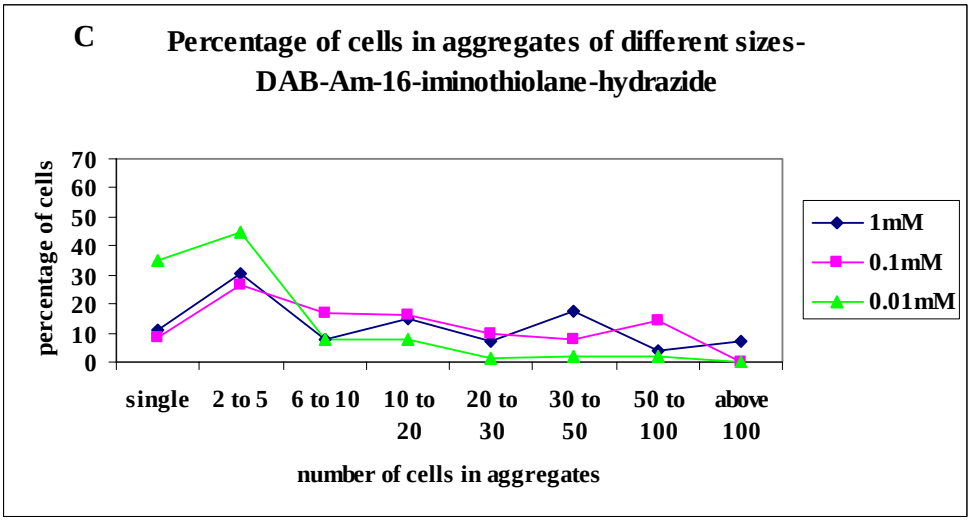
Fig 2.5 Cell aggregates by different kinds of chemical linkers. Cells were modified by 1 mM $NaIO_4$ in dark at 4 ^oC for 15 minutes, followed by incubated in different chemical solution for 30 minutes. A: 1 mM DAB-AM-4-iminothiolane-hydrazide. B: 1 mM DAB-AM-8-iminothiolane-hydrazide. C: 1 mM DAB-AM-16-iminothiolane-hydrazide. D: 1 mM PEI-600-iminothiolane-hydrazide. E: 0.1 mM PEI-2000-iminothiolane-hydrazide. From the images we can see that the shapes of the aggregates are random.

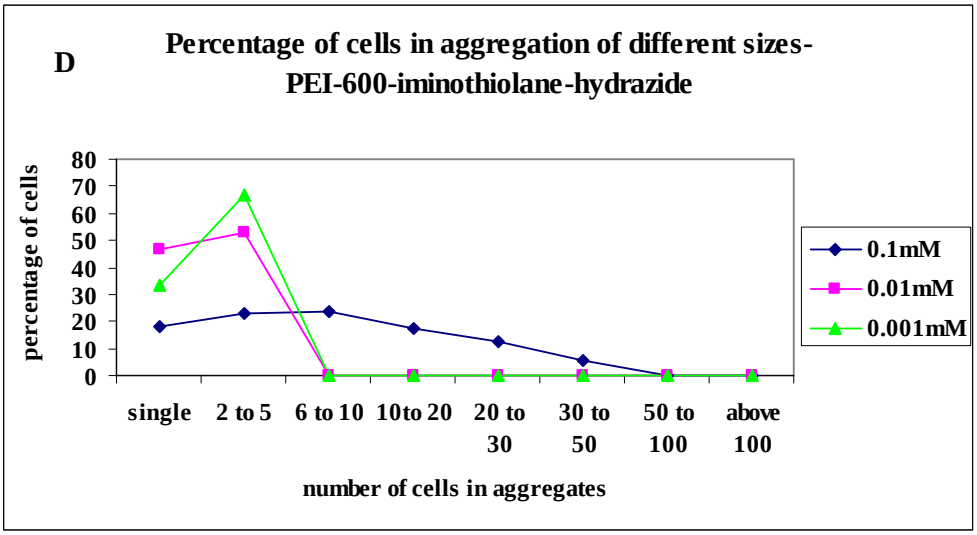
several images of the aggregates were taken for each sample. The images were then analyzed and percentage of cells in the aggregates of specific size was calculated. Results showed that in all the 15 conditions used for this experiment, we could never get aggregates of uniform size in the tube. Instead, we could plot out a distribution curve for each sample, correlating the percentage of cells in aggregates of specific size to the sizes of aggregates, as shown in Fig 2.6. From these curves we can see that, for the same kind of chemical linker, percentage of single cells in sample decreases and more cells appear in bigger sizes of aggregates as the concentration of linker increases, except that for PEI-600-hydrazide, the number of single cells in 0.01 mM solution is larger than that in 0.001 mM solution which might due to experimental error.

So the answer to the question in the beginning of this part is that we cannot control the size of the aggregates in this rotating tube system. However, we can change the percentage of cells in aggregates of specific size by fine-tuning the concentration or type of chemical used according to these distribution curves. For example, we can use 0.1 mM PEI-2000-hy in order to make sure over 50% of cells will be in aggregate larger than 50 cells


A
Percentage of cells in aggregates of different sizes-
DAB-Am-4-iminothiolane-hydrazide
percentage of cells
70
60
50
40
30
20
10
0
single
2 to 5
6 to 10
10 to 20
20 to 30
30 to 50
50 to 100
above 100
number of cells in aggregates
1mM
0.1mM
0.01mM


B
Percentage of cells in aggregates of different sizes-
DAB-Am-8--iminothiolane-hydrazide
percentage of cells
70
60
50
40
30
20
10
0
single
2 to 5
6 to 10
10 to 20
20 to 30
30 to 50
50 to 100
above 100
number of cells in aggregates
1mM
0.1mM
0.01mM


C
Percentage of cells in aggregates of different sizes-
DAB-Am-16-iminothiolane-hydrazide
percentage of cells
70
60
50
40
30
20
10
0
single
2 to 5
6 to 10
10 to 20
20 to 30
30 to 50
50 to 100
above 100
number of cells in aggregates
1mM
0.1mM
0.01mM



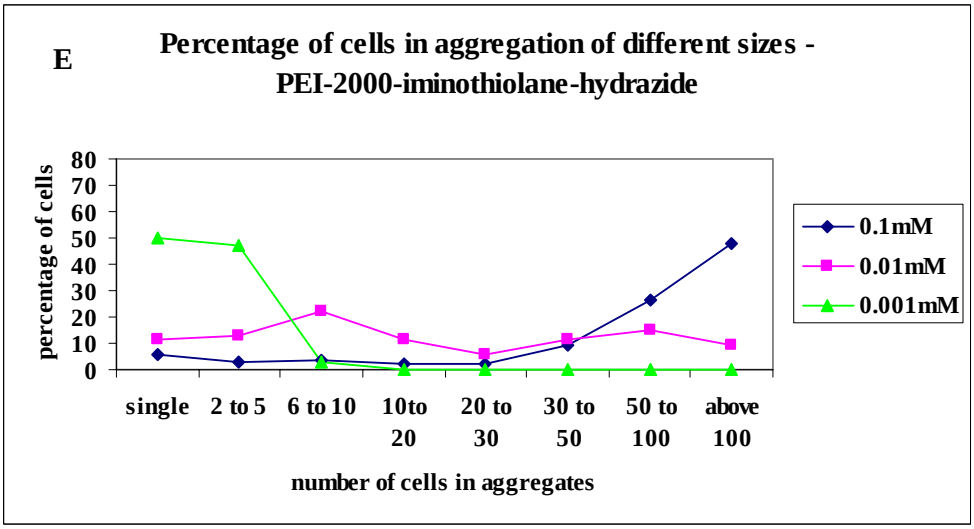


Fig 2.6 Distribution curves of cells in different sizes of aggregates. Five types of chemical linkers were used to aggregate the cells. Phase contrast images were taken and analyzed to plot these curves. A: DAB-Am-4-iminothiolane-hydrazide, B: DAB-Am-8-iminothiolane-hydrazide, C: DAB-Am-16-iminothiolane-hydrazide, D: PEI-600-iminothiolane-hydrazide, E: PEI-2000-iminothiolane-hydrazide. Concentrations for A, B, C are 1 mM, 0.1 mM and 0.01 mM whereas for D and E are 0.1 mM, 0.01 mM and 0.001 mM.

2.6 Live and Dead Assay of aggregates

It has been proven quite clearly that all of the synthesized chemicals above are efficient for aggregate formation. However, in order to prove the feasibility of this new system as a possible method for tissue engineering, it is imperative that this system is non-toxic to the cells. Because of the importance of carbohydrate residues on cell surface, it is possible that the introduction of aldehyde groups to cell surface followed by tagging with chemical linkers may compromise many functions of the cells. Previous work on surface modification of myoblast cells showed that periodate exposure had no gross effect on myoblast cells [146]. Our previous MTS assay also tells the cytotoxity of the chemical to unmodified cells. In order to assess the overall effect of this gluing system, which includes cell surface modification and aggregation provided by chemical linkers, we used the live and dead assay to distinguish the live cells from the dead ones, which told us the final viability of the aggregates (Fig 2.7). The concentration of all linkers here is 0.1mM. Alive cells were stained with Cell Tracker green. The nuclei of dead cells were stained with propidium iodide. In order to have a better idea of the viability of cells in the images, we quantified the number of alive and dead cells by ImagePro Plus, which is an image processing software and got the result in Fig 2.8. Result showed that for DAB series hydrazide, viability of cells increases as the concentration of chemical and the number of arms in chemical decrease, which agree with previous MTS data. For PEI series hydrazide, the viability does not change much when the type and concentration of the chemical used change

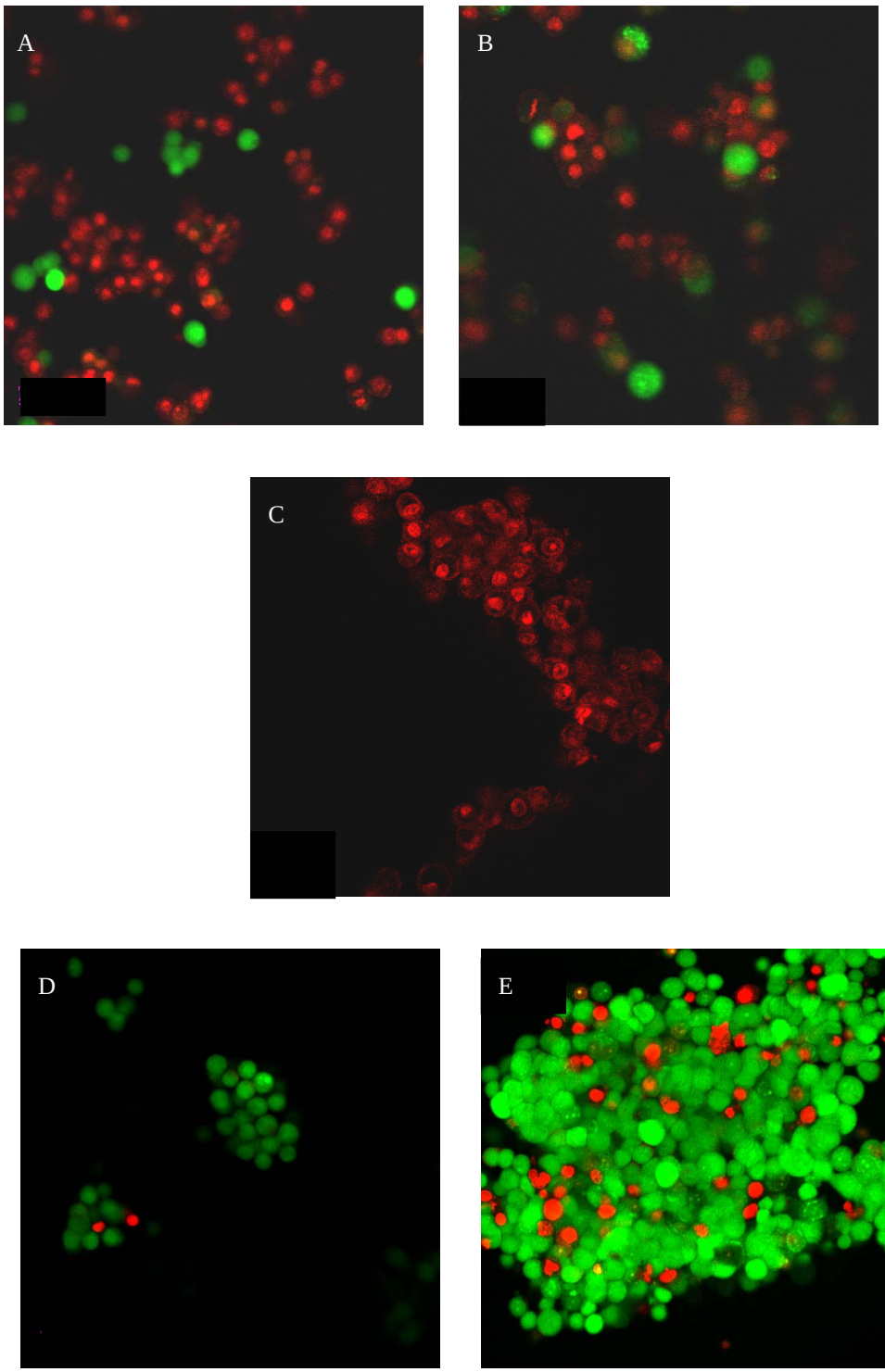


Fig 2.7 Live and dead cells in aggregations. The cells in aggregations were stained by CTG, green and PI, red. A: DAB-Am-4-iminothiolane-hydrazide, B: DAB-Am-8-iminothiolane-hydrazide, C: DAB-Am-16-iminothiolane -hydrazide, D: PEI-600-iminothiolane-hydrazide, E: PEI-2000-iminothiolane-hydrazide.

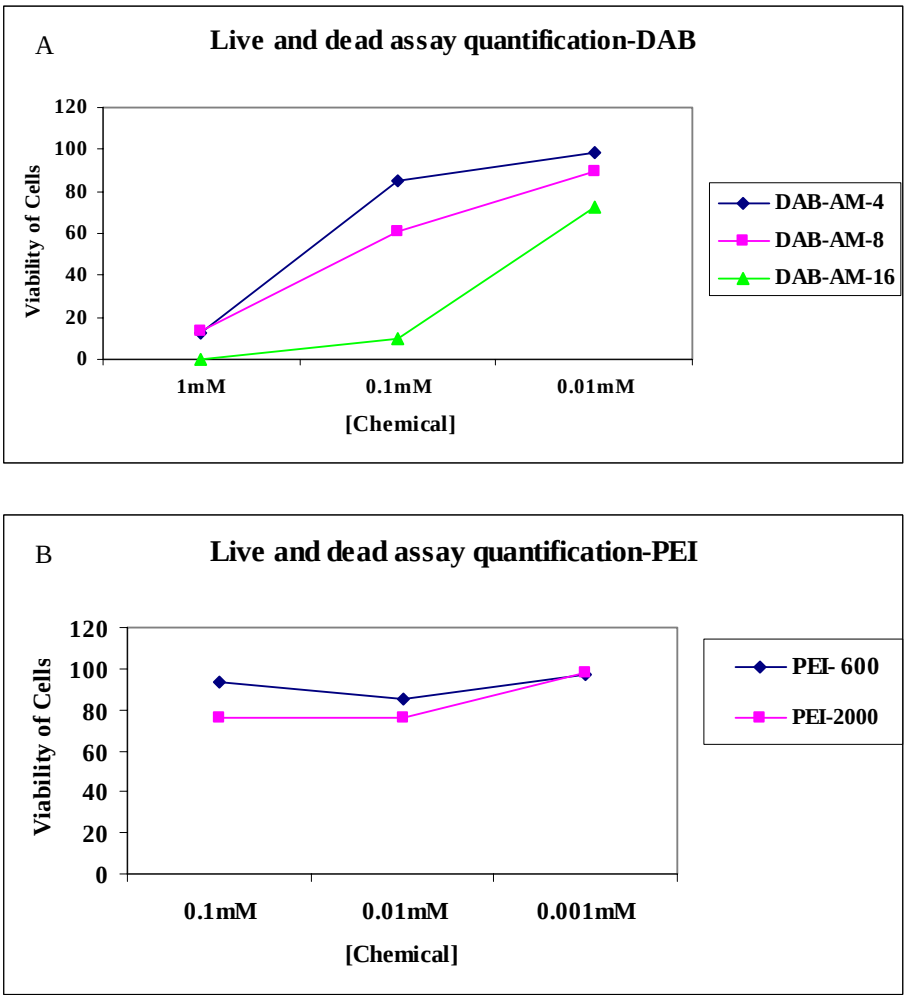


Fig 2.8 Quantification of live and dead images in Fig 2.7. Images were processed by ImagePro Plus and the curves were plotted according to the calculated results. The names of the chemicals were abbreviated as the initial reagent, for example DAB-AM-4 here stands for DAB-AM-4-iminothiolane-hydrazide

2.7 Comparison of the different types of chemical linkers.

Up till now, we have successfully synthesized several types of chemicals which can be used to aggregate cells into multi-cellular structures. However, the mechanism underlying this gluing process and further study of the aggregates are pending. Considering the possible large amount of workload and time limit, it is impossible to work on all five types of chemicals. In addition, since all the five types of chemicals are similar in chemical properties, cytotoxicity and aggregation ability from preliminary results, it is wise to choose one of them as the model system to study.

Since the aggregates will be the objects for study, the model chemical should be good at aggregating cells. Besides this, the aggregates should have high enough viability for the multicellular structures to be used in tissue engineering. In order to pick out this model chemical, we compared the aggregating ability of the five chemicals as well as the viability of aggregates from the chemicals, which are the two important properties for characterizing chemicals in this aggregating system. Concentrations of 0.1 mM and 0.01 mM were used separately for all five chemicals. In Fig 2.9A, PEI-2000-hy PEI-600-hy and DAB-Am-16-hy have better aggregation efficiency than other two chemicals. However, the viability of cells in aggregates from 0.1 mM DAB-Am-16 is so low (<20%) that it cannot be used for cellular system. In Fig 2.9B, PEI-2000-hy has much better aggregation efficiency than other four types of chemicals although viability of aggregates from all 0.01mM chemical were more than 70%. Based on

these, PEI-2000-hy was chosen as the chemical for detailed study in the following chapter.

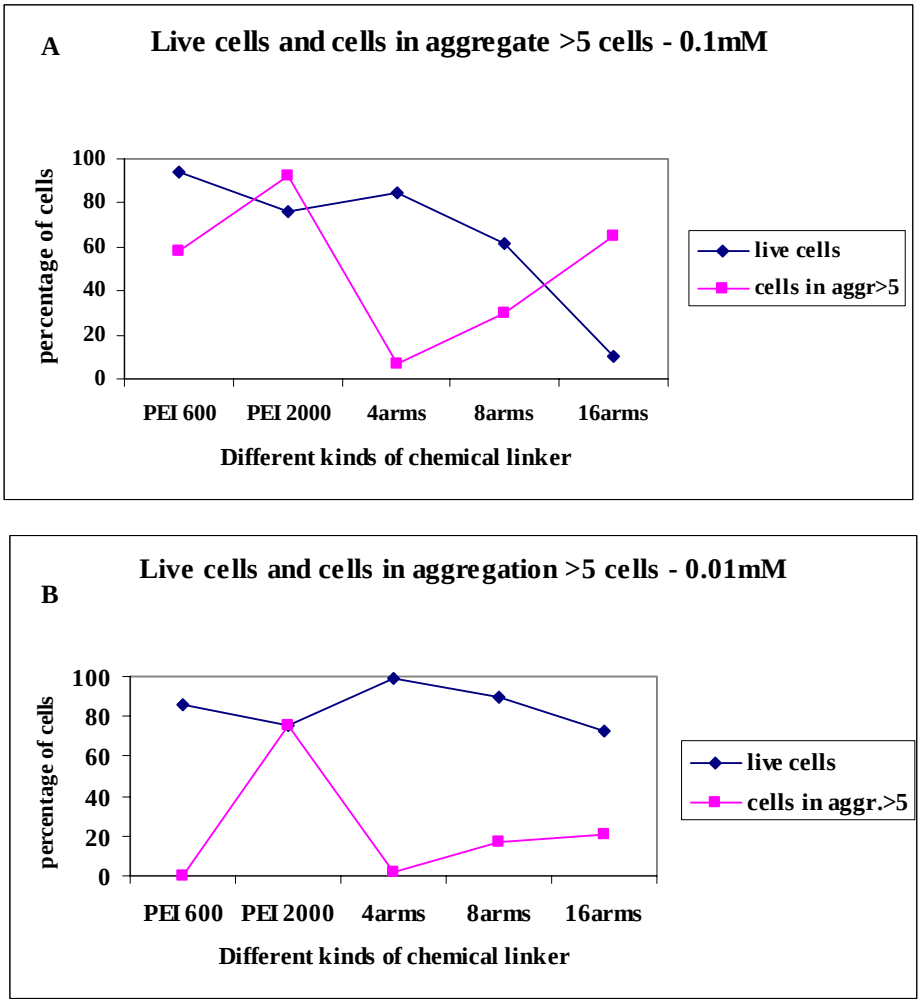


Fig 2.9 Comparison of five types of chemicals. Two properties were compared here. The blue dots show the viability of cells in aggregates and the red dots show the percentage of cells in aggregates the size of which are bigger than five cells. Two concentrations were tested here, A is 0.1 mM and B is 0.01 mM. Y-axis here shows the percentage of cells.

Chapter 3 Engineering Aggregates in a Rapid, Non-toxic and Controllable way

3.1 Aggregation ability characterization of the chemical linker

3.1.1 Number of hydrazide groups tested by Ellman's test

In order to synthesize chemicals which can react with several aldehyde groups on cell surface and thereby aggregate cells from individual ones, we designed a two-step reaction. In the first step, PEI-2000, which bears around 11 primary amino groups reacted with 2-iminothiolane. The product of first step with sulfhydryl groups will react with EMCH in the second step reaction. Since PEI-2000 has far more than 1 primary amino group in the molecule, it is possible to conjugate 2 or more sulfhydryl groups, which will be replaced into hydrazide groups by EMCH, resulting in the molecules with at least two hydrazide groups. More details of this reaction can be found in previous chapter 2.2.

In previous work we tried to use NMR to characterize the structure of the resultant molecule and we could conclude that the structure of the product is different from the initial reagents. The complex structure of PEI further increases the difficulty to characterize its structure by NMR data. So we tried to find an alternative quantitative method. Since the intermediate products bear sulfhydryl groups, the Ellman's test [202] which was used to calculate concentration of free thiols, was applied to

characterize the hydrazide groups indirectly. Based on protocol mentioned in "Materials and method", it was found that there are 3.85 sulfhydryl groups/molecule conjugated to PEI-2000 after the first thiolation reaction. It is not a surprising result since previous people have found that the use of 2-iminothiolane is an easy way to introduce lower amounts of sulfhydryl groups to HAS compared with other method [203]. In addition, the branched structure of PEI may hide some primary amino groups inside rather than expose them on the chemical surface, which may hinder the reaction happening. After EMCH reacted with sulfhydryl groups and replace free thiol groups into hydrazide groups at one-to-one ratio, only 0.064 sulfhydryl groups/molecule remained. So it was estimated that there were roughly 3.8 hydrazide groups on each molecule.

3.1.2 Formation of aggregates by PEI-2000-hy in a rapid way

Cell surface engineering used in this research modifies sialic acid into ketone bearing one (Fig 2.1). Our original chemicals bear amino groups on the surface. Although only a part of the amine groups were transversed into hydrazide groups according to Ellman's test result, the PEI-2000-hy with roughly 3.8 hydrazide groups functioned well in aggregates formation. Fig 3.1 shows the phase contrast images of the multi-cellular structures and illustrates the specificity of the aggregation ability of PEI-2000-hy from modified cells. Both the unmodified and modified HepG2 cannot be glued by PEI-2000-hy. The modified HepG2 can form very small clumps by PEI-2000, whereas only the modified cells can be glued by the modified PEI-2000-hy

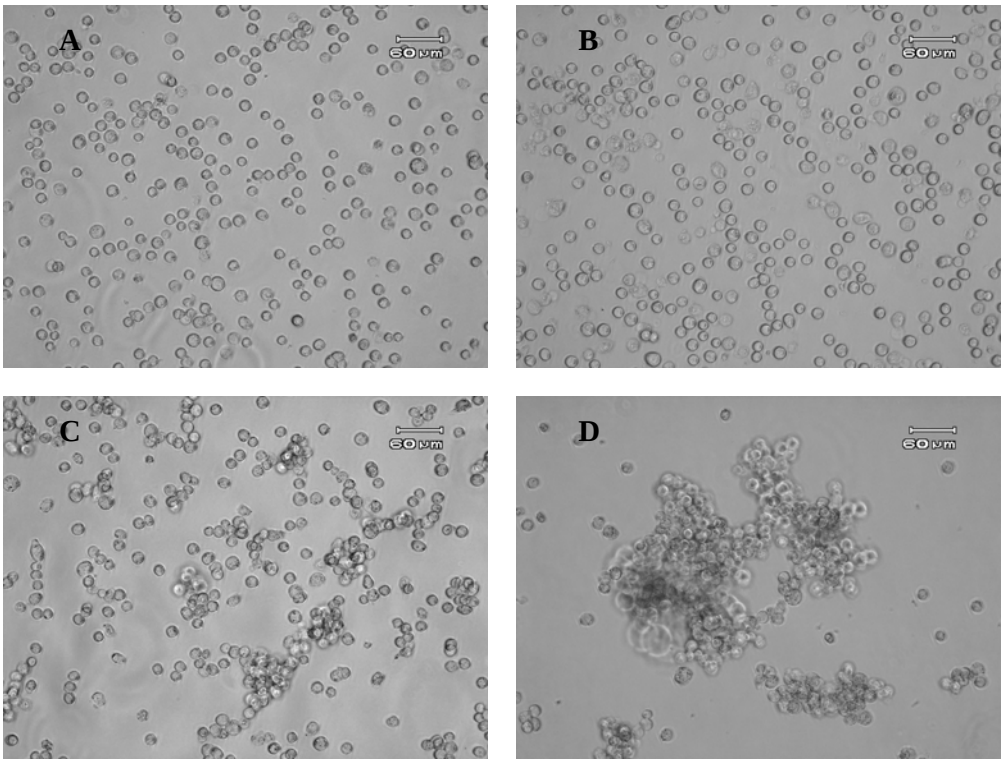


Fig 3.1 Aggregation formation of HepG2 cells from PEI-2000-hy. Cells were firstly treated by PBS or 1 mM sodium priodate at 4 ^{o}C for 15 minutes. Cells were resupspended in chemical solution and rotated for 30 minutes. A. Unmodified cells suspended in PEI-2000. B. Unmodified cells suspended in PEI-2000-hy. C. Modified cells in PEI-2000. D. Modified cells in PEI-2000-hy

[PEI-2000-hy]	**$NaIO_4$**			
	1 mM	0.5 mM	0.25 mM	0.125 mM
0.1 mM	5 + 10	5 + 10	5 + 10	10 + 30
0.05 mM	5 + 10	5 + 10	5 + 10	10 + 30
0.025 mM	5 + 10	5 + 10	10 + 10	15 + 30
0.01 mM	5 + 10	5 +20 / 10 + 10	5 + 30 / 15 + 20	> 15 + 30

Table 3.1 The shortest time for formation of aggregates > 10 cells. The first and second number shows the incubation time of $NaIO_4$ and linker separately. For example, 5+10 means that cells were incubated in $NaIO_4$ for 5 minutes followed by incubation in linker solution for 10 minutes.

efficiently. The clumps of modified HepG2 by PEI-2000 are probably due to the unspecific reaction between ketone group and amino groups on cell surface. Actually when people used PEI as important materials for gene delivery, they also found that bare PEI could also aggregate erythrocyte [204].

Besides the specificity of PEI-2000-hy to aggregate the modified cell, we also found that our new designed chemical is able to form aggregation much more rapidly. Comparing with the speed of previous method for aggregation formation by biotin hydrazide and avidin with the present new chemical linker, our new method shortens the time for aggregation formation from 2.5 hours [205] to as short as 30 minutes. Table 1 shows the shortest time for the aggregates of more than 10 cells forming in this system. We can see that aggregates can be formed by our method in 30 minutes from single cells. This finding stimulates our interest to explore the reason why chemical linker such as PEI-2000-hy can act more rapidly in gluing cells.

3.1.3 Efficiency of this aggregating system

Previous study has already showed the ability of PEI-2000-hy to glue cells in a rapid way. In addition to this, we have to calculate the aggregation efficiency of this chemical linking system to make sure the feasibility of using chemical linker for aggregating cells. In our experiment, we took 100 images for each sample and count the single cells and cells in aggregates one by one. Then we calculated the percentage of cells in aggregates, which we named “Aggregation Efficiency”. Although this method is not accurate because it becomes more and more difficult to see clearly the

number of cells as aggregates increases in size, this provides us a rough idea of the approximate percentage of single cells remaining in this rotating system.

From Fig 3.2, we can find that the aggregation efficiency of all the three conditions used was above 90%. In addition, there is no significant difference among these three conditions although the average value increases as higher concentration of PEI-2000-hy was used. Combining this result with previous data, we can conclude that by using $NaIO_4$ modification and PEI-2000-hy linkers, we can get 90% of cells into aggregates within one hour, which proves this way to be rapid and efficient in gluing cells.

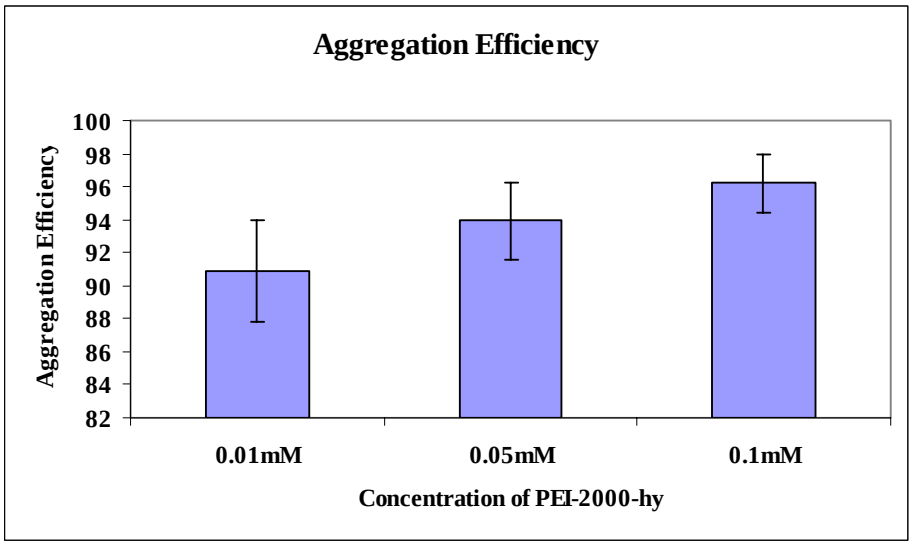


Fig 3.2 Aggregation efficiency under different concentration PEI-2000-hy. Cells were modified by 0.5 mM $NaIO_4$ for 15 minutes, followed by incubation in different concentrations of chemical solution for 30 minutes.

3.1.4 Importance of positive charge for PEI-2000-hy as an efficient linker

Initially the reaction between ketone group and hydrazide group was considered the key player underlying the formation of cell aggregates. However, this cannot explain why PEI-2000-hy act much faster and more efficiently than biotin hydrazide and avidin. When we compared the two methods of aggregating cells, we found that one and a half hours is required for biotin hydrazide to react with cell surface ketone groups, whereas 10 minutes is enough for PEI-2000-hy to aggregate the cells. The first possible reason is that PEI-2000-hy has 3.8 hydrazide groups on each molecule, whereas biotin hydrazide only has one. However, even we decrease the concentration of PEI-2000-hy to one tenth of that of biotin hydrazide, PEI-2000-hy still acts faster (data not shown here). So there should be other factors besides covalent reaction between hydrazide and ketone groups that play role in aggregating cells.

PEI has been widely used in gene transfection because of the favored interactions between negatively charged cell membranes and the positively charged polymer surfaces, which enables the polymers to adhere and assist the endocytosis of DNA/PEI complexes by cells. These facts provide us a hint that charge may also play an important role in the formation of aggregation. In order to find more support for this hypothesis, we synthesized another linker with no positive charge but bearing four hydrazide groups according to NMR data *. The ability of the chemical to

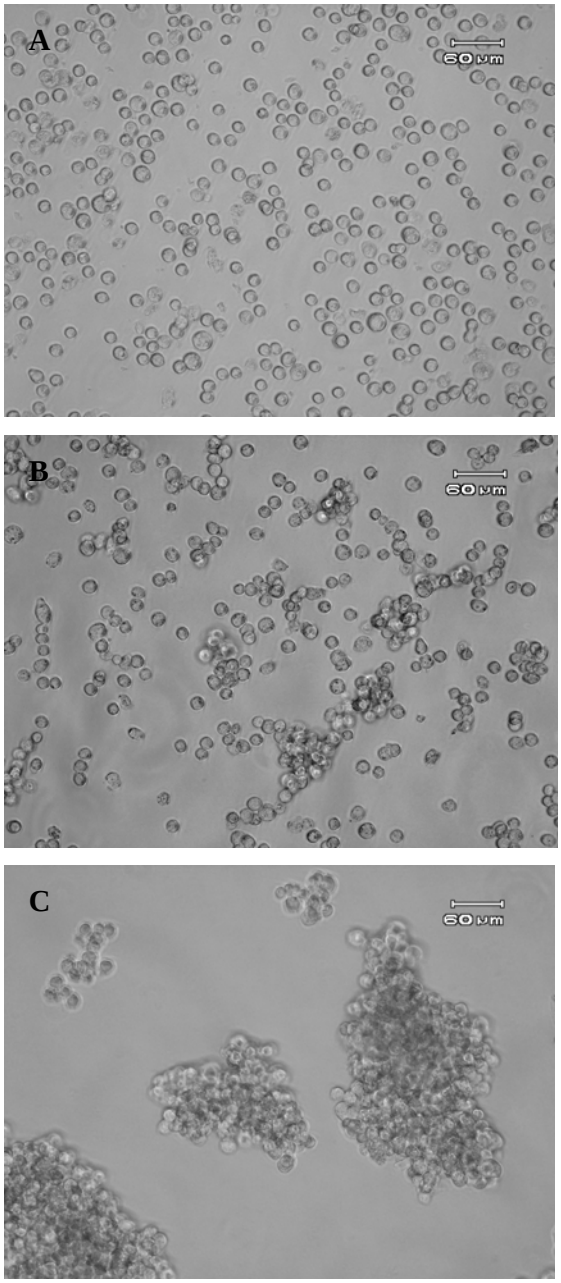


Fig 3.3 Positive charge is necessary of fast formation of the multi-cellular structure by chemical linker. A. Neutral linker with 4 hydrazide groups each molecule. B. PEI-2000 with no hydrazid groups. C. PEI-2000-hy with about 3.8 hydrazide groups each molecule. Only PEI-2000-hy, which is rich in positive charge due to the presence of many amino groups and hydrazide groups, is effective glue for modified cells. All of the cells were modified with 0.5 mM $NaIO_4$, and the concentration for all the chemicals were 0.1 mM.

* The chemical was kindly synthesized and characterized by Nguyen Thi Thuy Linh from Department of Chemistry.

aggregate cells was compared with PEI-2000-hy, which is highly positively charged. Fig 3.3 shows the huge difference of these two types of chemical in their capabilities to form aggregates. When the cells were modified to the same extent by $NaIO_4$, neutral chemical of the same concentration which has four hydrazide groups cannot aggregate cells at all whereas PEI-2000-hy is quite efficient for multi-cellular structure formation. This result proves our hypothesis correct.

3.2 Cytotoxicity of this aggregating system

3.2.1 Cytotoxicity of modification by $NaIO_4$

Although we have showed that PEI-2000-hy can be used to aggregate modified cells, the cytotoxicity of this system should be studied before it can be used in tissue engineering application. Since cells are treated with two types of chemical in this system, both of them should be considered for cytotoxicity study. In our present work, the method for modifying cell surface is the same as was used previously [205]. Because of the importance of cell surface carbohydrate residues, modifying sialic acid into ketone groups has the potential to compromise cellular function. Although previous work on surface modification of myoblast cells showed that periodate exposure had no gross effect on myoblast cells [205]; it is still necessary for us to test the toxicity of $NaIO_4$ to HepG2 in our experiment. One reason is that as we change the cell type, the effect of chemical on cells may change as well. The other reason is that we doubt the sensitivity of trypan blue exclusion as a test for viability of cells from previous experience. So instead of using trypan blue test alone, we used three

different ways, MTS, trypan blue exclusion, live and dead assay, in order to get conclusive result (Fig 3.4).

Fig 3.4A shows the result of MTS data after sodium periodate treatment. From the result, we can find that there should be something wrong with this method because it showed that the cells treated by PBS had lower reading than the 1 mM $NaIO_4$ treated ones. In MTS test, cells were firstly treated with PBS or $NaIO_4$ at 4 ^oC for 15 minutes. It is well known that the cytoskeleton of cells will decompose inside cells at 4 ^oC, resulting in the detachment of cells from substratum. So the number of cells in the well incubated with PBS decreased after 15 minutes treatment. However, $NaIO_4$ was a strong oxidant and cells treated by $NaIO_4$ on coverslips were found to be hard to be washed from substratum. Since MTS reading is highly dependent on the number of cells available, it is not hard to explain the strange results.

Our trypan blue results (Fig 3.4B) were quite similar to previous people's work. When the concentration of $NaIO_4$ was lower than 1mM, this treatment showed no significant effect on the viability of cells. However, the results from live and dead assay showed that even when 0.1 mM $NaIO_4$ was used, there is a significant drop of viability ($P<0.01$) to around 80% according to Fig 3.4C, although there is no significant difference among three different concentrations of $NaIO_4$, 1 mM, 0.5 mM and 0.1 mM. The low sensitivity of trypan blue test to dead cells can be further proved if we compare the results of cell viability after 10mM sodium periodate treatment. Trypan

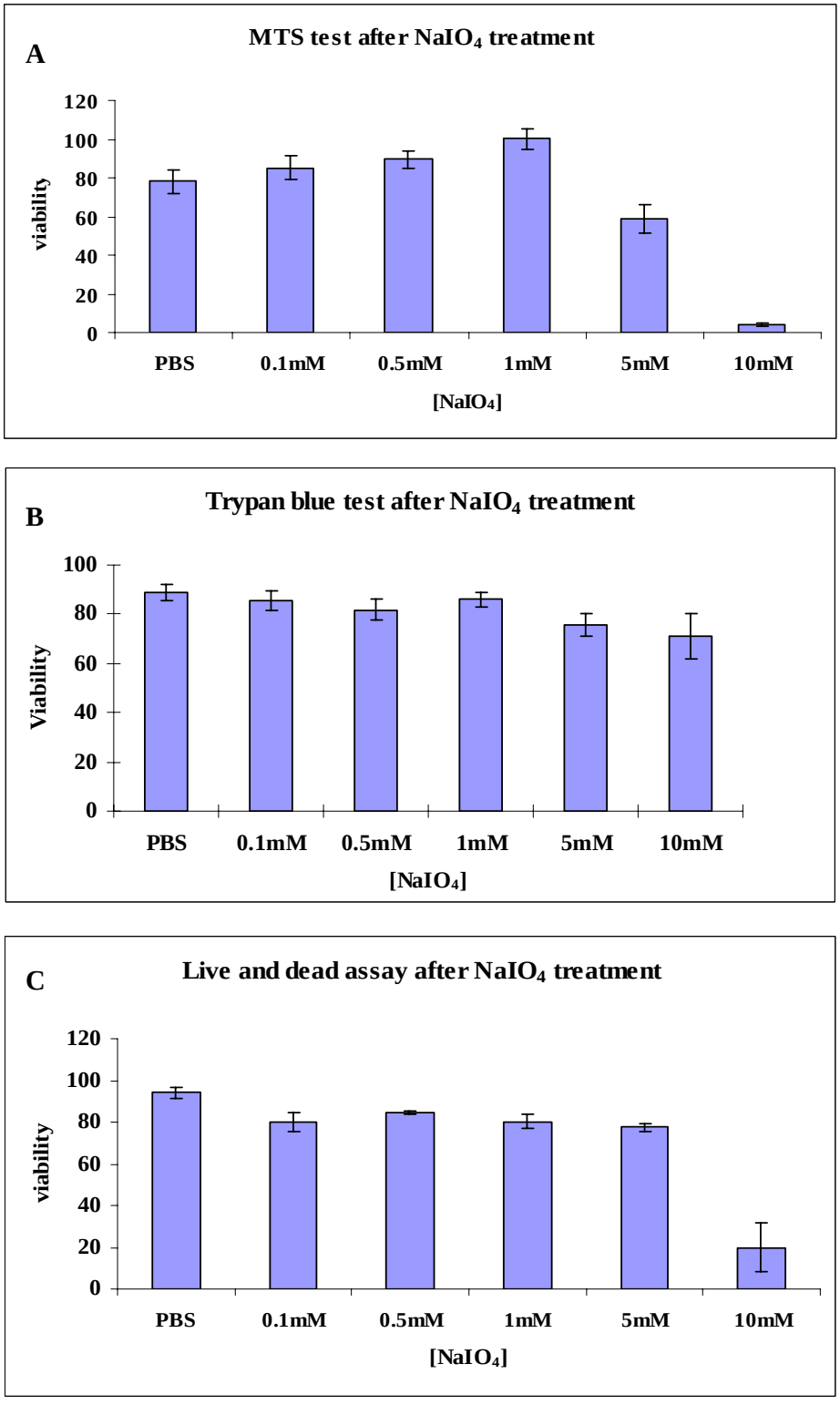


Fig 3.4 Cytotoxicity test of $NaIO_4$ treatment to cells. A is MTS test, which is not applicable to this system. B is trypan blue test, which is not sensitive to indicate the toxicity of $NaIO_4$. C is live and dead assay which was shown to be the most effective method. Result from C showed that $NaIO_4$ does influence the viability of cells.

blue test showed that around 70% cells were still alive whereas live and dead assay showed about 80% of cells were dead.

Based on previous results, we can conclude that trypan blue is not a good way to provide the viability data of cells. If we use other more sensitive ways, we can find that $NaIO_4$ treatment does affect the viability of cells, which is different from previous people's work. However, we can also conclude that the toxicity of $NaIO_4$ to cells does not vary much when the concentration is between 0.1 mM and 1 mM, below which sialic acid can be selectively oxidized.

3.2.2Cytotoxicity of PEI-2000-hy(PEI-2000-iminothiolane-hydrazide)

Although PEI was widely used in gene transfection and their cytotoxicity was well known and tested [208], cytotoxicity of PEI-2000-hy, which was seldom synthesized and characterized, has not been tested before. In order to prove the designed chemical can be used on HepG2 cells, we used MTS assay to check the cytotoxicity of PEI-2000-hy. Cells were incubated in different concentrations of hydrazide solution in serum free DMEM for 24 hours followed by incubation with MTS for 3 hours, by correlating the number of live cells with the absorption of the resultant solution, we calculated the percentage of live cells under different conditions. From results in Fig 3.5 we can find that when the concentration of chemical is below 0.01 mM, PEI-2000-hy possesses little toxicity to HepG2 cells in the experiment condition. Since PEI-2000 has about 11 amino groups, the result coincided with previous arts

[209] that lysosomal disruption at PEI concentrations above 0.001 M (amines), whereas nearly no effects on lysosomal stability when the concentration of PEI is at or below 0.0002 M. Considering the fact that concentration of PEI-2000-hy also affects the aggregation formation speed according to Table 3.1, we decided to use PEI-2000-hy between the concentration of 0.1 mM and 0.01 mM for further study, since the viability of the cells is about 80%, which is acceptable if the concentration of PEI-2000-hy increases to 0.1 mM.

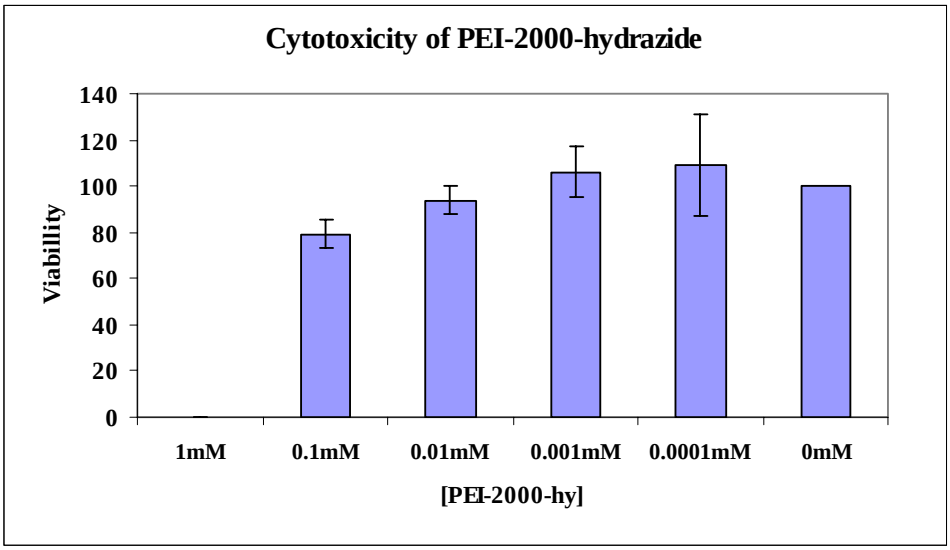


Fig 3.5 Cytotoxicity of PEI-2000-hy. The cytotoxicity of chemicals was tested by MTS assay. The viability of the cells was calculated based on the absorption of the resultant solution.

3.2.3 Live and Dead Assay of the Aggregates

Although we have characterized the cytotoxicity of $NaIO_4$ and PEI-2000-hy to HepG2 cells in this engineering system, both of the tests were performed independently of each other. The $NaIO_4$ treatment of cells was not followed by incubation with

PEI-2000-hy, which may further increases the toxic effect on cells. MTS test of PEI-2000-hy was done on natural cells without modification, which cannot represent the effect of linker on modified cells. In addition, the aggregates were produced in a rotating tube system, the effect of which cannot be represented by previous cytotoxicity test. In order to get complete information about the cytotoxicity of this system, we must find another way to test the viability of cells in aggregates after all of the chemical and engineering processes. Staining cells with CellTracker Green and PI, as we named live and dead assay was used to quantify the overall viability of cells in aggregates. CellTracker dyes are thiol-reactive fluorescent dyes and they can freely diffuse into the cell, where its weakly thiol-reactive moieties react with intracellular thiols, generating the green fluorescent product. Propidium iodide can only enter damaged cell membranes and undergoes a fluorescence enhancement upon binding to nucleic acids, promoting a red fluorescence in dead cells only. Stained cells can be observed and imaged using CLSM as shown in Fig 3.6.

We used 0.5 mM $NaIO_4$ to modify cells, followed by treated with 0.1 mM, 0.05 mM and 0.01 mM PEI-2000-hy respectively. The control cells were treated with PBS but using the same rotating system. In order to quantify the viability of cells, ten random areas of view were taken and processed by ImagePro Plus. The cell viability was calculated based on the number of live and dead cells. Results in Fig 3.7 tell us that even the cells were treated by PBS in rotating tubes; the average viability of cells reduces to 90%, which shows the inherent toxic effect of this system. When cells were

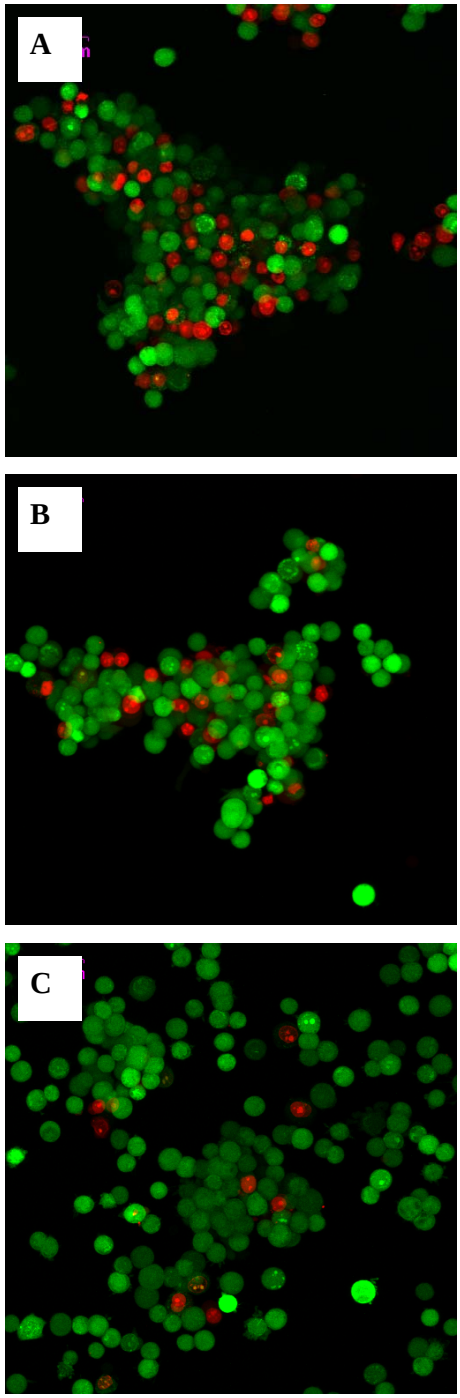


Fig 3.6 Live and dead assay of aggregates from PEI-2000-hy. Live cells were stained by cell tracker green and dead cells were stained by PI. The images were taken by confocal laser scanning microscopy FV 300. Cells were modified by 0.5mM $NaIO_4$, followed by linked with 0.1 mM (A), 0.05 mM (B), and 0.01 mM (C) PEI-2000-hy.

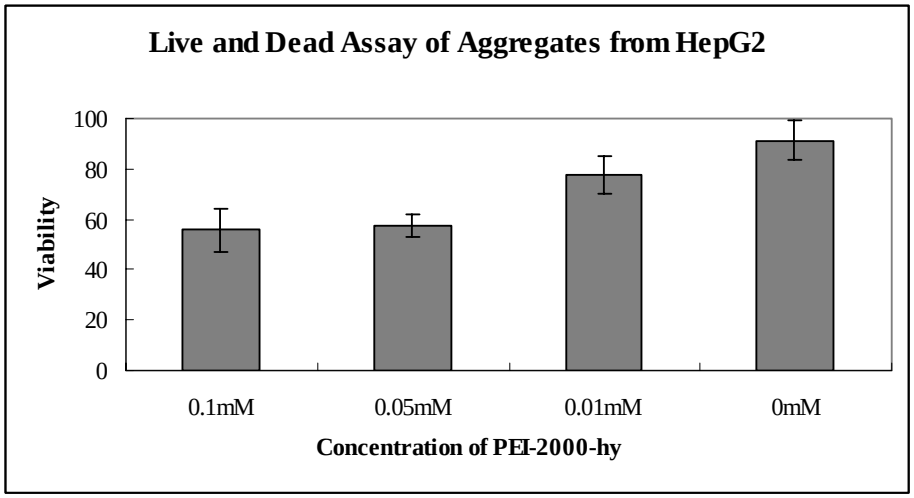


Fig 3.7 Quantification of Live and Dead assay in Fig 3.6. Ten random areas of view were taken and were processed by Imagepro Plus. The cell viability calculated as the mean ± SEM of three experiments.

treated by 0.5 mM $NaIO_4$ followed by 0.01 mM PEI-2000-hy, the average viability of cells reduces to around 75%, which coincidence with previous data the 0.5 mM NaIO4 treatment results in 80% viability of cells in Fig 3.4C. In addition, there is not significant difference between this condition and control. When the concentration of PEI-2000-hy increases to 0.05 mM, the viability of cells drops significantly to less than 60%.

Based on the above results, we can conclude that the rotating system also contributes to the death of cells during aggregates formation. We can find out some condition by adjusting the concentration of chemical linkers to minimize the overall cytotoxicity of the system.

3.2.4 Culture of the aggregates

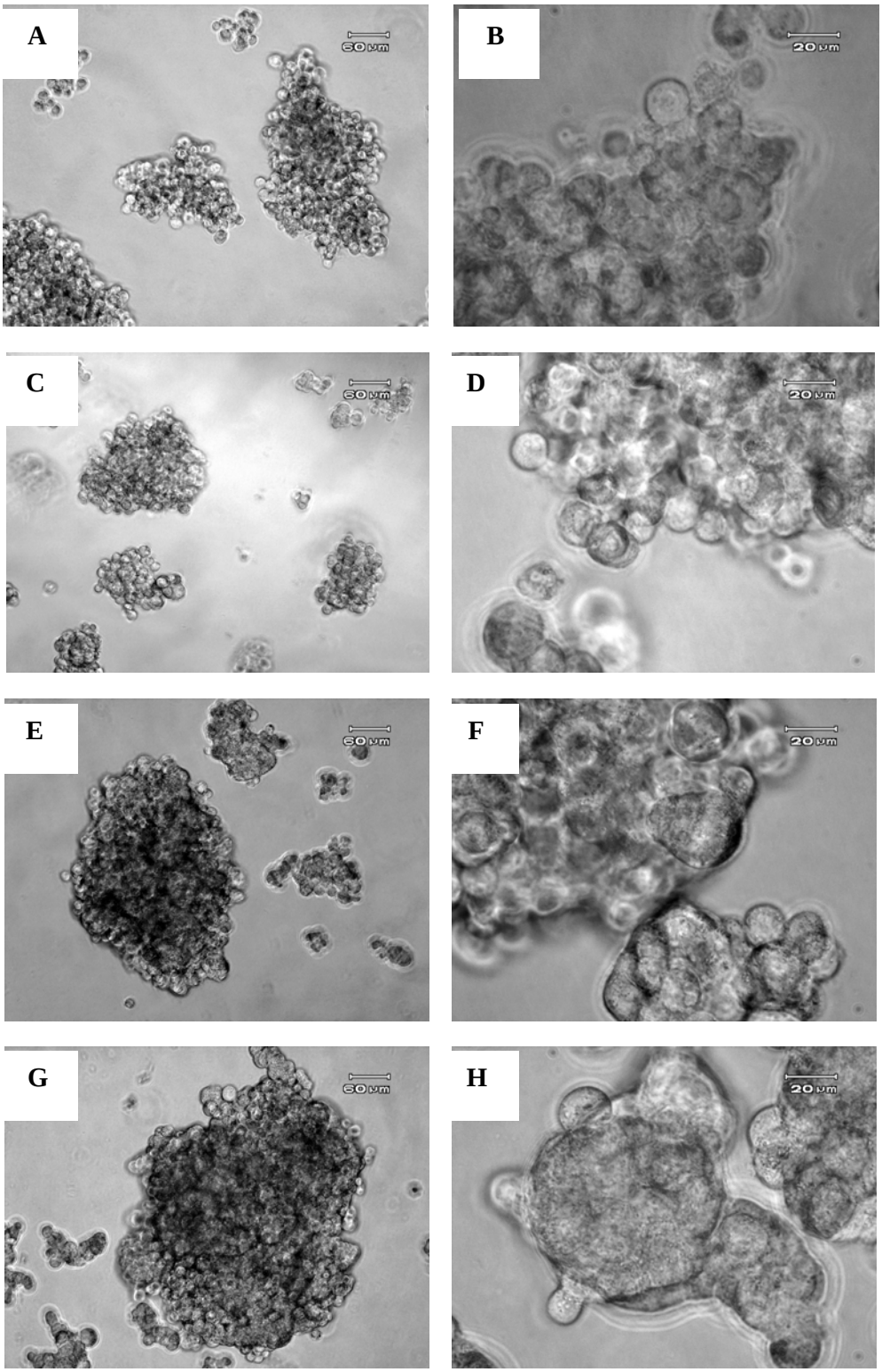
After we were able to engineer aggregates in a fast, efficient and low-toxic manner, we have to answer several questions before this system can be used for long-term application. For example, how can we culture the aggregates? How does the engineering process affect the long term viability of the cells? Is the proliferation ability of the cell line affected?

Based on previous results, we chose the condition of 0.5 mM NaIO4 and 0.01 mM PEI-2000-hy. We tried to answer the three questions above by culturing the aggregates in suspension up to 7 days. During the initial stage of this work, we even tried to culture the aggregates in bacterial culture dish and negatively-charge dish, both of which have low adhesion to cells. However, we found that the aggregates still adhered to the substratum and spread as normal cells. Later on, we cultured the aggregates in suspension by keeping them rotating. After we optimized the rotating speed at which the aggregates would not fall down, we decided to culture the aggregates in the plate on the rotating machine. When we observed the phase contrast images of aggregates taken on different days as they were cultured in suspension (Fig 3.8); we find that the cells in aggregates tend to grow compact and even grow into spheroid structure with smooth surface on the second or the third day. These phenomena are quite similar to those observed from natural aggregates from rotating system in our experiment (data not shown here), which hints the tendency of the engineered aggregates growing from engineered ones to natural ones.

In order to assess the viability changes of the aggregates in suspension culture, we characterized it by live and dead assay (Fig 3.9 & 3.10). Quite promisingly, we found that although the initial viability of the aggregates is only around 70%, which may not be satisfying in many biologists' opinion, the viability of the aggregates started to increase in suspension culture. After 7 days culture, the viability of cells in aggregates is about 95%. The increase of viability may be due to the proliferation of live cells. From the live and dead images, we can also find that there are fewer and fewer dead cells in the aggregates, which may also because of the detachment of dead cells from aggregates.

Besides Live and Dead assay, we also use MTS to test the mitochondrial activity of multi-cellular structure, which is one of the functional tests for cells. Curves in Fig 3.11 show quite promising result that the mitochondrial activity of aggregates also increases according to time. Both the increase in number of cells and in mitochondrial activity of single cells can contribute to this positive result.

Up till now, we are still not sure about the exact reason for increase of viability and MTS test data. However, we can find that the toxic effect of the engineering system on cell aggregates does not long as they are cultured in suspension, although the cells were treated with $NaIO_4$ and PEI-2000-hy in an unfavorable rotating system. By taking advantage of this fast, efficient and low-toxic aggregating approach as well as this suspension culture system, we maybe able to engineer aggregates with defined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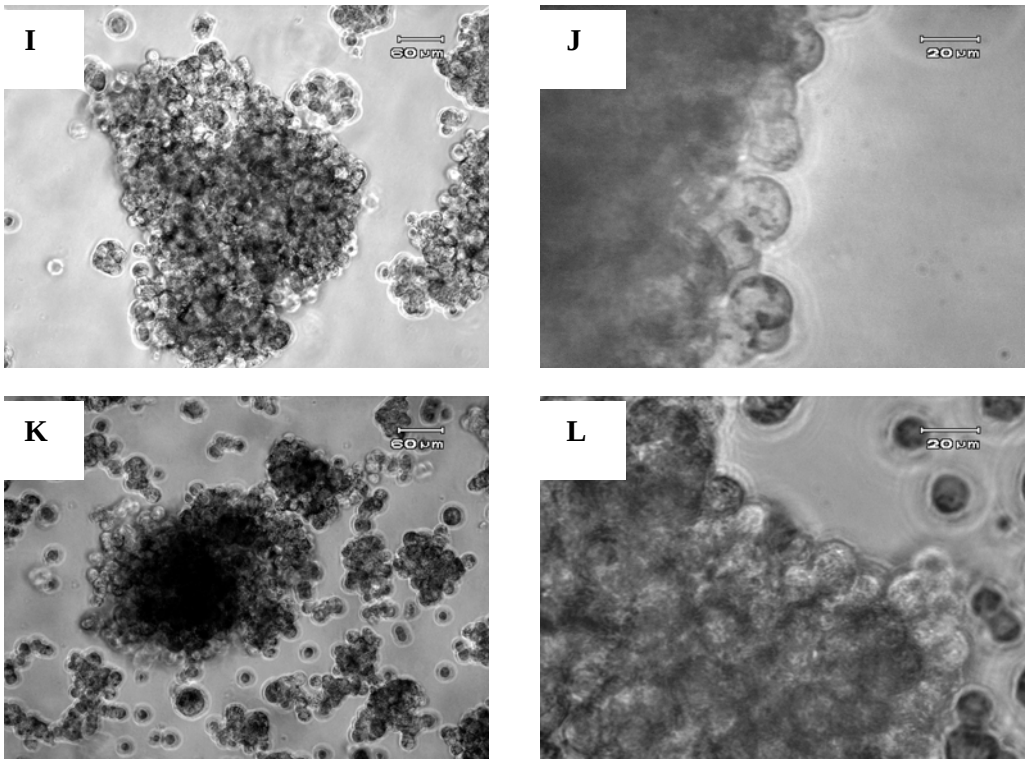

Fig 3.8 Phase Contrast images of the aggregates on different days during culture in suspension up to 7 days. Cells were modified by 0.5 mM $NaIO_4$ for 15 minutes at 4 ^oC in dark, followed by in incubating in 0.01 mM PEI-2000-hy in rotating tubes for 30 minutes. Aggregates were cultured in 6-well plate on rotating shaker. Images were taken on different days. A, B: 100 X and 400 X images on day 0; C, D: 100 X and 400 X images on day 1; E, F: 100 X and 400 X images on day 2; G, H: 100 X and 400 X images on day 3; I, J: 100 X and 400 X images on day 5; K, L: 100 X and 400 X images on day 7.

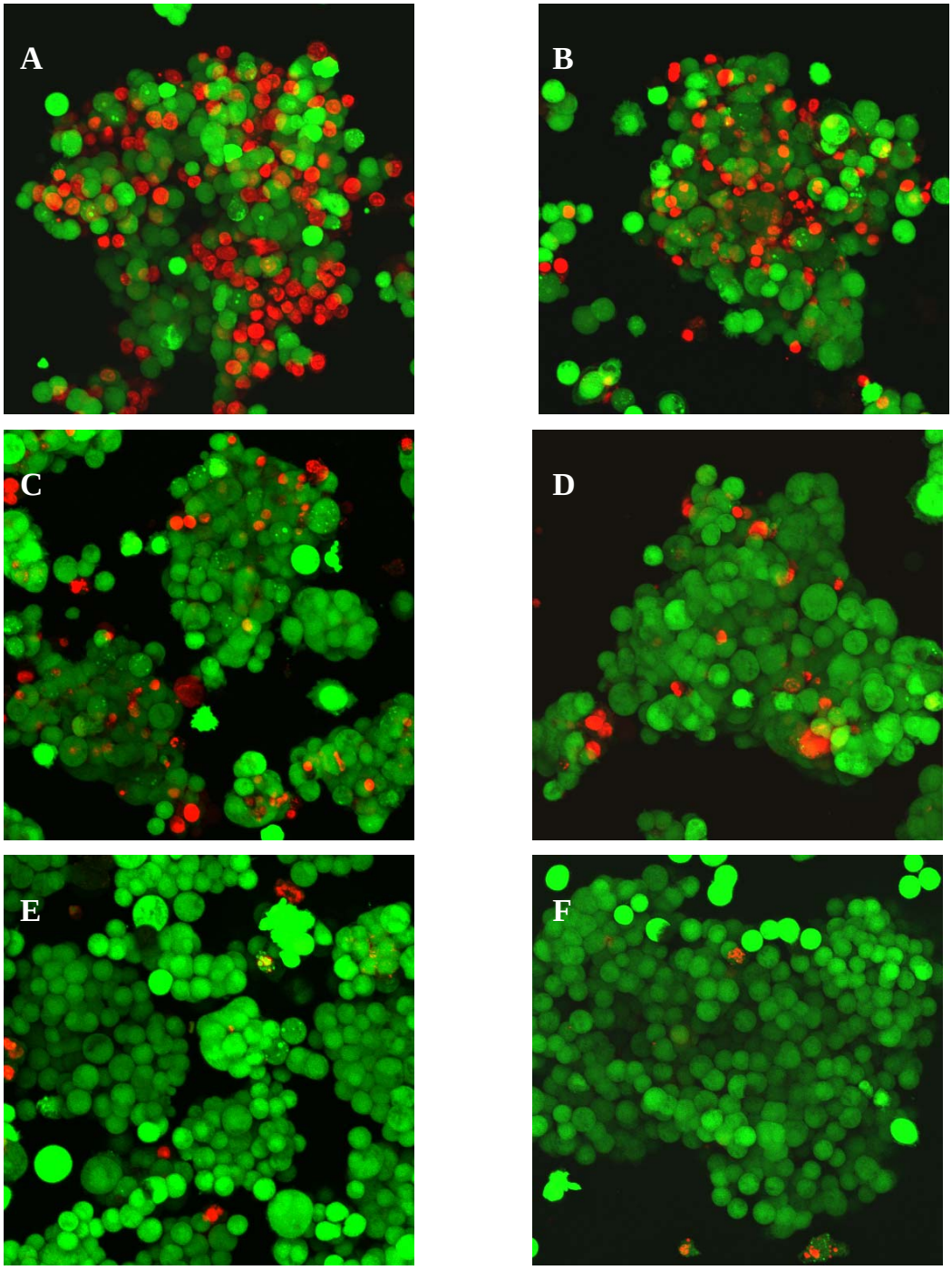

Fig 3.9 Live and Dead assay for culture of aggregates. The cells were modified with 0.5 mM $NaIO_4$ at 4 ^oC for 15 minutes followed by linked with 0.05 mM PEI-2000-hy. Cell aggregates were cultured in suspension up to 7 days. Cells in aggregates were stained with CTG and PI. Live and dead images taken by Confocal microscopy (Olympus Fluoview 500). A: Day 0, B: Day 1, C: Day 2, D: Day 3, E: Day 5, F: Day 7.

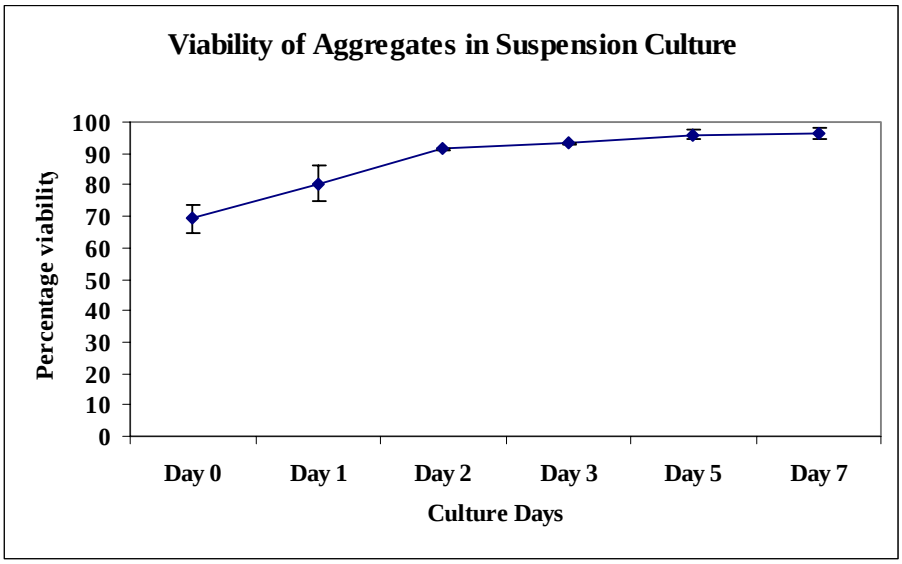

Fig 3.10 Quantification of images in Fig 3.9. Data were analyzed with ImagePro Plus. Three sets of experiment were done to make sure the feasibility of the results.

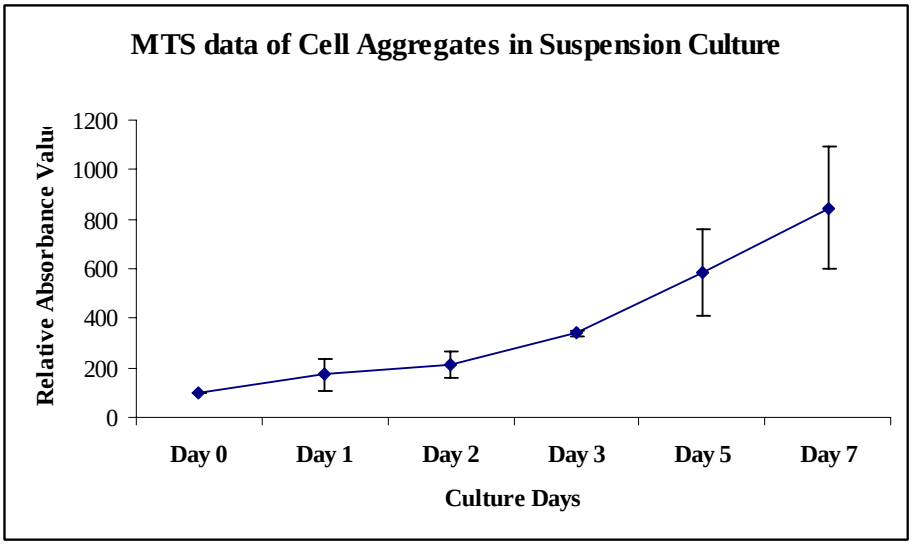

Fig 3.11 MTS data of cell aggregates in suspension culture. Data are presented as means+ SEMs (n=3).

shape and advanced functionality for tissue engineering application.

Since viability and mitochondrial activity are only two aspects of biological characteristics, functionality such as albumin secretion of HepG2 cells in aggregates can be studied in next phase. In addition, the cellular mechanism underlying this phenomenon also requires further exploration. Are the chemical linkers still on the cell surface after several days? Is there any up-regulation of cell adhesion molecules stimulated by signals proved by neighbor cells? Can we find other ways to aggregate the cells without modification? How can we control the shape and size of the multi-cellular structure? All of these are important topics to study to further validate the feasibility of this new system.

3.2.5 Fate of chemical linker

3.2.5.1 Observation of fluorescence- chemical linker on cell surface

Up to now, we have proved that by taking advantage of the positive charge and covalent reaction between ketone and hydrazide groups, we were able to aggregate cells in a rapid, efficient and low toxic way. In addition, the chemicals bear no adverse effect on the cells viability and functionality in culture. However, both aggregation formation process and the mechanism underlying the increased viability of aggregates are still unrevealed to us. We may be able to understand more about the system if we could observe the chemical linker directly. In order to achieve this goal, fluorescence tag was conjugated to PEI-2000-hy through the reaction between ester and amino groups. Similar method was used to track the intracellular fate of poly

(ethylenimie)/DNA complexes for gene delivery [206]. Un-reacted Oregon Green ester was removed by dialysis. Fluorescence linker was used in the same way as normal PEI-2000-hy to glue the cells. In the reaction, PEI-2000-hy was firstly synthesized followed by tagging with Oregon Green. Although the reaction capability between hydrazide and ester is higher than that between amine and ester in neutral or acidic buffer, the synthesized PEI-2000-hy only contains 3.8 hydrazide and more than 7 amine groups [207]. So the chance for ester to react with amine groups is still very high. Although we don't have data to show that the fluorescence tag is indeed on amine group instead of hydrazide group, aggregation data showed that the fluorescence linker can also aggregate the cells efficiently (not shown here). Since the purpose of synthesizing the linker is to track the fate of the linker on cell aggregates, we don't care much about which functional group the fluorescent probe is conjugated to as long as the linker can both aggregate the cells and shows fluorescence under confocal microscopy.

After cells were aggregated by the fluorescence-tagged-PEI-2000-hydrazide, we washed the aggregates with PBS buffer and observe them directly with confocal microscopy (Fig 3.12A). We find that most of the fluorescence enclosed the cell surface into a ring. However, some of the linkers were also inside cells. We thought it might be due to the endocytosis of cells. In order to further distinguish live and dead cells in aggregates from fluorescent linkers, we dually stained the cells with Cell Tracker Blue and PI. Images showed that both live and dead cells were enclosed by

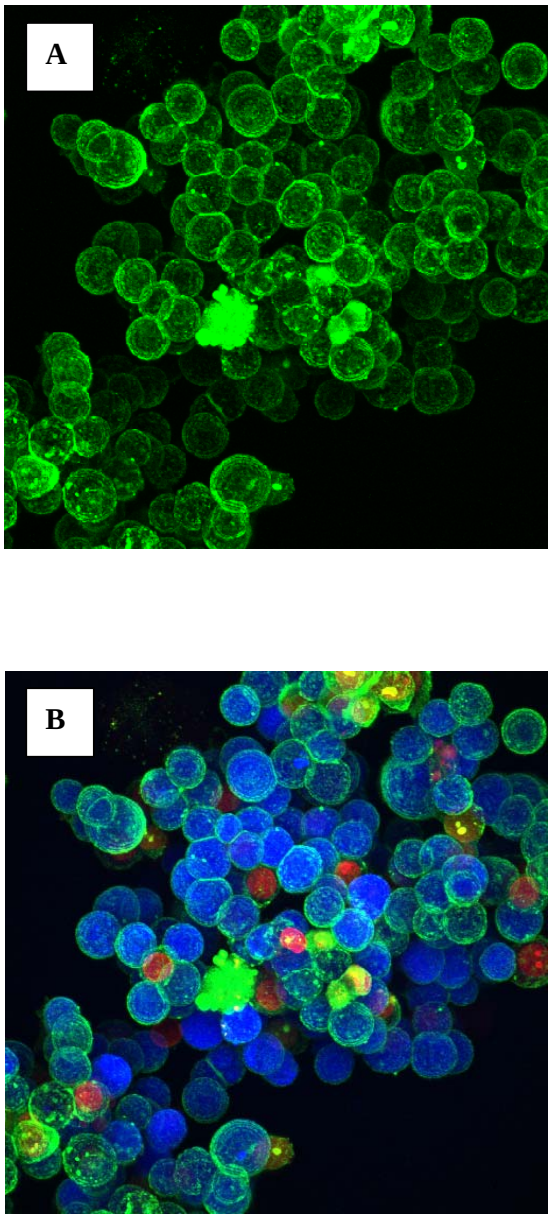

Fig 3.12 Fluorescence linker observed by Olympus Fluoview 500. A: The green color on the cell surface is the fluorescence linker. B: Live cells were stained with Cell Tracker blue and dead cells were stained with PI so that we can see all of the cells were enclosed by green color, which is from Oregon Green tagged-PEI-2000-hy. A: Cells were not stained. Only the fluorescence linker can be observed. B. Cells were dually stained with Cell Tracker Blue and PI.

linkers (Fig 3.12B).We can also see that most of the cells in aggregates are still alive, making it possible to track the fate of fluorescence linker in aggregates during culture in later study.

3.2.5.2 Tracking the chemical linker in suspension culture

In order to find out the mechanism underlying the increased viability of cell aggregates in suspension culture, we cultured the aggregates formed by fluorescence linker, using the same aggregation and culture system as above, only changing the normal PEI-2000-hy into Orgen Green tagged PEI-2000-hy. In negative control experiment, we found that, if all of the cells in aggregates were dead (by fixation), the fluorescence linker will continue to stay on cell surface during 7 days culture (data not shown here). We stained the aggregates with Cell tracker Blue and PI in order to make sure most of the cells are alive after the cells were modified by 0.5 mM $NaIO_4$ and aggregated with 0.05 mM fluorescent linkers (Here we changed the concentration because preliminary data showed that we can hardly get clear images of fluorescence if 0.01 mM fluorescent linker was used).

From Fig 3.13 we can find that on Day 0, the fluorescence is clearly distributed on cell surface and enclosed the cell to a ring. The fluorescence keeps disappearing from cell surface according to the images taken on different days and some of the cells even endocytosed the linker into the cells. From Day 5 on, there is almost no fluorescence linker on the cell surface or inside the cells. In order to get a clearer idea of the fate of

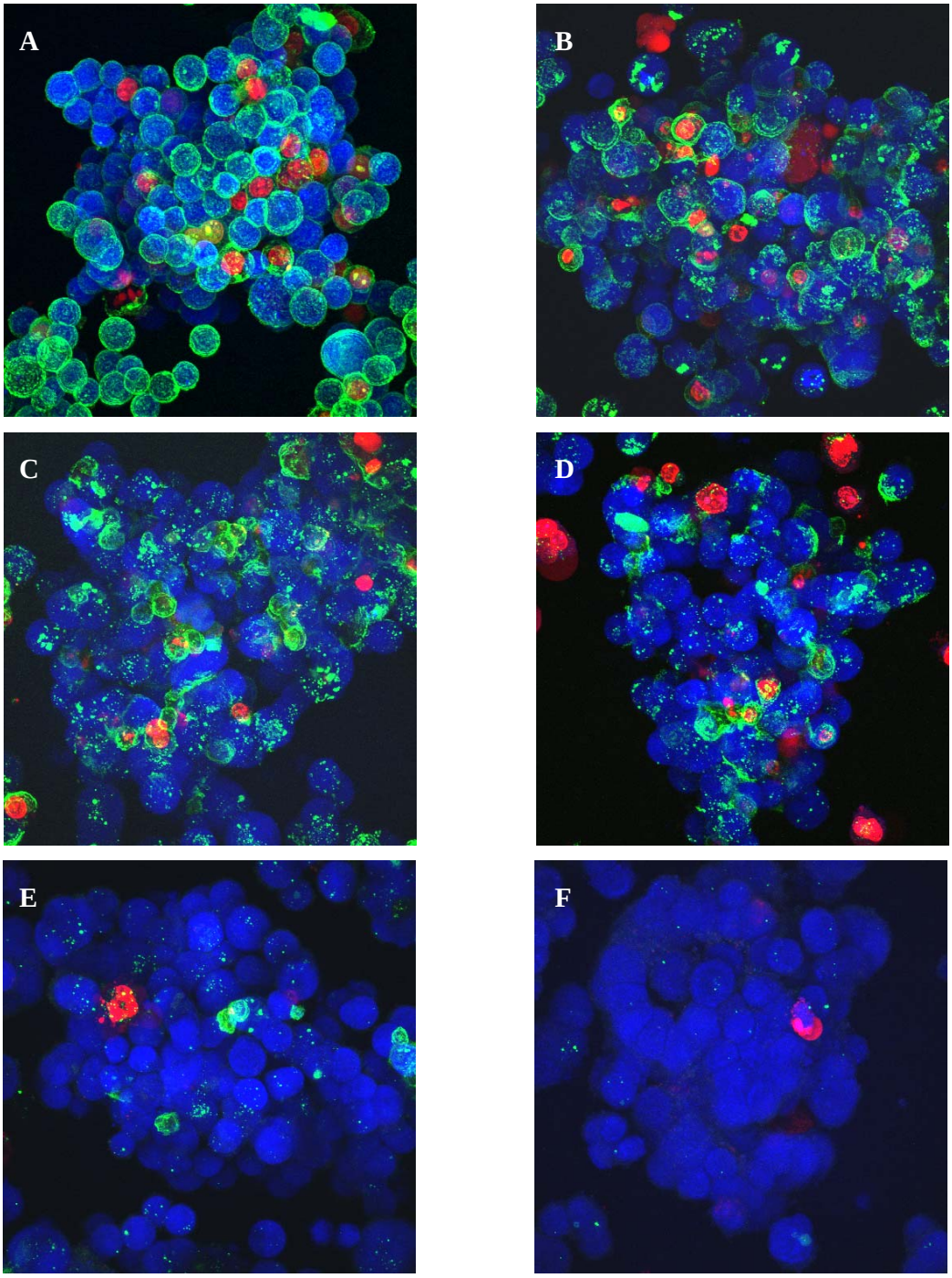

Fig 3.13 Fate of chemical linker on cell surface in culture. Cells were dually stained by Cell-tracker blue (live cells) and PI (dead cells). Linker can be directly observed. Images were taken on different days. A. Day 0. B. Day 1. C. Day 2. D. Day 3. E. Day 5. F. Day 7.

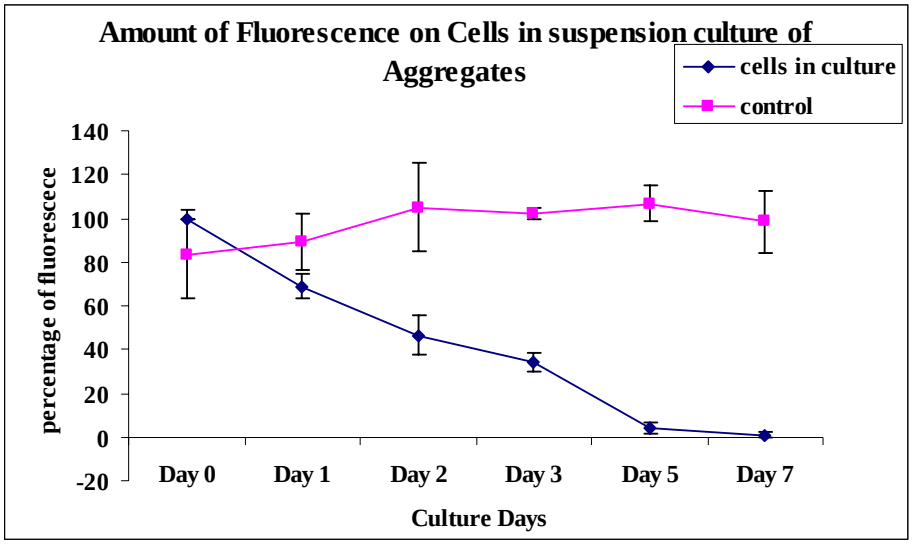

Fig 3.14 Quantification of amount of fluorescence remaining on cell surface. Images in Fig 3.13 were quantified by ImagePro Plus.

fluorescent linker on the cell surface, we quantified the images using ImagePro Plus (Fig 3.14). Compared with control, in which the cells in aggregates were killed, we can see that the disappearance of fluorescent linkers is because of the cellular activity instead of the properties of the linkers. This result stimulates our further interest to stain the cell adhesion molecules on the cell surface, which may be responsible for gluing the cells after chemical linkers disappear.

3.3. Ability of controlling aggregates using chemical linking system

3.3.1 Control the size distribution by linker concentration changes.

The advantage of our system is that aggregates can form in rapid, efficient and low-toxic way. So if we could define the structure of the multi-cellular strucuture, we might be able to achieve functional structures with proper structure. However, from discussion in previous chapter, we already know that aggregates formed in this tube

rotating system are not uniform in size or shape. What we can do in this system is to control the size distribution of aggregates by varying concentration of chemical linkers. So the distribution curve is quite useful when we want to collect aggregates of specific sizes. Based on this, using the same method as previous chapter, we plotted the aggregates distribution curve of PEI-2000-hy at 0.05 mM and 0.01 mM separately (Fig 3.15). From this Fig, we can find that when 0.01 mM PEI-2000-hy was used, more than 50% cells formed aggregates smaller than 30 cells, whereas, around 60% cells appeared in aggregates larger than 50 cells if 0.05 mM PEI-2000-hy was used. These distribution curves could be used as indicators when we want to get specific sizes of aggregates. However, in both situations, we cannot get aggregates of the same size, not to mention the defined structure.

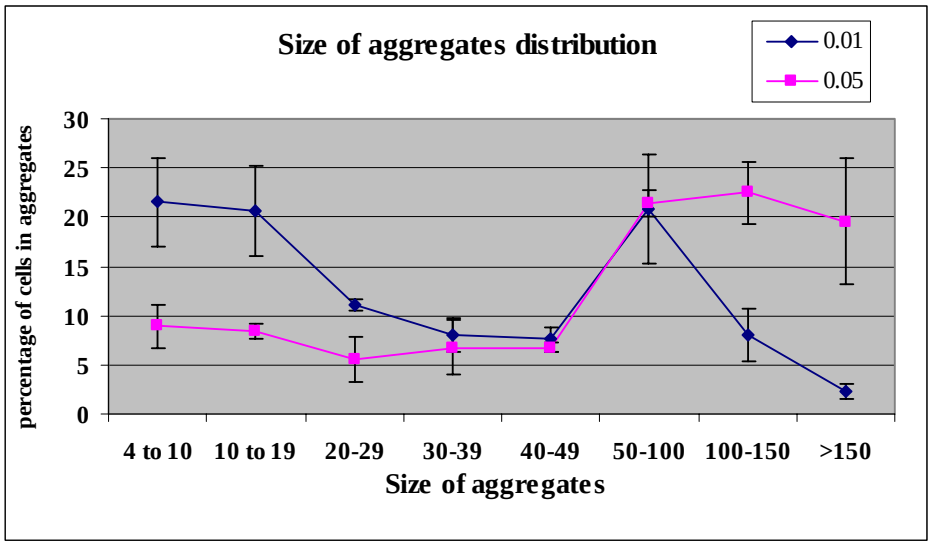

Fig 3.15 Distribution curves of the sizes of aggregates from PEI-2000-hy. Two concentrations were compared, 0.01 mM and 0.05 mM. The percentage of cells in specific size of aggregate changed when the concentration of chemical linker changed. More than 50% cells were in sizes < 30 cells for 0.01 mM PEI-2000-hy, whereas, fewer than 30% cells were in sizes < 30 cells when 0.05 mM PEI-2000-hy was used, in which most cells (more than 60%) formed aggregates > 50 cells.

3.3.2 Manipulating cells into defined structure by stenciling and micromanipulation

Previous data has shown that we can change the size distribution of aggregates by changing the concentration and type of chemical linkers, but we could never be able to achieve multi-cellular structure of uniform size in this rotating tube system. As can be seen from phase contrast images, the shapes of the aggregates are random as well, which are mostly like branches or grapes. In order to achieve standard building blocks for tissue engineering, we used micropatterning and micromanipulation to control the size and shape of the multi-cellular structure. In our experiment, we were able to pattern multi-cellular structure by stenciling (Fig 3.16A) as well as define the shape more precisely with micromanipulation (Fig 3.16B), which hinted us the possible use of this system in precisely manipulating cells into 3D structures. It will not only assist the research on relationship of structure and function, but also provided a novel way to reconstruct tissue *in vitro.* The reason why micropattern can be used to control the shape of the aggregates is because cells can be glued very fast and efficiently using our system. If it took longer time, the cells might adhere to the PDMS substratum instead of sticking to each other. Aligning cells by micromanipulation cannot be used for other aggregating methods either. Because it is impossible to hold two cells together until they stick to each other after several hours.

However, there are still several problems for both methods. The problems involved in stenciling include, firstly, difficulty in getting a complete thin layer of PDMS because

of the fragility of the PDMS layer; secondly, difficulty in peeling off the layer from substrate with the cell aggregates intact. The main problem for micromanipulation is that the whole process took such a long time that it is rather difficulty to keep the viability of cells. Further work is required before better conditions are worked out to manipulate cells in a controllable and biocompatible way.

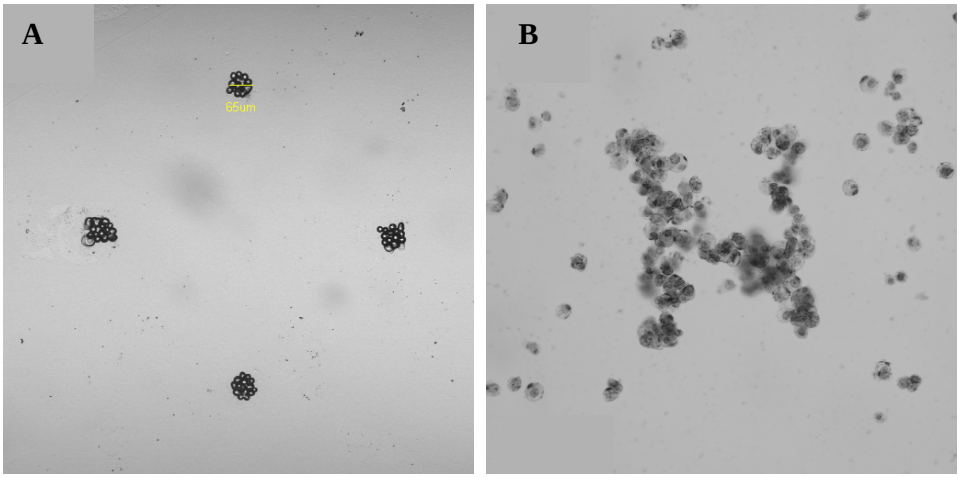

Fig 3.16 The structure of the aggregates can be controlled by stenciling or micromanipulation.
A. PDMS film with diameter of 50 μm circles were molded from silicon wafers. Modified cells (by 1 mM $NaIO_4$) settle down into the holes and incubated in the chemical solution for 30 minutes. After the PDMS film was peeled off, cell aggregates in round shape remained on the substrate.
B. Cells were modified by 1 mM NaIO4 for 15 minutes. With the presence of PEI-2000-iminothiolane-hydrazide at the concentration of 0.1 mM, cells were glued with single cell resolution with the aid of micromanipulation. Images were taken by FV 300.

Chapter 4 Conclusion and Future Work

Although cell aggregates has been used since several decades ago as a powerful tool for understanding cell-cell adhesion, cell-matrix interaction and cell sorting, not until recently have the cell aggregates been treated as building blocks in tissue engineering. Previous work has been done to prove the concept of self-assembling of aggregates, which justified the use of cell aggregates in organ printing to create 3-D tissues. However, an efficient and controllable way to build cell aggregates is still lacking. Using 2-step chemical conjugation reaction, we successfully synthesized two series of chemical linkers with poly (ethyleneimine) and dentrimers as the initial reagent separately. All of the five types of linkers can glue the cells efficiently and specifically.

In order to further prove the feasibility of this chemical linking system in the area of tissue engineering, we need to sort out a sample chemical to prevent repeating work and heavy workload. Aggregation efficiency and viability of aggregates from the linkers were used as two parameters to justify choice of PEI-2000-hy. By working deeper into this aggregating system using PEI-2000-hy as a model, we found that the way to use chemically controlled linker to build multi-cellular structures is faster compared with previous biotin hydrazide and avidin method. We also discovered that the charge interaction between the linker and the negatively charged cell surface is as important as the covalent nature of chemical reactions. Under the guidance of this

principle, we could find an optimal condition that would form multi-cellular structure efficiently and keep relatively good viability. In addition, we found the cells in the aggregates showed increased viability over time in suspension culture although the initial viability is not promising enough. With the help of fluorescence tag, we find that the fluorescence linker gradually disappeared from cell surface as aggregates were cultured, when the aggregate became more compact. This stimulated our interest into studying the fate of cell adhesion molecule to understand the mechanism underlying these phenomena.

However, it was found that the shape and size of the aggregates could not be controlled by this random-rotating system. In order to really engineer aggregates with defined shape and size, we applied microstenciling and micromanipulation to control the shape of the multi-cellular structures. The interesting preliminary results validated the possible application of this system into contructing functional 3-D tissues *in vitro*.

Despite the promising results above, there are some inherent problems in this chemical linking system. Firstly, sodium periodate treatment of sialic acids into ketone sialic acids on cell surface does affect the viability of the cells, from the result we have got. It is imperative to find alternative way to treat the cells more gently in order to reduce the adverse effect of the engineering process on cells. Secondly, the mechanism underlying the aggregating process has not been clarified; although we have found out the importance of covalent binding and positive charge the linker

provides, the kinetics is still a mysterious. Thirdly, although the aggregates could restore viability in continual culture, it usually took several days to achieve this. The system will have more application if we could find ways to shorten the recovery time of aggregates. In addition, HepG2 is a kind of cell line which keeps proliferating, which contributes to the increased viability of aggregates. So whether similar result can be achieved on non-proliferating cells such as primary hepatocytes is still a question. Last but not the least one, although we managed to control the size and shape of the multi-cellular structures by micropatterning and micromanipulation, how to manipulate the cells in more compatible way is still a pending question.

Chapter 5 Materials and Methods

All reagents were purchased from Sigma-Aldrich unless stated otherwise.

5.1 Cell culture

HepG2 cells (ATCC, VA, US) were cultured in 75 cm^2 tissue culture flask (NUNC, Denmark) with Dulbecco's modified Eagle's medium (GIBCO, Invitrogen,) supplemented with 10% fetal bovine serum (Hyclone, Utah, US), 1.5 g/L glucose, 100 units/mL penicillin, 100 g/mL streptomycin, 1.3 g sodium bicarbonate and 1.2 g Hepes (Gibco BRL, Life technology, Maryland, US). The cells were cultured at 37^oC in a humidified environment with 5% CO_2.

5.2 Determination of surface modification by $NaIO_4$ on HepG2 cell surface

Coverslips were put into 24-well tissue culture plate (NUNC, Denmark). HepG2 were grown into confluence on the coverslip, washed twice with phosphate-buffered saline (NUMI, National University of Singapore) at room temperature and incubated in the dark at 4 °C for 15 min with cold PBS or a solution of sodium periodate in cold PBS. Wash cells with biotin buffer (PBS with 0.1% FCS, pH = 6.5) twice, followed by incubating them in 5 mM biotin hydrazide (dissolved in biotin buffer) at room temperature for 90 minutes. Wash cells with cold avidin buffer twice (PBS with 0.1% FCS, pH = 7.4). Then treat cells with Streptavidin-FITC (5 μg/ml, Dako, Denmark) for 20 minutes at 4 °C in the dark. Cells surface fluorescence was then analyzed by

fluorescent confocal microscopy (Olympus Fluoview 300) with the magnification of 400 X. The excitation wavelength is 488 nm.

5.3 Synthesize of the chemical linkers

4-DAB-Am-hydrazide:

8 mg 4-DAB-AM (Mw=316, 25 μmol) was added to 2-iminothiolane (15 mg, Mw=137.6, 100 μmol) in distilled-water (2 ml) at room temperature. The mixture was stirred for 2 hr. EMCH (22.5 mg, 100 μmol, Pierce, IL, US) was added in DMSO (0.5-1 ml, J.T. Baker, NJ, US.) to react for 4hr. The finally product was isolated by eluting it through a PD-10 column (Amersham Pharmacia Biotech AB Piscataway, NJ, US.) with distilled-water, freeze-dried 48hr.

8-DAB-Am-hydrazide:

8 mg 8-DAB-Am (Mw=773, 12.5 μmol) was added to 2-iminothiolane (15 mg, Mw=137.6, 100 μmol) in distilled-water (2 ml) at room temperature. The mixture was stirred for 2 hr. EMCH (22.5 mg, 100 μmol) was added in DMSO (0.5-1 ml) to react for 4 hr. The finally product was isolated by eluting it through a PD-10 column with distilled-water, freeze-dried 48 hr.

16-DAB-Am-hydrazide:

11 mg 16-DAB-Am (Mw=1686.6, 6.5 μmol) was added to 2-iminothiolane (13.5 mg, Mw=137.6, 100 μmol) in distilled-water (2 ml) at room temperature. The mixture was stirred for 2 hr. EMCH (22.5 mg, 100 μmol) was added in DMSO (0.5-1 ml) to react for 4 hr. The finally product was isolated by eluting it through a PD-10 column with distilled-water, freeze-dried 48 hr.

PEI-600-hydrazide:

15 mg PEI-600 (Mw=600, 25 μmol) was added to 2-iminothiolane (12.5 mg, Mw=137.6, 90 μmol) in distilled-water (2 ml) at room temperature. The mixture was stirred for 2 hr. EMCH (21 mg, 90 μmol) was added in DMSO (0.5-1 ml) to react for 4 hr. The finally product was isolated by eluting it through a PD-10 column with distilled-water, freeze-dried 48 hr.

PEI-1200-hydrazide:

15 mg PEI-1200 (Mw=1200, 12.5 μmol) was added to 2-iminothiolane (25 mg, Mw=137.6, 180 μmol) in distilled-water (2 ml) at room temperature. The mixture was stirred for 2 hr. EMCH (40 mg, 180 μmol) was added in DMSO (0.5-1 ml) to react for 4 hr. The finally product was isolated by eluting it through a PD-10 column with distilled-water, freeze-dried 48 hr.

PEI-2000-hydrazide:

20 mg PEI-2000 (Mw=2000, 10 μmol) was added to 2-iminothiolane (15 mg, Mw=137.6, 110 μmol) in distilled-water (2 ml) at room temperature. The mixture was stirred for 2 hr. EMCH (25.6 mg, 110 μmol) was added in DMSO (0.5-1 ml) to react for 4 hr. The finally product was isolated by eluting it through a PD-10 column with distilled-water, freeze-dried 48 hr.

5.4 Characterization of the chemical linkers – Ellman's test

In an appropriate cuvette (Plastibrand®, BRAND, Germany), add 50 μl of the DTNB solution (50 mM sodium acetate (NaOAc) with 2 mM DTNB in H_2O (refrigerate, DTNB is 5, 5'- Dithiobis (2-nitrobenzoic acid), Molecular Probes, CA, US), 100 μl

Tris solution, and water up to (1000µl – sample µl). Mix solution carefully using pipette. Place cuvette into UV-Vis spectrophotometer (UV-1201, Shimadzu, Japan) and take a background scan using the solution as background. Introduce sample solution into cuvette with a syringe, keeping the cuvette in the instrument. Carefully mix solution with a pipettemen without disturbing the cuvette. Incubate 5 min at room temperature. Scan sample and record Absorbance at 412 nm. Calculate absorbance for each sample and then average the results, divide this by 13600 M^{-1} cm^{-1} (the extinction coefficient of the reagent) to get the molarity of the solution. After PEI-2000 reacts with 2-iminothiolane for 2 hr, product was eluted by PD-10 column. The amount of free thiol group on PEI was tested by Ellman's test. The first step product was put back together with EMCH to react another 4 hrs. Final product was eluted through PD-10 and the remaining free thiol group was tested again. By comparing the amount of thiol groups on the products of two steps, the number of hydrazide group on each PEI molecule was estimated.

5.5 Cytotoxicity test of the chemical linkers

Culture HepG2 cells on 96-well microtitre plates (Nunc, Denmark) at a density of 1.2 × 10^4 cells/well. Incubate for 24 hours, remove medium and add the chemical solutions in serum free medium at different concentrations (0.1 µM, 1 µM, 10 µM, 100µM, 1mM), with a final volume of 200 µl. After 24 hours of incubation, the medium was removed and the cells were rinsed by 160 µl sterile PBS for twice. 200 µl CellTiter96 ® Aqueous One Solution Reagent (Promega, Madison, USA) diluted

by 5 times PBS was added to each well. After incubation for 3 hours, the absorbance will be measured at 490 nm using a Sunrise^{TM} microplate absorbance reader [Tecan, Switzerland].

5.6 Synthesis of fluorescence PEI-2000-hy

PEI-2000-hy synthesized as described above was diluted to a concentration of 10 mg/ml with 0.1 M sodium bicarbonate. The PEI-2000-hy was then transferred to microcentrifuge tubes (Axygen, Union City, CA, US) in 1-ml aliquots. While stirring, 50 ml of prepared tag [Oregon Green 488 carboxylic acid, succinimidyl ester (Molecular Probes), in DMSO at 10 mg/ml] was added to each of the microcentrifuge tubes, after which stirring continued for 1 hour in the dark at room temperature. The stirring was then followed by an additional incubation of at least 1 hour in the dark. Unreacted fluorescence probes were removed by Spectra/Por® Cellulose Ester (CE) dialysis membranes with MWCO 1000 (Spectrum Laboratories, Inc**,** CA, USA). The labeled PEI could then be used for aggregation or stored at 4°C.

5.7 Cytotoxicty of $NaIO_4$ on cells

1. MTS assay of cells treated by $NaIO_4$

Culture HepG2 cells on 96-well microtitre plates (NUNC, Denmark) at a density of 1.2×10^4 cells/well. Incubate for 48 hr; remove medium and wash cells with 1 X PBS twice. Different concentrations of $NaIO_4$ (10 mM, 5 mM, 1 mM, 0.5 mM, 0.1 mM and PBS) were used to incubate cells in dark at 4 ^{o}C for 15 minutes. Cells were

then washed with PBS twice again and incubated in CellTiter96 ® Aqueous One Solution Reagent (Promega, Madison, USA) solution diluted 5 times by PBS for 3 hours in normal culture environment. The absorbance will be measured at 490 nm using a Sunrise^{TM} microplate absorbance reader [Tecan, Switzerland].

2. Trypan blue assay of cells

Cells were cultured in 75 cm^2 tissue culture flask (NUNC, Denmark) in the same medium mentioned above. The cells in flask were washed with phosphate-buffered saline twice at room temperature. Cells were suspended by incubation with 3ml pre-warmed Trypsin/EDTA solution at 37 °C. Trypsin was neutralized with DMEM medium and the cells were centrifuged at 800 g for 2 minutes. Cell pellets were washed twice with PBS, pelleted and resuspended in cold PBS or different concentrations (10 mM, 5 mM, 1 mM, 0.5 mM, 0.1 mM) of sodium periodate solution. After incubation at 4 °C in dark for 15 minutes on rotary shaker, cells were pelleted and washed with cold PBS twice. The final cell pellet was resuspended and incubated with trypan blue solution (Fluka, Switzerland) for 5 minutes. The cells were then imaged using microscope equipped with an Olympus IX50 microscope. Cells were counted in a cell counter [Hirschmann EM techonology]. Four random fields of view were counted per assay condition, and the cell number and viability calculated as the mean ± SEM of three experiments.

3. Live and dead assay of cells

HepG2 cells were cultured, trypsinized, pelleted, washed with PBS and incubated with PBS or sodium periodate solution using similar method mentioned above. Cells were then washed with PBS twice and dually stained with CellTracker™Green (CTG, Molecular Probes, CA, US) and propidium iodide (PI). The cells were resuspended in double staining solution (50 μmol/ml CTG and 25 μg/ml PI in DMEM). Incubate for 30 minutes at 37 ^{o}C. Wash the cell with PBS another twice. Put the cells on ice until observation by fluorescent confocal microscopy (Olympus Fluoview 500). CTG was observed at the wavelength of 488nm and PI was at 543nm. Images were analyzed by ImagePro Plus and viability of the cells was calculated based on the number of live cells and dead cells which were counted separately by the software on at least 10 images for each sample. The result was calculated as the mean ± SEM of three experiments

5.8 Formation of cell aggregates by modified cells and chemicals

HepG2 cells were cultured and trypsinized using similar method mentioned above. Then the cells were centrifuged at 800 g for 2 minutes. Cell pellets were washed twice with PBS, pelleted as described above and resuspended in cold PBS or sodium periodate solution. After incubation at 4° C in dark for 15 minutes, cells were pelleted and washed with cold PBS twice. Chemical linkers with specific concentration were added and cells were kept in rotation to prevent from settling down. Pipet approximately 25 μl of the cell suspension into 12-well plate (NUNC, Denmark) and

dilute it with 1 ml PBS after 35 minutes incubation at 4 °C. Observe the aggregation by phase contrast light microscope (Olympus IX50) at the magnification of 100 X.

5.9 Statistics on aggregates size distribution

After the aggregates were formed, 500 ul of the cell suspension was taken out and put into 12-well plate. The images on aggregation were taken by phase contrast light microscope. Then counting was carried out on the images. The number of individual cells, the number of aggregates as well as the number of cells in each aggregate was counted according to the image taken. The percentage of cells in aggregates was worked out based on those data.

5.10 Live and dead assay of the aggregates

Cell aggregates were loaded into 8-well chamber (LapTek; Nunc, Naperville, IL) and allowed to settle down. Remove the supernatant and wash the aggregates with cold PBS twice. Resuspend the cell aggregates in double staining solution (50 μmol/ml CTG and 25 μg/ml PI in Dulbecco's modified Eagle's medium). Incubate for 30 minutes at 37 ^{o}C. Wash the cell aggregates with PBS another twice. Fix the cell aggregates with 3.7% PFA for 30 minutes at 37 ^{o}C. Then wash with PBS twice and keep the cells in PBS until observation. Images of cell aggregates were taken by fluorescent confocal microscopy (Olympus Fluoview 500) at the magnification of 200X. CTG was observed at the wavelength of 488 nm and PI was at 543 nm. Images were analyzed by ImagePro Plus and viability of the aggregates was calculated based

on the number of live cells and dead cells which were counted separately by the software. The cell number and viability were calculated as the mean ± SEM of three experiments.

5.11 Culture of cell aggregation

After aggregation formation, chemical linkers were removed from supernatant. Culture medium was added into aggregates pellets. Resuspend the aggregates in Dulbecco's modified Eagle's medium with all the necessary additions as mentioned previously and culture them in 6-well tissue culture plate (NUNC, Denmark) on top of rotary machine (Spectra-teknik, US) at the speed of 50 rpm at 37 ^{o}C in a humidified environment with 5% CO_2.

5.12 MTS assay of the aggregates

Centrifuge aggregates at 800 g for 1 minute. Washed the pellet once with 1X PBS. Add 2 ml of 37 ºC CellTiter96 ® Aqueous One Solution Reagent (Promega, Madison, USA) (5 X dilution in PBS) into the tube, and transfer the solution into a 12-well plate. Incubate at 37 ºC for 3 hr in the dark on the rotary machine (Spectra-teknik, US). Transfer the solution into a 1.5 ml tube. Spin down at 4 ºC, 800 rpm for 2 minutes. Remove 100 µl of MTS solution into 96-well plate for absorbance reading at 490 nm using a Sunrise^{TM} microplate absorbance reader [Tecan, Switzerland]. Track the fate of chemical linker by fluorescence tagged PEI-2000-hy.

5.13 Track the fate of chemical linker by fluorescence tagged PEI-2000-hy

Aggregates were formed using 0.05 mM Oregon green-tagged PEI-2000-hy on HepG2 cells modified by 0.5 mM $NaIO_4$. Aggregates were dually stained with CellTracker™ Blue CMAC (CTB) and PI (50 μmol/ml CTG and 25 μg/ml PI in Dulbecco's modified Eagle's medium) to distinguish live and dead cells. Aggregates were cultured using the same way as mentioned above. Fluorescence of the linker can be directly observed using Olympus Fluoview 500 at the excitation wavelength of 488 nm. CTB was observed at the wavelength of 412 nm and PI was at 543 nm. Images of the fluorescence on different days were taken and exported to the image processing program of ImagePro Plus. The fluorescence intensity was calculated and divided by the number of cells in the image, resulting in the average fluorescence intensity per cell.

5.14 Micropatterning

The method mentioned in previous literature [210] was used to fabricate the reusable PDMS film with holes. The PDMS film are fabricated by casting and curing an elastomeric polymer, Sylgard 184 (Dow Corning, MI, US), against a photoresist micropatterned silicone master, which was kindly provide by Khong Yuet Mei and Ng San San from Graduate Program in Bioengineering at NUS. Both the diameter and depth of the circle is 50 μm. PDMS film was applied to the surface of tissue culture dish (NUNC, Denmark) and sealed simultaneously against the surface. Cells were

modified by 1 mM $NaIO_4$ at 4 ^oC in dark for 15 minutes and suspended in PBS followed by seeding onto the culture dish. PDMS film prevented the cells from attaching to the surface of dish except on the holes area. After a few minutes, the cells settled down into the holes. Then chemical linker solution (1mM PEI-2000-hy) was poured onto the surface and the cells were incubated in solution for 30 minutes. After the PDMS film was peeled off, circular multi-cellular structure remained on the dish and the structure was rather stable which could sustain the wash of PBS. Images were taken by confocal laser scanning microscopy (Olympus Fluoview 300).

5.15 Micromanipulation

Microcapillary with the tip diameter of around 10 μm is used to manipulate the HepG2 cells. Cells were modified by 1 mM $NaIO_4$ at 4 ^oC in dark for 15 minutes and suspended in 1 mM PEI-2000-hy. Cell suspension was transferred into a tissue culture dish (NUNC, Denmark). Micromanipulator (Eppendorf 5171, Hamburg, Germany) and CellTram (Eppendorf, Air) were used to manipulate the HepG2 cells into defined structure. Images were taken by confocal laser scanning microscopy (Olympus Fluoview 300).

5.16 Statistical analysis

Statistical calculations were carried out by function of T-Test in Microsoft Excell. All values are presented as the mean $\pm$standard deviation unless otherwise noted. Probability values $P<0.05$ were considered significant.

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