Development and study of biological coating materials for enhancement of host-graft interfacing affinity and biocompatibility

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**DEVELOPMENT AND STUDY OF BIOLOGICAL
COATING MATERIALS FOR ENHANCEMENT OF
HOST-GRAFT INTERFACING AFFINITY AND
BIOCOMPATIBILITY**

TAO CHAO

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

2020

**DEVELOPMENT AND STUDY OF BIOLOGICAL
COATING MATERIALS FOR ENHANCEMENT OF
HOST-GRAFT INTERFACING AFFINITY AND
BIOCOMPATIBILITY**

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School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2020

Statement of Originality

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The contributions of the co-authors are as follows:

Prof. Wang provided the initial review direction and edited the manuscript drafts.

I prepared the manuscript drafts. The manuscript was revised by Dr. Chuah and Prof. Xu.

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The contributions of the co-authors are as follows:

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I prepared the manuscript drafts. The manuscript was revised by Prof. Xu.

I co-designed the study with Prof. Wang and performed all the laboratory work at School of Chemical and Biomedical Engineering. I also analyzed the data.

Dr. Zhu assisted in synthesis of dopamine modified albumin.

Prof. Iqbal assisted in pathological analysis in Singapore General Hospital.

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SUMMARY

Extracellular matrix (ECM) based grafts are promising in tissue regeneration since ECM is a hospitable 3D microenvironment for cells to adhere and proliferate. However, there are two issues that need to be addressed for clinical translation of ECM based grafts including immunocompatibility of xenografts and interfacing affinity of grafts with host tissues. In this thesis, we proposed a novel and facile method by coating natural biological materials to furnish the grafts with functions such as immune evasion or adhesion.

For ECM based xenografts, autologous red blood cell (RBC) membrane or stabilized albumin coatings were investigated for attenuation of immune and inflammatory responses. Cell membrane is embedded with macromolecules such as sugars and proteins that govern cell functions or interactions including self-recognition. Autologous cell membrane coating could function as a disguise on xenografts which will be recognized as “self” by the host. Therefore, the host immune system will not attack the implants with autologous cell membrane as a disguise. RBC was chosen due to its abundance in human bodies. On the other hand, inflammation was initiated by absorption of proteins on foreign bodies. From this perspective, albumin could be used due to its passivation nature on xenografts to eliminate absorption of other proteins. In this case, the delivery of signals is hindered, which will affect the induction of immune and inflammatory responses. In this thesis, porcine-source living hyaline cartilage graft (LhCG), which contains mainly porcine

chondrocytes and ECM, and decellularized LhCG (dLhCG), which is composed of porcine derived ECM, were chosen as xenograft models. Both autologous cell membrane disguise coating and stabilized albumin passivation coating were stable on grafts and showed better immunocompatibility on cell, protein and gene levels with less inflammatory cells involving neutrophils, macrophages and lymphocytes recruited and lower inflammation-related gene expression level.

It is worth noting that in order to enhance adhesion of albumin on xenografts mentioned above, a mussel inspired adhesive, dopamine, was grafted on albumin. Dopamine is popular as a tissue adhesive, the catechol groups on which could form chelating bonds with metal ions. Inspired by this dopamine modified albumin coating on ECM based grafts, we considered developing an adhesive coating on ECM based grafts which could crosslink *in situ* with ferric ions for fixation with surrounding tissues after implantation. Therefore, dLhCG with dopamine based natural biological materials coatings was manufactured to address the interfacing affinity issue between dLhCG and cartilage. A macromolecules backbone was needed for the coating material to avoid the formation of a rigid crosslinking system and cytotoxicity caused by dopamine monomers. In this thesis, chondroitin sulfate (CS), a cartilage derived sulfated GAG, was chosen as backbone to fabricate dopamine modified CS (CSD) with no impurities introduced to joint. Dopamine modified serum albumin (BCD) was also chosen for comparison. Both dLhCG coated with CSD and dLhCG coated with BCD showed enhanced adhesive strength with cartilage after chelating with ferric ions *in situ* compared to dLhCG and further

potential in improving interfacing affinity of dLhCG with cartilage. However, CSD coated dLhCG is superior in stronger adhesive strength at optimized working concentration, better performances in cell migration and no impurities introduced in joint.

In summary, in this thesis, natural biological materials were investigated on ECM based grafts as functional coatings for enhanced immunocompatibility or interfacing affinity with surrounding host tissues.

Chapter 1 Introduction

1.1 Xenogeneic extracellular matrix (ECM) based implants for tissue engineering

Approaches based on natural extracellular matrix (ECM) have attracted more and more attention for tissue engineering[1–3]. ECM is the 3D microenvironment of resident cells in tissue, consisting of cell secreting structural and functional molecules[4,5]. It's reported that ECM based implants have been successfully used in regeneration of heart valves[6], abdominal walls[7] and even commercially used (*eg.* AlloDerm[®], Oasis[®])[8]. For example, cartilage ECM-derived particles[9] and decellularized ECM sheets[10] could be used as matrix for cartilage tissue regeneration. On the other hand, ECM derived from *in vitro* cultured cells has also drawn the attention of researchers. Chen *et al.* engineered an autologous ECM scaffold by culturing autologous cells onto a poly(lactide-*co*-glycolide) (PLGA) 3D template and subsequent decellularization and template removal[11]. Furthermore, they studied 3D stepwise chondrogenesis-mimicking ECM graft by controlling the stages of chondrogenic differentiation[12]. Our group has been studying porcine source ECM cartilage tissue engineering implants for years and developed Living Hyaline Cartilage Graft (LhCG) for cartilage tissue regeneration[13–16]. Decellularized LhCG (dLhCG) was further developed and studied[17–19]. Briefly, porcine hyaline chondrocytes were extracted from porcine cartilage and encapsulated with gelatin microspheres in alginate. The cells outgrew and filled up

the cavities, forming pure micro-tissues. After the 3D ECM was secreted and stable, the alginate hydrogel was removed by rinse in sodium citrate solution for a cartilaginous ECM and chondrocyte-based graft LhCG. LhCG was decellularized by physical, chemical and enzymatic treatments to harvest dLhCG. The cell-derived ECM is superior to synthetic biomaterials derived hydrogels to better mimic the healthy situation so that the cells are more willing to attach and proliferate on the lesion site[4]. Moreover, degradation rate of synthetic biomaterials scaffolds is difficult to control and scaffold residues can interfere cell-cell signaling and further tissue growth or morphogenesis[8].

Implants for tissue engineering could be categorized into xenografts, derived from heterologous animals, allografts, derived from another individual of the same species with a different genotype, autografts, derived from the same individual, and synthetic biomaterials, according to the source. However, in order to address the problem of limited autografts and allografts source, reduce surgery cost and relieve the pain of patients[20], more and more researchers have been focusing on xenografts such as implants based on ECMs secreted by *in vitro* cultured porcine or bovine cells[1,21]. Moreover, the fabrication of autografts or allografts might need a pre-surgery on patients which cause pain or damage to patients' tissues or organs. For example, autologous chondrocyte implantation (ACI), a scaffold-based delivery of chondrocytes to cartilage defects involving collecting and implanting autologous chondrocytes, needs two operations and long recovery time[20]. Therefore, xenogeneic source ECM based grafts are promising candidates for tissue

regeneration. However, there are still obstacles that hindered the clinical translation of xenografts. Immune and inflammatory responses elicited in recipient are the most severe issues that need to be addressed.

1.2 Inflammatory and immune responses elicited by xenografts

It is inevitable that xenografts accompany with concerns of their biocompatibility[22], which threatens long term survival of the implants and even the life of patients. The immune and inflammatory responses to xenografts were initiated in various of ways due to the diverse antigens present in grafts which led to recognition of foreign bodies by the host. Moreover, novel infectious organisms were possibly transmitted to recipient and brought more antigens[23]. Since tissue cellular antigenicity is one of the main stimulations of recipient immune responses, decellularization of xenografts has been widely studied as a simple approach toward clinical use. Decellularized porcine cornea[24,25], decellularized bovine pericardial scaffold[26,27] as well as other decellularized ECM based grafts[28,29] showed enhanced performance in acceptance by host immune system compared to fresh xenografts, some of which were even put into clinical trial[30]. However, besides tissue cellular antigenicity, acellular antigens in ECM, such as α -gal antigen[31] which binds with natural anti-gal antibodies in human, and antigens from homologous proteins[32] of diverse sequences, would also contribute to rejection from recipient and further failure of grafts. Other approaches for elimination of recipient immune responses included genetic modifications by

genome editing tools[33], glutaraldehyde cross-linking[32], removal of antigens[34,35] and post-surgery treatment[36]. However, current existing methods were complicated and might sacrifice ECM construct, leading to compromised regenerative effect. In this case, facile and effective alternatives for immune evasion of xenografts worth further investigating. Coating natural biological materials such as cell membrane is simple and safe with moderate operation environment and at the same time reserves nature of biological materials[37,38].

1.2.1 Cell membrane coating for immune evasion

Biocompatibility is a widely discussed issue not only in tissue engineering field but also in other fields such as drug delivery and biomedical devices[39–41]. Cell membrane coated on nanoparticles has shown its potential for evasion from immune system in drug delivery [33,34]. Cell membrane is a phospholipid bilayer embedded with biomacromolecules such as sugars and proteins which govern cell functions including interactions with biological materials or biomaterials. Therefore, the biological functions could be transferred and performed by coating cell membrane onto biomaterials. Cell membrane-derived materials have been utilized to increase biocompatibility[44]. CD47 is a “marker of self” glycoprotein which is expressed on cell membranes of all mammals. CD47 could interact with CD172a, also known as signal regulatory protein- α (SIRP α), on macrophages to inhibit phagocytes[45]. Furthermore, it is reported other membrane proteins on RBC membrane such as complement receptor 1 and membrane cofactor protein could defense against attack

from complement system[46]. Nowadays, cell membrane coated nanoparticles have been widely studied for drug delivery and shown potential in immune evasion[46–48]. In addition, cell membrane coating has been successfully applied onto nanomotors[49] and nanofiber scaffold[50]. In this thesis, we plan to transfer and investigate the immune evasion application of cell membrane coating to tissue engineering field.

1.2.2 Albumin coating for immune evasion

Albumin, the most abundant (35-50g/L) plasma protein, is produced in liver with a molecular weight of around 66.5kDa (584-590 amino acids), and plays an important physiological role in maintaining the colloid osmotic pressure of plasma and transporting endogenous compounds such as proteins, cholesterol and bile pigments[51,52]. Due to the advantageous intrinsic properties such as biocompatibility, biodegradability, low immunogenicity and nontoxicity[51,52], commercially available albumins such as bovine serum albumin (BSA) and human serum albumin (HSA) have been widely utilized for a variety of clinical and biomedical research applications[53]. It is worth noting that albumin is a potent stealth coating for immune evasion since albumin is capable of passivating biomaterial surfaces[54–56]. Albumin on the surface could block the other proteins, the absorption of which is the activation of blood coagulation and further inflammation[57]. Albumin has been successfully applied as a camouflage coating on a variety of nanoparticles such as virus-based nanoparticles[58 – 60] and DNA

origami[61]. For instance, Albumin was modified through Michael addition and conjugated to Tobacco mosaic virus (TMV) nanoparticles for immune evasion, at the same time exhibiting better pharmacokinetics than PEG coatings and increased circulation half-life of $t_{1/2}$ -100 min by 10 times[58 – 60]. BSA was conjugated to the second generation dendron (G2) through cysteine-maleimide bond, after which BSA-G2 was anchored to DNA origami via electrostatic interaction. The resulted BSA coating enhanced transfection rates by 2.5 folds, protecting DNA origami from DNase I endonuclease and evade from immune recognition[61]. Endogenous or exogenous albumin has also been either covalently or noncovalently coated on polymer[62], inorganic nanoparticles[63] and liposomes[64–67] to attenuate immune responses and prolong blood circulation time. Nowadays, albumin for immune evasion was mainly studied in drug delivery area. Therefore, we plan to transfer and investigate the immune evasion application to tissue engineering field. Our group has successfully synthesized dopamine modified albumin called BCD[68] which could be used in this thesis for albumin coating with dopamine, a mussel-inspired adhesive[69], enhancing adhesion on grafts.

1.3 Mussel-inspired adhesive for enhancement of interfacing affinity

1.3.1 Adhesives for biomedical applications

Existing biomaterials studied for adhesives covered proteins such as fibrin[70], gelatin[71,72], albumin[68], polysaccharides[73,74] such as dextran and chitosan and synthetic polymers such as poly (ethylene glycol) (PEG)[75–77], polyurethanes

(PU)[78] which are biocompatible with sufficient mechanical strength. Moreover, the adhesives should be capable of adhering to surrounding tissues by chemical bonds, interpenetration or physical linkage such as hydrogen bonds[79]. Binding of adhesives to tissues could be ascribed to covalent bonds formed, for example, between cyanoacrylate adhesives[80] or aldehydes on dextran[81] and amines on the lysine of the tissue proteins. And the adhesion could also be achieved by an interpenetrating network formed between the polymerized or crosslinked adhesives and extracellular proteins in the tissue[82]. The synthetic polymers or functionalized natural polymers with reactive groups could polymerize or crosslink *in situ* initiated by photo, heat, hydrolysis or crosslinkers and generate an interpenetrating network with tissue proteins, achieving the adherence with surrounding tissues. However, irradiation is usually needed for polymerization and crosslinking, resulting in limitation in applicable areas in the bodies. And the free radicals produced in polymerization would be harmful to healthy tissues.

Bio-inspired adhesives including mussel-inspired, Gecko-inspired and endoparasitic worm inspired adhesives have been paid attention to in recent years[79]. Nanopillars resembling Gecko feet were fabricated to achieve adhesion by enlarging contact area as well as van der Waals forces[83]. In addition, microneedle tissue adhesives were studied inspired by endoparasitic worm proboscis[84]. The association of catechol and amine was believed to contribute to the adhesion of mussel, which inspired synthesis of various kinds of catecholamine. Mussel-inspired adhesives have drawn more and more attention due to their

biocompatibility, strong and non-selective adhesion to various surfaces.

1.3.2 Polydopamine-based adhesives for biomedical applications

Among mussel-inspired catecholamines, dopamine was widely used due to its low cost and simplicity. Although not well established, according to existing theories, the cohesion of dopamine originated from self-polymerization/oxidation to polydopamine in mild alkaline environment initiated by oxygen[85]. The adhesion was widely believed to be resulted from π - π stacking, van der Waals forces and hydrogen bonding[86]. Moreover, it is reported that there formed chelating bonds between catechol groups on polydopamines and metals or metal oxides that would strengthen the adhesion[86].

Based on its adhesive property, polydopamine could work as coatings on nanoparticles, nanofibers, graphene oxide or grafts for immobilization of ions or biomolecules or other modifications to be further used in biosensing[87], drug/gene delivery[88], antifouling field[89] and tissue engineering[90]. Besides, polydopamine could self-assemble into micro/nanospheres for bioimaging, drug delivery or cancer photothermal treatment[91]. Particularly, application of polydopamine in tissue engineering was mainly focused on enhancing healing by enhanced cell adhesion and proliferation[92], improving tissue regeneration by supporting cell proliferation and differentiation[93], and bone tissue engineering based on its capability of binding with calcium ions[94] and attachment of bioactive molecules[95].

Moreover, polydopamine is a promising candidate as a tissue adhesive material. Our group has developed two kinds of dopamine-functionalized tissue bio-adhesives with bovine serum albumin (BCD)[68] and chondroitin sulfate (CSD)[96] as backbones, respectively. The oxidation of dopamine in mild basic environment and chelating bonds between catechol groups and ferric ions furnished them with adhesive nature. The tissue bio-adhesives showed sound seroma prevention and liver hemorrhage repair with excellent biocompatibility and potential in diverse surgical applications such as incision closure. However, polydopamine based adhesives were mainly investigated for wound closure currently.

1.4 Host-graft interfacing affinity

Integration or fixation of grafts or devices to tissues is a big challenge for successful implantation from a clinical perspective. For example, it is reported that dislocation of implanted mesh in a preperitoneal mesh hernia repair would remarkably increase the recurrence rate[97]. Furthermore, incomplete immobilization in transplantation will result in graft failure[98]. Unlike wound closure, it is crucial to preserve porous structures of the implanted grafts when adhesives for fixation of grafts are applied. Therefore, current commercially-available and commonly used fibrin glue, TISSEEL, with a mechanism inspired by clotting with two components, thrombin and fibrinogen[99], might not be sufficient for the fixation of ECM based grafts.

Various kinds of absorbable or non-absorbable sutures[100–105], staples[106]

and adhesive tapes were available for fixation of grafts or medical devices such as skin grafts, vascular grafts and cardiac grafts. However, sutures and staples are limited in damaging surrounding tissues, eliciting inflammatory responses, scar tissue formation and possible anesthetics administration[79]. Moreover, sutures and staples are not applicable for the porous, sponge-like ECM based grafts due to the damage caused to the structure and challenge of the surgeon skills. Adhesive tapes are flawed in limited application in wet and hairy areas[107].

Grafts or devices fixation using adhesives or glues has also been reported. For example, a swellable polystyrene-*block*-poly(acrylic acid) microneedle adhesive was designed for fixation of skin grafts by mechanical interlocking with tissues[84]. In addition, adhesives based on chitosan[108] or fibrin[70] were also developed for skin graft fixation. Mussel adhesive protein or phenolic-derived adhesives were reported for fixation in cartilage[109,110] or bone repair surgery[111,112]. Moreover, self-adhesive grafts such as a polydimethylsiloxane-based soft skin adhesive elastomeric wound scaffold[113], or devices such as permanently implantable adhesive bioelectrodes based on graphene 3D printed onto resorbable polyester substrates[114] were investigated.

ECM-derived xenografts are proved to be desirable tissue engineering scaffolds with a porous structure, a hospitable microenvironment for cells to attach and proliferate and abundant donating sources. However, in addition to inflammatory and immune responses, interfacing affinity of grafts with surrounding

host tissues is a barrier for their clinical translation[115].

1.5 Objectives and organization of this dissertation

Xenogeneic ECM derived grafts are reported to be promising in tissue engineering but prone to elicit inflammatory and immune responses which interferes their translation to clinical use. Inspired by development in immune evasion in drug delivery, we considered transferring cell membrane or albumin coatings in tissue engineering on xenografts for suppression or alleviation of immune responses.

On one hand, red blood cell (RBC), due to its abundance in human bodies and accessibility, is believed to be an appropriate choice for the autologous cell membrane source. The basic idea for clinical use is collecting blood from the patients before surgery, after which the RBCs are separated and lysed for cell membrane which is further coated as a disguise onto xenografts for implantation, so that the xenografts could be recognized as autografts.

On the other hand, passivation property of albumin was taken advantage of and transferred to tissue engineering field. A facile method for evasion from immune rejection elicited by xenografts was developed through coating albumin. Adhesion of albumin onto grafts were achieved by introducing dopamine, a mussel inspired adhesive which would polymerize under mild alkaline environment, via chemical reaction.

Furthermore, inspired by dopamine modified albumin coated xenografts and considering chelating bonds could be formed between catechol groups on polydopamine and metal ions to provide affinity, whether dopamine modified polymer coating like BCD coating could further act as a glue between graft and tissues by adding metal ions has drawn our interests. Two kinds of dopamine modified macromolecules (chondroitin sulfate-based CSD and albumin-based BCD) were applied, respectively, as additives on grafts to achieve further adhesion between graft and surrounding host tissues. Ferric ions were added *in situ* to form chelating bonds with catechol on dopamine for enhancement of affinity.

In summary, we applied two kinds of natural biological materials coatings, cell membrane coating and stabilized albumin coating, for attenuation of immune and inflammatory responses elicited by xenografts. Inspired by BCD coated ECM based grafts, we further investigated two kinds of dopamine modified macromolecules coating on ECM based grafts for enhanced interfacing affinity between grafts and host tissues.

This dissertation consists of five chapters which were summarized as follows:

Chapter one reviews current progress and existing problems in ECM based implants, especially immune and inflammatory responses elicited by xenografts and host-graft interfacing affinity. We herein highlight the potential approaches of coating natural biological materials, especially cell membrane coating and stabilized albumin coating on xenografts for immune evasion. And we further

demonstrate the advantages and potential of dopamine-based adhesives in enhanced interfacing affinity between grafts and host tissues. The objectives and organization of this dissertation are presented.

Chapter two describes investigations on the immune evasion effects of autologous cell membrane coating on xenogeneic ECM based tissue engineering grafts. Porcine sourced LhCG and dLhCG established by our group were chosen as model xenografts. Cell membrane is embedded with membrane proteins for regulation of cell interactions including self-recognition and could work as a disguise on xenografts.

Chapter three describes investigations on the immune evasion effects of stabilized albumin coating on xenogeneic ECM based tissue engineering grafts. Porcine sourced LhCG and dLhCG established by our group were chosen as model xenografts. Albumin was firstly modified by dopamine to enhance its adhesion on graft surfaces and could work as a stealth coating on grafts through passivation of the biomaterial surfaces.

Chapter four extends the dopamine modified macromolecules coating to enhanced interfacing affinity between ECM based grafts and surrounding host tissues. It compares two kinds of dopamine-based adhesives (chondroitin sulfate-based CSD and albumin-based BCD) for improvement of interfacing affinity between dLhCG and host tissues. CSD and BCD could coat on dLhCG at the first place and subsequently bind with ferric ions *in situ* to form chelating structures.

Chapter five summarizes the current work in this dissertation and suggest possible future work.

**Autologous cell membrane coatings on tissue engineering
xenografts for suppression and alleviation of acute host
immune responses**

Chao Tao, Xiaolei Nie, Wenzhen Zhu, Javed Iqbal, Chenjie Xu, Dong-An Wang

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Chapter 2 Autologous cell membrane coatings on tissue engineering xenografts for suppression and alleviation of acute host immune responses

2.1 Introduction

As is mentioned in chapter 1, xenografts were derived to address the shortage of human donated organs or other allografts or autografts. However, it is inevitable xenografts accompany with concerns about their immunocompatibility due to both cellular and acellular antigenicity[22]. Autografts, which will be recognized as self after implantation, are believed to be less likely to induce immune and inflammatory responses than allografts and xenografts[116,117].

Cell membrane is embedded with membrane proteins for regulation of cell interactions including self-recognition and potent in reducing foreign body rejections. Therefore, biomaterials with cell membrane coating on surfaces have potential to suppress or alleviate immune and inflammatory responses. Cell membrane coating has been successfully applied on nanoparticles or nanofibers for immune evasion. Therefore, in this study, the immune evasion property of cell membrane was transferred to tissue engineering field.

In this chapter, a novel and facile method for evasion from immune system was developed by coating autologous red blood cell (RBC) membrane as a disguise on xenogeneic extracellular matrix (ECM) based tissue engineering graft surface.

Porcine source living hyaline cartilage graft (LhCG), which contained porcine chondrocytes and ECM, and decellularized LhCG (dLhCG), which was mainly composed of porcine derived ECM, were chosen as the xenograft models. The basic idea is collecting blood from patients firstly for lysis of autologous red blood cell membrane, followed by coating autologous RBC membrane onto xenografts for implantation relying on the absorption of cell membrane on solid surface driven by high surface energy of cell membrane vesicles. RBC was chosen as cell membrane source because it is abundant and easily accessible. The experimental design of chapter 2 was illustrated in [Figure 2.1](#). Briefly, LhCG and dLhCG were derived from chondrocytes of porcine cartilage and coated with RBC membrane collected from rats, after which the grafts were implanted into rats' omentum for investigations on immunocompatibility. Omentum was chosen for its sensitivity to foreign bodies. Autologous RBC membrane would work as a disguise on LhCG and dLhCG and help xenografts to be identified as autografts.

Figure 2.1 Schematic illustration of the design of RBC membrane coated xenografts[118].

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2.2 Materials and methods

2.2.1 Materials

3,3'-dioctadecylox-acarbocyanine perchlorates (DiO) were purchased from Sigma Aldrich and dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) before use. Porcine chondrocytes for LhCG and dLhCG fabrication were extracted from commercially available porcine bone from market. Whole blood for RBC membrane extraction was collected from rat's tails.

2.2.2 Derivation of cell membrane vesicles

RBC membrane vesicles were prepared according to a published protocol[42]. Briefly, whole blood was centrifuged at 800×g at 4°C for 5min, after which the serum and fluffy precipitation on the surface was carefully removed. The collected RBCs were washed with cold 1×phosphate buffered saline (PBS, Gibco, USA) for three times. Then the RBCs were suspended in 10mmol/L Tris-HCl buffer (pH=7.4, 1st Base Pte Ltd, Singapore) at 4°C for 1h for hemolysis treatment. The white pellet (RBC membrane) was collected after centrifuge at 800×g for 5min and washed with the hypotonic buffer. The finally collected RBC membrane was sonicated for 5min with a sonicator (XUB5, Chemoscience Pte Ltd, Singapore) at 60Hz and 250W to promote the self-assembly of cell membrane before coating.

2.2.3 Characterization of cell membrane vesicles and coatings

Dynamic light scattering (DLS) was performed on a Malvern Nano-ZS for

measurement of RBC membrane vesicles' size and zeta potential. Samples were prepared by suspending cell membrane vesicles with deionized (DI) water.

RBC membrane vesicles were suspended and diluted in DI water and spread on a slide of silicon wafer. The samples were dried at room temperature and scanned by atomic force microscopy (AFM) (CSPM5500A, Being NanoInstruments Ltd., China).

Attenuated total reflectance-fourier transform infrared reflection (ATR-FTIR) (Smart iTR, Thermo Fisher Scientific Inc., Singapore) was carried out for chemical structure of cell membrane components. Cell membrane sample was freeze-dried for ATR-FTIR measurement.

For verification of the feasibility of coating formation, cell membrane suspended in DI water was coated onto silicon (Si) (SUNSON, China) and coverslip (CellPath Ltd, UK) surfaces by drip coating then drying overnight at room temperature. Water contact angle was conducted on a Kruss DSA25 Contact Angle Analyzer with a 5 μ L DI water drop. Moreover, RBC membrane was also coated onto natural cartilage surface and characterized for water contact angle. Five samples were measured and averaged for the final water contact angle value.

2.2.4 Cell membrane coating on grafts

LhCG and dLhCG were engineered based on an established protocol in our previous publications as model xenografts[13–16]. dLhCG is a promising candidate

for cartilage tissue engineering[17–19]. LhCG was also chosen as a control as well as a template for illustration of the RBC membrane effect. Briefly, porcine hyaline chondrocytes were extracted from porcine cartilage and encapsulated with gelatin (gelatin from bovine skin, Sigma Aldrich, USA) microspheres in alginate (Alginic acid sodium salt, Sigma Aldrich, USA). The cells outgrew and filled up the cavities, forming pure micro-tissues. After the 3D ECM was secreted and stable, the alginate hydrogel was removed by rinse in sodium citrate (Sigma Aldrich, USA) solution (55mM in 0.15M NaCl) for a cartilaginous ECM and chondrocyte-based graft LhCG. LhCG was decellularized by sequential physical, chemical and enzymatic treatments to harvest dLhCG.

LhCG and dLhCG samples were rinsed in RBC membrane vesicles which were suspended in Dulbecco's Modified Eagle's Medium (DMEM) or 1×PBS, respectively for 1h for coating, followed by rinse in pure culture medium or DI water respectively for 30min to wash the uncoated cell membrane. A certain amount of whole blood was used to extract cell membrane components to coat LhCG and dLhCG which is around 0.5cm in diameter. Finally, we got the cell membrane coated LhCG (CM-LhCG) and cell membrane coated dLhCG (CM-dLhCG).

RBC membrane concentration, or whole blood volume applied, was optimized before coated on LhCG and dLhCG for further *in vivo* studies. It was achieved by evaluating effective RBC membrane content in CM-dLhCG and CM-LhCG with RBC membrane coating collected from gradient whole blood volume. For dLhCG,

gradient volumes of whole blood (0.2mL, 0.4mL, 0.6mL, 0.8mL, 1mL) were used for lysis of RBC membrane respectively, after which the collected RBC membrane was diluted in 1mL DI water and coated on dLhCG with a diameter of ~0.5cm. Then CM-dLhCG was digested by papain (1mg/mL in DI water) at 60°C overnight. Phosphorus content in the solution, which reflects RBC membrane coating contents, was tested by inductively coupled plasma mass spectrometry (ICP-MS) (Elan-DRCe, PerkinElmer SCIEX, USA). dLhCG was also digested and tested as a control. For LhCG, gradient volumes of whole blood (0.2mL, 0.4mL, 0.6mL, 0.8mL, 1mL) were collected and used for lysis of RBC membrane respectively, after which the collected RBC membrane was diluted in 1mL culture medium and coated on LhCG with a diameter of ~0.5cm. Then CM-LhCG was stained by CD47 monoclonal antibody (miap301), FITC (anti-CD47-FITC, Invitrogen) after blocked by 5% bovine serum albumin (BSA). CD47 in RBC membrane could be stained by anti-CD47-FITC and emit green fluorescence after excited by blue light. The fluorescence intensity was measured by Image J based on the fluorescent images of each group. LhCG was also stained and measured as a control.

2.2.5 Visualization and detection of cell membrane coating on grafts

For visualization of cell membrane coatings on LhCG and dLhCG, DiO, a lipophilic tracer, was used to label cell membrane and observed under a fluorescent microscope (Olympus IX71S1F-3, Japan). The dyed samples were embedded in paraffin (Surgipath[®] Paraplast[®], Leica, USA) and sectioned on a slicer (RM2255,

Leica, USA) before observation.

X-ray photoelectron spectroscopy (XPS) was operated on a Kratos AXIS Supra spectrometer with a bandpass energy of 40eV to detect carbon (C), oxygen (O), nitrogen (N), phosphorous (P) and sulfur (S). The high-resolution spectrum of C was fitted by Origin 9.0 software. dLhCG and CM-dLhCG samples were freeze-dried (BT48, Millrock Technology Inc, USA), while LhCG and CM-LhCG samples were fixed in 2.5v/v% glutaraldehyde (Sigma Aldrich, USA), dehydrated in gradient ethanol, followed by dried in a critical point dryer (K850, Quorum Technologies Inc., England).

2.2.6 Stability of cell membrane coatings

dLhCG is decellularized ECM consisting of glycosaminoglycan (GAG) and glycoproteins. Cell membrane is a phospholipid embedded with membrane proteins. Therefore, almost all phosphorus come from cell membrane components. In this case, the phosphorus content could reflect the cell membrane content on the surface. Phosphorus (P) of CM-dLhCG samples was measured by ICP-MS as quantification of cell membrane coatings. First, CM-dLhCG was digested by papain (1mg/mL in DI water, Sigma Aldrich, USA) at 60°C overnight. Then the solution was diluted 50 times in DI water with 1% HCl. CM-dLhCG samples, which were rinsed in DI water for 1d, 7d, 14d and 28d, were measured to investigate the stability of the cell membrane coatings. DI water was chosen as the medium to avoid the interference of possible P content in the solution. P content of dLhCG was also measured.

For LhCG samples, RBC membrane dyed with DiO beforehand during the extraction process (CM-DiO) was coated onto LhCG and fixed in 4% paraformaldehyde on 1d, 7d, 14d and 28d, respectively. The fixed samples were further embedded in paraffin, sectioned into 10µm thick and observed under fluorescent microscope.

2.2.7 Morphology of grafts

The morphology of LhCG, CM-LhCG, dLhCG and CM-dLhCG was observed under scanning electronic microscopy (SEM) (JEOL JSM 6700F, Japan). dLhCG and CM-dLhCG samples were freeze-dried, while LhCG and CM-LhCG samples were fixed in 2.5v/v% glutaraldehyde, dehydrated in gradient ethanol, followed by dried in a critical point dryer. All samples were coated with platinum by sputtering for 150s at 10mA (JFC-1600, JEOL Asia Pte. Ltd., Japan) before SEM observation.

2.2.8 In vivo rat omentum implantation model

All animal experiment protocols have been approved by Institutional Animal Care and Use Committee (IACUC), Singapore according to the local law.

The *in vivo* studies on immune and inflammatory responses were performed on sixty-four Sprague Dawley female rats (n=4, 250-300g) by an omentum implantation model. Omentum was chosen due to its sensitivity to foreign bodies. If the grafts could survive in omentum, it will be more convincing for the grafts not to elicit immune responses in other organs or tissues, especially the immune

privileged sites such as skin and cartilage. All the rats were randomly divided into eight groups. Two groups of rats were implanted with dLhCG (Xeno-d) and LhCG (Xeno), respectively. Rats implanted with CM-dLhCG of which RBC collected from other or the same rats were labeled as Allo-d and Auto-d, respectively. Similarly, rats implanted with CM-LhCG of which RBC collected from other or the same rats were labeled as Allo and Auto, respectively. Then the grafts (~0.5cm in diameter) were washed with sterile PBS before embedded into rats' omentum. Before surgery, blood was collected from tails of rats and coated onto dLhCG and LhCG for the fabrication of CM-dLhCG and CM-LhCG samples. An optimized amount of whole blood was used for harvest of cell membrane coating for one graft with size of around 0.5cm in diameter. During the surgery, an approx. 2cm incision was firstly made on the rats' abdomen. Two grafts of the same group were then embedded into each rat before closure of the incision with 4-0 Nylon sutures and stainless-steel clips. On post-surgical day 1 and day 14, after sacrifice of the animals, the two implanted grafts in each rat, as well as the surrounding tissues were collected from the surgical sites, and kept in 4% paraformaldehyde (Sigma Aldrich, USA) or liquid nitrogen respectively for the following experiments. Samples collected on day 1 were labeled as Xeno1, Xeno-d1, Allo1, Allo-d1, Auto1 and Auto-d1, respectively. Similarly, samples collected on day 14 were labeled as Xeno14, Xeno-d14, Allo14, Allo-d14, Auto14 and Auto-d14, respectively. Natural xenogeneic cartilage slices with 0.5 cm in diameter were punched from porcine articular cartilage, decellularized, embedded into rats and collected on day 1 (PC1)

and day 14 (PC14) post-operation according to the same protocol mentioned above as a positive control. Natural autologous cartilage was obtained from rat ear, embedded into the same individual and collected on day 1 (RE1) and day 14 (RE14) post-operation according to the same protocol mentioned above as a negative control.

2.2.9 Histological and immunohistochemical analysis

The collected samples fixed in 4% paraformaldehyde were embedded in paraffin and sectioned into 10µm thick on a slicer (RM2255, Leica, USA). The sectioned samples were deparaffined and rehydrated before staining.

Hematoxylin and Eosin (H&E) staining (Sigma Aldrich, USA) was carried out according to a standard protocol of the manufacturer. Immune and inflammatory responses on cell level were evaluated based on numbers of inflammatory cells by scoring[119–122] according to a five-point grading scheme by a pathologist who was blinded to experimental design and groups.

Immunohistochemistry (IHC) staining of MPO, CD68 and CD3 was performed with UltraVision™ Quanto Detection System HRP DAB kit (Thermo Fisher Scientific, UK) based on the standard protocol of the manufacturer for detection of neutrophils, macrophages and T cells, respectively. The details of antibodies were listed in [Table S2.1](#). The slides were finally mounted with Surgipath® Micromount (LeicaBiosystems, USA).

2.2.10 Gene expression

Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to evaluate inflammatory responses on a gene level by detecting gene expression of inflammation-related cytokines. The target gene expression was also confirmed by agarose gel electrophoresis.

Total RNA of the graft with surrounding tissues was isolated by TRIzol[®] reagent (Life Technology, USA) from samples kept in liquid nitrogen according to the manufacture's protocol and the RNA concentration was tested by NanoDrop 2000c (ThermoFisher Scientific, USA). Then total RNA was reverse transcribed to cDNA. A 12µL reaction volume containing 500ng of RNA, 1µL Oligo dT was used for first-strand cDNA synthesis reaction. The mixture was heated to 70°C for 5min and quickly cooled on ice for 15min. Afterwards, 5×reaction buffer (5µL), dNTP Mix (1.25µL, 10mM), M-MLV RT RNase (H-) Pt Mutant (0.5µL) and DI water were added. All the reverse transcription reagents were purchased from Promega, USA. The reaction mixture was incubated at 40°C for 10min, 50°C for 50min and then 70°C for 15min, after which the first-strand cDNA was generated.

qRT-PCR was conducted by SYBR Green chemistry on a CFX Connect Real-Time PCR System (BIO-RAD, USA) with a 20µL reaction mixture containing 1µL cDNA, 1.2µL primers, 10µL SsoAdvanced[™] Universal SYBR[®] Green Supermix (BIO-RAD, USA) and DI water. The primers used for qRT-PCR were purchased from Integrated DNA Technologies, Singapore and listed in [Table S2.2](#). The qRT-

PCR results of target genes were normalized against a housekeeping gene (*GAPDH* gene) and calculated by the comparative $2^{-\Delta\Delta C_T}$ method.

On the other hand, cDNA was amplified using Taq DNA polymerase (NEW ENGLAND BioLabs, England) for semi-quantitative PCR. Thermal cycle was performed as follows: 95°C for 30s followed by 30 cycles of 95°C for 30s, annealing at 55°C for 30s, 68°C for 30s, and a final extension at 68°C for 5min. The products were visualized on 1.5% agarose gels in Tris-Borate-EDTA Buffer (Vivantis Technologies, Malaysia) with SYBR[®] Safe DNA gel stain (Invitrogen, USA).

2.3 Results

2.3.1 Derivation of cell membrane vesicles and coatings

First of all, cell membrane was extracted and cell membrane vesicles were characterized by DLS and AFM. The chemical structure of the extracted cell membrane was confirmed by ATR-FTIR.

As shown in ATR-FTIR spectrum of RBC membrane (Figure 2.2a), peaks at 3282cm^{-1} and 1635cm^{-1} belong to C-H stretching vibration and C=O stretching vibration, respectively. The peaks of P=O stretching vibration (1224cm^{-1}) and P-O-C stretching vibration (1081cm^{-1}) belong to the hydrophilic phosphate head of phospholipids and confirm the successful extraction of cell membrane.

According to DLS results, the cell membrane vesicles were $52.32\pm 3.77\text{nm}$ in size with a PDI of 0.61 ± 0.04 (Figure 2.2b), which is in consistent with the AFM

results (Figure 2.2c). And the zeta potential was $-28.97 \pm 0.82 \text{ mV}$, in accordance with the literature[123].

Figure 2.2 (a) ATR-FTIR spectrum of RBC membrane, (b) DLS result and (c) AFM images of RBC membrane vesicles, water contact angle of (d) coverslip and (e) Si surfaces respectively with and without RBC membrane coating[118]. Reproduced with permission from © 2020 Elsevier Ltd.

In order to confirm the feasibility of cell membrane coating formation, RBC membrane was firstly coated onto coverslip and Si surfaces, respectively. Water contact angles were measured and shown in Figure 2.2d and Figure 2.2e. Coverslip

has a superhydrophilic surface whose water contact angle is below 5° . Water contact angle of coverslip increased to $43.4^\circ \pm 0.4^\circ$ with RBC membrane coating on the surface (Figure 2.2d). In addition, Si surface became more hydrophilic with water contact angle decreasing to $38.1^\circ \pm 1.4^\circ$ from $59.4^\circ \pm 1.8^\circ$ after coated with RBC membrane (Figure 2.2e). The above water contact angle results confirmed successful coating formation on the two different kinds of surfaces.

Figure 2.3 Water contact angles of cartilage surface and cell membrane coated cartilage (CM-cartilage) surface[118]. Reproduced with permission from © 2020 Elsevier Ltd.

Contact angle was measured as an indicator of surface energy. LhCG and dLhCG were derived from extracted porcine chondrocytes from porcine bone and reconstructed the microenvironment of cartilage. The porous and rough surfaces of dLhCG and LhCG were not suitable for contact angle test and would lead to inaccurate results. Therefore, natural porcine cartilage was used for measurement of water contact angle instead. Water contact angle of cartilage with cell membrane coating surface decreased from $71.3^\circ \pm 1.0^\circ$ to $37.6^\circ \pm 2.0^\circ$ (Figure 2.3), indicating relatively high surface energy cell membrane vesicles adhered to a more hydrophobic surface. After interaction mechanism was confirmed, RBC membrane was further coated onto dLhCG and LhCG surfaces, respectively.

2.3.2 Characterization of cell membrane coating on grafts

The visualization and characterization of RBC membrane coating on grafts were achieved by fluorescent microscopy and XPS. DiO, a lipophilic tracer which will emit green fluorescence when conjugated with cell membrane and excited by blue light, was used to label RBC membrane in advance. Fluorescent image ([Figure 2.4a and 2.4b](#)) confirmed the successful coating of cell membrane.

Figure 2.4 Fluorescent image of (a) CM-dLhCG and (b) CM-LhCG with DiO-dyed RBC membrane coating[118]. Reproduced with permission from © 2020 Elsevier Ltd.

Elemental information on the surface within a depth of 10nm could be detected by XPS. In order to investigate the difference in surface chemistry among the four kinds of grafts, XPS was carried out ([Figure 2.5](#)).

Table 2.1 Surface elemental composition of dLhCG, CM-dLhCG, LhCG and CM-LhCG[118].

	C (%)				O (%)	N (%)	P (%)	S (%)
	C-C/C-H	C-N/C-S/C-O	C=O	-O-C=O				
dLhCG	17.8	16.0	4.0	3.9	32.0	22.3	0.7	3.3
CM-dLhCG	29.7	19.9	9.2	0.7	18.3	17.3	2.4	2.5
LhCG	18.0	15.5	5.0	2.8	28.9	21.5	2.9	5.4
CM-LhCG	15.7	17.6	6.4	3.5	28.6	20.0	3.4	4.8

Figure 2.5 (a)XPS spectra of dLhCG, CM-dLhCG, LhCG and CM-LhCG, P 2p and S 2p spectra were zoomed in and shown by side, XPS spectrum of C 1s for (b) dLhCG, (c) CM-dLhCG, (d) LhCG and (e) CM-LhCG[118]. Reproduced with permission from © 2020 Elsevier Ltd.

dLhCG which had been decellularized constituted primarily glycoproteins and GAG [124], while cell membrane was mainly made of phospholipids. Therefore, LhCG and CM-LhCG had a relatively higher level of P content, which came from cell membrane, compared to dLhCG and CM-dLhCG (Table 1). As shown in Figure 2.5a, P 2p peak at around 132 eV of dLhCG was almost invisible, indicating an absence of cell membrane in dLhCG. Moreover, P content of dLhCG increased from 0.7% to 2.4% after coated with RBC membrane (Table 1), further showing

successful coating of RBC membrane on dLhCG surface. There was also a slight increase in P content of CM-LhCG (3.4%) compared to LhCG (2.9%), indicating successful coating on LhCG. According to our previous studies[19], there is a loss of GAG during decellularization of LhCG, resulting in a decrease of S content in dLhCG (3.3%) compared to that of LhCG (5.4%), which is also reflected in S content in CM-dLhCG (2.5%) and CM-LhCG (4.8%).

Furthermore, the high resolution of C 1s spectra were fitted with peaks at 284.6eV (C-C, C-H), 286.0eV (C-N, C-S, C-O), 287.8eV (C=O) and 288.7eV (-O-C=O) and shown in [Figure 2.5b-e](#).

2.3.3 Morphology of grafts

SEM was used to detect the morphology of the grafts with and without cell membrane coatings, respectively.

Figure 2.6 SEM images of (a) dLhCG, (b) CM-dLhCG, (c) LhCG and (d) CM-LhCG[118].

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As shown in Figure 2.6, the coating of cell membrane did not change morphology of dLhCG or LhCG (Figure 2.6a and 2.6b). The coating process did not destroy the porous structure of dLhCG. For LhCG (Figure 2.6c) and CM-LhCG (Figure 2.6d) samples, chondrocytes were observed aggregating under SEM in the dried samples. LhCG and CM-LhCG samples showed a relatively densely packed structure compared to the porous structure of dLhCG (Figure 2.6a) and CM-dLhCG (Figure 2.6b).

2.3.4 Optimization of RBC membrane content

RBC membrane was collected from whole blood. And the collected RBC membrane content was proportional to whole blood volume. RBC membrane content used for rinsing and coating dLhCG and LhCG was optimized through detection of effective RBC membrane content coated on dLhCG and LhCG.

Figure 2.7 P content in dLhCG and CM-dLhCG with RBC membrane coating collected from 0.2mL, 0.4mL, 0.6mL, 0.8mL and 1mL whole blood, respectively.

As shown in Figure 2.7, there was an increase in effective RBC membrane

coating content as whole blood used increased from 0 to 0.6mL. However, effective RBC membrane content on dLhCG almost remained the same with 0.6mL and above whole blood used for RBC membrane coating. Therefore, RBC membrane lysed from 0.6mL whole blood was the optimized value for coating dLhCG with a diameter of ~ 0.5 cm.

Figure 2.8 Fluorescence intensity of anti-CD47-FITC stained LhCG and CM-dLhCG with RBC membrane coating collected from 0.2mL, 0.4mL, 0.6mL, 0.8mL and 1mL whole blood, respectively.

As shown in [Figure 2.8](#), fluorescence intensity increased as RBC membrane content increased but reached a platform when the whole blood volume used for RBC membrane collection was 0.6mL and above. It indicates there was no increase in effective RBC membrane content on LhCG with a diameter of around 0.5cm after whole blood volume for RBC membrane collection reached 0.6mL. Therefore, RBC membrane lysed from 0.6mL whole blood was the optimized value for coating LhCG with a diameter of ~ 0.5 cm.

2.3.5 Stability of cell membrane coating on grafts

After the graft is implanted into the injured sites, it takes time for the cells to migrate in and grow. So how long cell membrane would stay on the graft, that is, the stability of cell membrane coating is a following issue that should be taken into consideration.

Figure 2.9 (a) ICP-MS results of P in CM-dLhCG rinsed in DI water for 1d, 7d, 14d and 28d, respectively and that of dLhCG; (b) fluorescent intensity of CM-DiO coated LhCG and fluorescent image of CM-DiO coated LhCG for (b) 1d, (c) 7d, (d) 14d and (e) 28d, respectively[118]. Reproduced with permission from © 2020 Elsevier Ltd.

For CM-dLhCG samples, ICP-MS was used to detect the P element content to

reflect the cell membrane content and stability[125,126]. dLhCG, consisting mainly of glycoproteins and GAG, barely contains phosphorus. However, cell membrane was mainly made of phospholipids. Therefore, almost all phosphorus is supposed to come from cell membrane coatings in CM-dLhCG. In this case, the amount of cell membrane coatings could be reflected by P content detected by ICP-MS, which could be accurate to ppt level. As shown in [Figure 2.9a](#), the cell membrane coating was quite stable and no obvious decrease in P content was observed from 1d to 28d. This implied the strong affinity between cell membrane coatings with dLhCG surface and guaranteed the long-term use of graft *in vivo*. This high affinity might also result from the high surface energy of cell membrane vesicles in aqueous environment. The cell membrane vesicles were so unstable that the adherence to graft surface was irreversible.

For CM-LhCG, RBC membrane was dyed by DiO beforehand (CM-DiO) during extraction process and detected by fluorescent microscope in order to investigate its stability. According to [Figure 2.9c-f](#), RBC membrane was detected coated on the surface of LhCG as well as adhering to some chondrocytes surface on day 1 and day 7. On day 14 and day 28, though the fluorescence was not as obvious, there was still cell membrane coating detected on surface of LhCG. According to fluorescent intensity measured by Image J ([Figure 2.9b](#)), the fluorescent intensities of CM-DiO coated LhCG on day 1, 7, 14 and 28 were all between 30 to 35. Only a slight decrease was observed of CM-LhCG 14d and CM-LhCG 28d compared to CM-LhCG 1d and CM-LhCG 7d.

2.3.6 Pathological analysis

Figure 2.10 (a) Photos of samples collected from sacrificed rats. The scale bar is 0.5cm, and quantitative assessment of immune response according to numbers of inflammatory cells including (b) neutrophils, (c) macrophages, (d) lymphocytes, and (e) the 5-point grading system based on which the samples were evaluated. 0=no inflammatory cells were seen, 1=singly dispersed sparse inflammatory cells visible in high power field, 2=infiltration of inflammatory cells visible in at least one low power field, 3=abundant inflammatory cells present in every low power field, 4=samples were badly infiltrated by inflammatory cells observed in low power field (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)[118]. Reproduced with permission from © 2020 Elsevier Ltd.

The quantitative assessment of acute inflammatory and immune responses was

determined by a pathologist who was blinded to the design and groups of the experiment according to a five-point grading scheme. White blood cells including neutrophils, macrophages and lymphocytes were evaluated. According to the pathologist, neutrophils have multiple nuclear lobes with usually no granules. Macrophages are recognized as small ovoid or indented nuclei with relatively abundant cytoplasm, while lymphocytes as round dark nuclei with scant cytoplasm. The results and 5-point grading system were listed in [Figure 2.10b-e](#).

Photos of grafts collected from sacrificed rats were shown in [Figure 2.10a](#). LhCG with and without cell membrane coatings remained cylinder in shape. Coagulation could be observed on Xeno graft on day 14. dLhCG with and without cell membrane coatings showed change in shape after collected from rats' omentum due to the sponge-like porous structures. Obvious tissues were observed grown surrounding grafts 14 days post-implantation.

First of all, all rats survived, indicating there was no hyperacute rejection. On day 1 post-implantation, Xeno-d and Allo-d grafts showed remarkably infiltration of neutrophils, while neutrophils infiltration was minimal in Auto-d graft. There were less neutrophils infiltrated in Auto graft compared to Xeno and Allo grafts which were mild in neutrophils infiltration. For positive control, PC sample showed a mild neutrophils recruitment. For negative control, RE sample induced minimal neutrophils invasion ([Figure 2.10b](#)). Furthermore, Allo and Allo-d grafts showed the most severe macrophages infiltration followed by Xeno, Xeno-d grafts and PC

sample with relatively moderate macrophages infiltration. There were less macrophages present in Auto, Auto-d grafts as well as RE samples (Figure 2.10c). All groups demonstrated a relatively slighter lymphocytes infiltration with no significant difference between each other. PC sample showed moderate infiltration which was the most severe in all the groups. Xeno, Allo, Xeno-d and Allo-d grafts were mild in lymphocytes invasion, while Auto, Auto-d grafts and RE sample showed minimal lymphocytes infiltration (Figure 2.10d).

On day 14 post-implantation, minimal neutrophils infiltration was observed in all grafts except Auto-d with rare neutrophils detected (Figure 2.10b). Macrophages infiltration was even more robust in Allo, Xeno-d and Xeno grafts compared to day 1 results, while it stayed minimal in Auto-d graft and RE sample. Moreover, there were moderate macrophages observed in Allo-d graft and PC sample (Figure 2.10c). Lymphocytes infiltration increased to moderate in Allo graft, PC sample and Xeno-d graft on day 14, became slightly worse in Xeno, Allo-d grafts and stayed minimal in Auto, Auto-d grafts and RE sample (Figure 2.10d).

2.3.7 Immunohistochemistry staining

Furthermore, immunohistochemistry staining of neutrophils (MPO), macrophages (CD68) and T cells (CD3) was carried out for further study on immune and inflammatory responses on a protein level. H&E and IHC staining results for grafts collected on day 1 and day 14 were listed in Figure 2.11, respectively.

Figure 2.11 H&E and IHC staining images for grafts collected on (a) day 1 post-implantation and (b) day 14 post-implantation, scale bar=250 μ m[118]. Reproduced with permission from © 2020 Elsevier Ltd.

IHC staining of neutrophils (MPO), macrophages (CD68) and T cells (CD3) of implanted grafts (Figure 2.11) as well as autologous rat ear (Figure 2.12) and decellularized porcine cartilage (Figure 2.13) was carried out. H&E staining was carried out for comparison. The IHC staining results were in accordance with the pathological analysis.

Figure 2.12 H&E and IHC staining images of autologous rat ear collected on day 1 (1d) and day 14 (14d) post-implantation. Scale bar=250 μ m[118]. Reproduced with permission from © 2020 Elsevier Ltd.

Figure 2.13 H&E and IHC staining images of xenogeneic natural porcine cartilage collected on day 1 (1d) and day 14 (14d) post-implantation. Scale bar=250 μ m[118]. Reproduced with permission from © 2020 Elsevier Ltd.

First, the staining results of grafts for day 1 which indicated inflammation in

an early immune response phase was shown in [Figure 2.11a](#). Xeno graft showed mild infiltration of neutrophils, macrophages and lymphocytes according to pathological analysis. As shown in [Figure 2.11a](#), neutrophils (MPO), macrophages (CD68) and T cells (CD3) which were stained in dark brown were found mainly on the surface of Xeno graft. There were mild neutrophils, moderate macrophages and mild lymphocytes present in Allo graft. Neutrophils (MPO) were observed accumulating on the surface of as well as surrounding the deficiency inside Allo graft. CD68 was remarkably stained both on the surface of and inside Allo graft, indicating a much more severe macrophages inflammation compared to Xeno graft. T cells (CD3) were found both on the surface and inside Allo graft. Minimal inflammatory cells were detected in Auto graft. As illustrated in [Figure 2.11a](#), only a small number of macrophages (CD68) were observed on the surface of Auto graft with a few T cells (CD3) distributed inside Auto graft. Inflammatory cells were much less in Auto graft than in Xeno and Allo grafts. Abundant neutrophils, mild macrophages and T cells were observed in Xeno-d graft. Neutrophils (MPO) were intensively infiltrated, while macrophages (CD68) distributed dot by dot in Xeno-d graft. Obvious dark brown areas or dots could also be observed in Xeno-d graft stained by CD3. Allo-d graft was graded moderate to severe in neutrophils and macrophages infiltration and mild in lymphocytes infiltration, which was in consistent with the staining results ([Figure 2.11a](#)). In addition, Allo-d graft displayed much more severe macrophages invasion than Xeno-d graft. In Auto-d graft, inflammatory cells were rarely visible.

Grafts collected on day 14 post-implantation were sectioned and stained for evaluation of intermediate phase of acute inflammatory and immune responses (Figure 2.11b). Neutrophils infiltration became minimal in all grafts except Auto-d graft which showed nearly no inflammation in neutrophils. MPO was detected inside Xeno and Allo grafts as well as in surrounding tissues of Xeno-d and Allo-d grafts. Macrophages and lymphocytes infiltrations in Xeno graft were between mild and moderate. According to Figure 2.11b, obvious CD68 (macrophages) and CD3 (T cells) were observed inside Xeno graft. Moderate macrophages and lymphocytes were recruited on day 14 and found randomly distributed inside Allo graft. There was still mild macrophages infiltration at the boundary between Auto graft and surrounding tissues according to IHC staining image of CD68. T cells were almost invisible in Auto graft, indicating minimal lymphocytes infiltration. Moderate macrophages, which distributed both in graft and its surrounding tissues, and moderate lymphocytes, which mainly distributed in graft especially at the border between graft and surrounding tissues, were observed in Xeno-d graft. The mild to moderate macrophages and lymphocytes recruited were mainly found in the boundary and surrounding tissues of Allo-d grafts. Nearly no inflammatory cells were detected in Auto-d graft.

2.3.8 PCR

After evaluating the immune and inflammatory responses on cell and protein levels and proving grafts with autologous RBC membrane coating were recognized

as “self” post implantation, we plan to figure out the performance on a gene level. In the early stage of biomaterial recognition, macrophages released monocyte chemoattractant protein-1 (MCP-1), Interleukin-6 (IL-6) and Interleukin-1 β (IL-1 β) [58]. MCP-1 regulates migration and infiltration of monocytes or macrophages, recruiting monocytes, memory T cells to the sites of inflammation[127]. IL-6 creates a pro-inflammatory response when signaling in monocytes or macrophages[128]. IL-1 β is also a proinflammatory cytokine. Quantitative real-time PCR (qRT-PCR) was carried out on grafts for the evaluation of gene expression encoding inflammation-related cytokines, including *MCP-1*, *IL-1 β* and *IL-6*. In order to better compare the inflammatory cytokines mRNA values, we set the gene expression value of Xeno graft as 1 (Figure 2.14). The gel electrophoresis results were also shown in Figure 2.14 for further confirmation of qRT-PCR results.

On day 1 post-implantation, natural porcine cartilage (PC), Allo and Allo-d grafts showed the highest *MCP-1* expression, followed by Xeno graft and then Xeno-d graft. There was slight *MCP-1* expressed in Auto graft and autologous ear cartilage (RE), while *MCP-1* expression was nearly invisible in Auto-d graft. For *IL-6*, Allo graft had the highest expression followed by PC and Xeno, Allo-d grafts, while Xeno-d graft was relatively lower in *IL-6* expression. There was nearly no *IL-6* expression in Auto-d graft and RE. As for *IL-1 β* , Allo graft demonstrated the highest expression, followed by PC, Xeno-d, Allo-d and Xeno grafts with obvious *IL-1 β* expression. In addition, nearly no *IL-1 β* was detected in Auto graft, RE and Auto-d graft.

Figure 2.14 Gene expression of (a,e) *MCP-1*, (b,f) *IL-6* and (c,g) *IL-1β*, (d,h) gel electrophoresis of *GAPDH*, *MCP-1*, *IL-6* and *IL-1β* for samples collected on day 1 (a~d) and day 14 (e~h) post-implantation[118]. Reproduced with permission from © 2020 Elsevier Ltd.

Gene expression results of day 14 post-implantation was shown in [Figure 2.14b](#).

Allo and Xeno-d grafts showed the highest *MCP-1* expression, followed by PC and Allo-d graft. In addition, Xeno graft also showed obvious expression in *MCP-1*, while Auto, Auto-d grafts and RE were low in *MCP-1* expression. For *IL-6*

expression, Xeno and Allo grafts showed the highest value, followed by PC, Xeno-d and Allo-d grafts. *IL-6* expression in Auto, Auto-d grafts and RE was almost invisible. Allo and Allo-d grafts expressed the most *IL-1 β* , followed by PC, Xeno-d and Xeno grafts. Slight *IL-1 β* expression could be detected in Auto graft, while Auto-d graft and RE showed low value.

2.4 Discussion

In this study, xenografts were coated with autologous RBC membrane to alleviate immune and inflammatory responses, which is promising in enhancing immunocompatibility in tissue engineering.

The successful extraction of cell membrane was confirmed by ATR-FTIR (Figure 2.2a). The RBC membrane vesicles were around 50nm in diameter according to DLS (Figure 2.2b) and AFM (Figure 2.2c). Moreover, AFM image confirmed the self-assembly of cell membrane to a globular morphology. The vesicles were negative in zeta potential, which was probably due to the self-assembled cell membrane with hydrophilic phosphoric acid outside and hydrophobic fatty acids inside when suspended in an aqueous solvent. However, cell membrane vesicles were highly unstable with high surface energy and inclined to adhere to solid surface to reduce the free energy, which is the driving force for the successful coating of cell membrane onto grafts. Water contact angle of coverslip increased to $43.4^{\circ} \pm 0.4^{\circ}$ from around 0° after coated with RBC membrane, while that of silicon decreased to $38.1^{\circ} \pm 1.4^{\circ}$ from $59.4^{\circ} \pm 1.8^{\circ}$ after coated with RBC

membrane, indicating the feasibility of RBC membrane vesicles to be coated on solid surfaces (Figure 2.2d and 2.2e). Moreover, it is worth noting that the water contact angle of RBC membrane coating on coverslip was relatively higher than that on Si. When coated on superhydrophilic coverslip surface, RBC adhered to the surface with mainly hydrophilic head attach to the surface and fatty acids tails exposed. Conversely, RBC membrane coated onto Si with fatty acids inside and mainly hydrophilic phosphate head exposed. In addition, as a simulation of LhCG and dLhCG which were derived from chondrocytes extracted from porcine cartilage, natural porcine cartilage was coated with RBC membrane (CM-cartilage) after which water contact angle was measured. CM-cartilage showed a relatively hydrophilic surface with a water contact angle of $37.6^{\circ} \pm 2.0^{\circ}$ compared to that of cartilage surface with a water contact angle of $71.3^{\circ} \pm 1.0^{\circ}$ (Figure 2.3), indicating cell membrane vesicles would adhere to cartilage ECM which had a lower surface energy.

RBC membrane, which was dyed with DiO beforehand, was successfully coated onto dLhCG (Figure 2.4a) and LhCG (Figure 2.4b), respectively according to the fluorescent images. In addition, LhCG was mainly composed of chondrocytes surrounded with glycoproteins and glycosaminoglycan secreted by the cells, while dLhCG consisted of glycoproteins, mainly collagen, and glycosaminoglycan. Cell membrane was a phospholipid with fatty acid tails and a phosphate head. As a result, as shown in XPS results in Figure 2.5 and Table 1, LhCG had a higher phosphorus content than dLhCG. Similarly, there was more phosphorus in CM-LhCG than CM-

dLhCG. What's more, phosphorus ratio increased to 2.4% and 3.4% from 0.7% and 2.9% on the surface of dLhCG and LhCG respectively after coated with RBC membrane, implying successful coating of RBC membrane on dLhCG and LhCG grafts. It was reported in our previous study that during decellularization almost all collagen remained while there was a loss of glycosaminoglycan[51]. That is why there was more sulfur detected in LhCG (5.4%) and CM-LhCG (4.8%) compared to dLhCG (3.3%) and CM-dLhCG (2.5%), respectively.

Understanding the stability of the cell membrane coating on dLhCG is also crucial since it takes some time for cells to migrate in the grafts and proliferate after implantation for clinical purpose. For CM-dLhCG, no obvious decrease in P content was detected in 4 weeks. This implied the strong affinity between cell membrane coatings with dLhCG surface and guaranteed the long-term use of CM-dLhCG graft *in vivo* (Figure 2.9). This high affinity might also result from the high surface energy of cell membrane vesicles in aqueous environment. The cell membrane vesicles were so unstable that the adherence to graft surface was irreversible. During this time, the cell membrane coating could stay on the dLhCG surface as a disguise. Furthermore, it is worth noting that the coating process did not affect the porous structure of the dLhCG grafts (Figure 2.6a and 2.6b). For LhCG, CM-DiO was detectable under fluorescent microscope on day 1, day 7, day 14 and day 28, indicating the existence of RBC membrane coatings.

Furthermore, in order to evaluate its immunocompatibility performance *in vivo*,

a rat omentum implantation model was applied due to the sensitivity of omentum to foreign bodies. Xenografts with autologous RBC membrane coatings were proved to be superior to xenografts with and without allogeneic RBC membrane coatings in suppressing and alleviating inflammation on cell, protein and gene levels.

After implantation of biomaterials, platelets produced during coagulation secrete cytokines which attract neutrophils from blood stream. In an acute phase of inflammatory response, neutrophils are the first cells to migrate out of the blood stream and head to inflammation site. Then neutrophils release pro-inflammatory cytokines such as IL-6 and IL-1 β , promoting the recruitment of the macrophages which are involved in the later phase of inflammatory response and at the same time amplifying neutrophils recruitment. If there is a frustrated phagocytosis, the macrophages will fuse into multinucleated giant cells. Afterwards, those phagocytes present major histocompatibility complex II (MHC II) proteins on their surfaces, which will bind with receptors specifically on T cells and activate them. As the first inflammatory cells to migrate to inflammation site, neutrophils have a short lifespan.

On day 1 post-implantation, macrophages were dominating in the process of inflammation in Xeno and Allo grafts ([Figure 2.10](#)). Macrophages were recruited into tissues under the trigger of cytokines secreted by neutrophils for phagocytosis of the foreign bodies. For Xeno graft, the inflammatory and immune responses were

caused by porcine chondrocytes and antigenic properties of xenogeneic source ECM. Macrophages were observed mainly on the surface with some inside Xeno graft, while neutrophils and T cells mainly found on and near the surface (Figure 2.11a). For Allo graft, macrophages and T cells were found distributed uniformly inside the graft besides accumulating on the surface (Figure 2.11a), implying more serious inflammatory and immune responses than Xeno graft. It is probably caused by antigens on allogeneic RBC membrane. Xeno-d graft was undergoing an early phase of inflammation with neutrophils dominating (Figure 2.10). Neutrophils and macrophages distributed uniformly, while clusters of T cells were found inside Xeno-d graft (Figure 2.11a). In Allo-d graft, both neutrophils and macrophages were seriously infiltrated (Figure 2.10), indicating the recruitment of macrophages for clearance of foreign bodies and at the same time another initiation of neutrophils infiltration. Therefore, Allo-d graft showed the most severe inflammatory and immune responses on day 1 post-implantation. For positive control, macrophages and lymphocytes were the most prevailing inflammatory cells in PC (Figure 2.10), implying a later phase of inflammation had already taken place. In Auto graft, minimal to mild inflammatory cells were detected with relatively more T cells, which indicated slight inflammation occurred. Nearly no inflammation was observed in Auto-d graft and RE.

On day 14 post-implantation, as the first inflammatory cells to migrate in with short lifespan, minimal neutrophils were infiltrated in the intermediate and later phase of acute inflammation and immune responses (Figure 2.10b). Moreover,

neutrophils were rarely detected in Auto-d graft (Figure 2.10b). After 14 days, macrophages and lymphocytes were dominating in inflammatory and immune responses. Allo graft showed the most severe inflammatory cells infiltration on day 14, followed by Xeno graft. The porous structure of dLhCG made it easier for inflammatory cells to migrate in. At the same time the larger specific surface area resulted in a larger contact area with tissues and blood, causing more serious inflammatory and immune responses during an early stage in Allo-d and Xeno-d grafts. However, after two weeks, more antigens on LhCG were exposed and detected, resulting in an increase in macrophages and lymphocytes recruitment which were found mainly distributed inside Xeno and Allo grafts (Figure 2.11b). Furthermore, Allo-d graft was going through a later phase of inflammation with lymphocytes dominating and a slight decrease in macrophages infiltration, while Xeno-d graft undergoing an intermediate phase of inflammation with both macrophages and lymphocytes dominating. As a positive control, PC sample showed heavy infiltration of macrophages and lymphocytes (Figure 2.10). There was slight inflammation occurred in Auto graft with mild macrophages detected. Nevertheless, nearly no inflammation was observed in Auto-d graft as well as RE. Autologous RBC membrane coating on LhCG worked for immune evasion and contributed to better performance of Auto graft in inflammatory and immune responses than Xeno and Allo grafts. However, for Auto graft, due to the preference of RBC membrane coating onto chondrocytes surface and metabolism of chondrocytes, antigens located on LhCG ECM matrix and cells were presented,

inducing mild *in vivo* inflammatory and immune responses. dLhCG coated with autologous RBC membrane was recognized as “self” and evaded from immune system attack.

Inflammation is a complicated and highly orchestrated process involving many kinds of inflammatory cells and cytokines. The cytokines are produced and acts as a regulator and stimulator during inflammation process. Therefore, expression of inflammation-related cytokines could reflect severity of inflammatory and immune responses induced. Considering the inflammatory and immune responses have been evaluated on cell and protein levels, evaluation on a gene level, expression of mRNA encoding inflammation-related cytokines, was measured. MCP-1, one of the major chemoattractants for monocytes or macrophages, plays a significant role in inflammation. In addition, it is reported expression of proinflammatory cytokines involving IL-6 and IL-1 β was strongly upregulated during inflammation.

Expression of *MCP-1* (Figure 2.14) roughly correlated with macrophages infiltration (Figure 2.14). It is because MCP-1 played an important role in recruitment of macrophages. On day 1 post-implantation, compared to PC, Allo graft showed more serious inflammatory and immune responses with comparative *MCP-1* expression and higher *IL-6* and *IL-1 β* expression; Allo-d graft exhibited slightly lower inflammatory and immune responses with comparative *MCP-1* expression and lower *IL-6* and *IL-1 β* expression; Xeno and Xeno-d grafts were lower in inflammation-related gene expression value than PC. Autologous RBC

membrane was proved to help suppress or alleviate inflammatory and immune responses on gene level in that dLhCG and LhCG grafts with autologous RBC membrane coating showed an inflammation-related gene expression which was comparative to or slightly higher than autologous rat ear (RE), respectively. 14 days later, dLhCG and LhCG with and without allogeneic RBC coatings as well as PC also showed inflammatory and immune responses with relatively high *MCP-1*, *IL-6* and *IL-1 β* expression overall, while Auto, Auto-d grafts and RE with low value in inflammation-related gene expression. Compared to positive control PC, Allo graft showed more serious inflammatory and immune responses with higher inflammation-related gene expression; Allo-d graft exhibited comparative inflammation-related gene expression; Xeno graft showed a comparative *IL-6* expression and lower *MCP-1*, *IL-1 β* expressions; Xeno-d graft had a higher *MCP-1* expression, a comparative *IL-6* expression and a lower *IL-1 β* expression. Autologous RBC membrane coating was proved to help suppress or alleviate inflammatory and immune responses on gene level with dLhCG and LhCG grafts with autologous RBC membrane coating a slightly higher inflammation-related gene expression than the negative control RE.

2.5 Summary

This study introduced a novel and facile method to suppress or alleviate immune and inflammatory responses in tissue engineering field by coating autologous RBC membrane on xenografts as a disguise with dLhCG and LhCG as

model grafts. First of all, RBC membrane was successfully extracted, coated and quite stable on dLhCG and LhCG for at least 4 weeks. RBC membrane coatings showed no obvious decrease in amount on dLhCG and remained visible on LhCG within 4 weeks. Furthermore, the immune response *in vivo* was evaluated by a rat omentum implantation model. Both LhCG and dLhCG grafts coated with autologous RBC membrane (Auto and Auto-d) showed superior performance in immune evasion on both cell and protein level, especially Auto-d graft with nearly no inflammation detected. It showed enhanced immunocompatibility with less inflammatory cells infiltration in early and intermediate phase of acute host immune responses compared to xenografts with and without allogeneous RBC membrane coatings. Moreover, its enhanced immunocompatibility was also proved on a gene level with low gene expression encoding inflammation-related cytokines including *MCP-1*, *IL-6* and *IL-1 β* .

In conclusion, xenografts coated with autologous cell membrane could be recognized as “self” post implantation. This is proved to be a promising approach for suppression and alleviation of immune and inflammatory responses in tissue engineering field.

Supplementary data

Table S2.1. Antibodies used in the immunohistochemistry staining.

Primary antibody	Host	Species reactivity	Dilution	Company
Anti-CD3 (ab16669)	Rabbit	Mouse, Rat, Human, Pig	1:100	Abcam
Anti-CD68 (ab31630)	Mouse	Mouse, Rat, Human	1:100	Abcam
Anti-MPO (ab9535)	Rabbit	Mouse, Rat, Human, Pig, Monkey	1:50	Abcam

Table S2.2 Primer sequences for qRT-PCR and PCR

Gene name	PCR	Sequence (5'→3')		Product size (dp)
<i>MCP-1</i>	qRT-PCR	Forward primer	TGA ACT TGA CCC ATA AAT CTG AAG	121
		Reverse primer	AAG GCA TCA CAT TCC AAA TCA C	
	PCR	Forward primer	ACC TGC TGC TAC TCA TTC AC	325
		Reverse primer	CTA CAG AAG TGC TTG AGG TG	
<i>IL-1β</i>	qRT-PCR	Forward primer	GAG TCT GCA CAG TTC CCC AA	89
		Reverse primer	ATG TCC CGA CCA TTG CTG TT	
	PCR	Forward primer	GAC CTG TTC TTT GAG GCT GAC	578
		Reverse primer	TCC ATC TTC TTC TTT GGG TAT TGT T	
<i>IL-6</i>	qRT-PCR	Forward primer	TGC CCT TCA GGA ACA GCT ATG	102
		Reverse primer	TGA AGT AGG GAA GGC AGT GG	
	PCR	Forward primer	CTC TGA ATG ACT CTG GCT TTG	390
		Reverse primer	CTT GGG ACT GAT GTT GTT GAC	
<i>GAPDH</i>	qRT-PCR	Forward primer	CTCCCATTCCTCCACCTTTG	185
		Reverse primer	CTTGCTCTCAGTATCCTTGC	
	PCR	Forward primer	TGC CAT CAA CGA CCC CTT CA	569
		Reverse primer	TGA CCT TGC CCA CAG CCT TG	

Stabilized albumin coatings on engineered xenografts for attenuation of acute immune and inflammatory responses

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Chapter 3 Stabilized albumin coating on engineered xenografts for attenuation of acute immune and inflammatory responses

3.1 Introduction

In chapter 2, autologous RBC membrane, one of natural biological materials, was proved to be efficient as a disguise on xenografts for immune evasion due to the functional biomacromolecules embedded in cell membrane which made autologous cell membrane coated xenografts to be recognized as self. In this chapter, another natural biological coating material, serum albumin (SA), was applied for alleviation of immune and inflammatory responses. The immune evasion of albumin was based on the passivation of biomaterials surface, xenografts surface in this particular study.

Various albumin conjugates have been synthesized and utilized for coatings of biomedical devices for improved biocompatibility to obtain antithrombotic surfaces or other non-protein adsorption surfaces. For instance, Loh *et al.* loaded graphene oxide (GO) with albumin and demonstrated its capability as an antithrombotic coating for blood contacting devices[129]. Tessler *et al.* prepared a poly (ethylene oxide) (PEG)-bovine serum albumin (BSA) hybrid coating to reduce the non-specific adsorption of proteins on nanogel which improves the nanogel's function as a quantitative monitor of single-molecule protein[130]. Kawamura *et al.* applied the non-adhesive nature of BSA to switch the adhesive properties of modified

polystyrene to control the adhesion of cells[131].

On the other hand, since SA is blood-compatible and non-immunogenic, SA can be coated onto nanoparticles to evade immunogenic response, reduce colloidal aggregation and enhance biocompatibility[132 – 134]. Albumin is a potent candidate as “stealth” coating on various kinds of nanoparticles, overcoming uptake by the immune system[58,135]. Albumin camouflage coating has been applied on various nanoparticles including virus-based nanoparticles, DNA origami, liposomes and metal nanoparticles.

In this chapter, we took advantage of the passivation nature of albumin, transferred it to tissue engineering field and developed a facile method for evasion from immune rejection elicited by xenografts through coating albumin. The basic idea is making use of passivation nature of albumin to avoid adsorption of other proteins and further coagulation. Adhesion of albumin onto grafts were achieved by introducing dopamine, a mussel-inspired adhesive[69], via chemical reaction which avoids the introduction of free dopamine. Moreover, similar to chapter 2, living hyaline cartilage graft (LhCG)[13–16], which is composed of porcine chondrocytes derived extracellular matrix (ECM) with porcine chondrocytes, and decellularized LhCG (dLhCG)[17–19], which is mainly constituted by porcine chondrocytes derived ECM were applied as xenograft models in this study. The process was briefly illustrated in [Figure 3.1](#). Inflammation process could be divided into acute inflammation, which lasts within a few days to weeks, and chronic inflammation,

which is developed from persistent acute inflammation and may last for years[39,136]. We herein focused on studying attenuation of acute immune and inflammatory responses, the failure of which will lead to severe chronic inflammation and disease. The immunocompatibility of LhCG, dLhCG with and without albumin coatings were evaluated on cell, protein and gene levels, respectively.

Figure 3.1 Schematic illustration of BCD-LhCG and BCD-dLhCG[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

3.2 Materials and methods

3.2.1 Materials

BSA, citrate acid (CA), dopamine hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-Hydroxysuccinimide (NHS) and sodium hydroxide (NaOH) were purchased from Sigma Aldrich for preparation of dopamine functionalized albumin. Chondrocytes were extracted from market-available porcine bone for fabrication of LhCG and dLhCG. Gelatin from bovine

skin, alginate acid sodium salt and fluorescein isothiocyanate (FITC) were also purchased from Sigma Aldrich. 1×phosphate buffered saline (1×PBS, pH=7.4, Gibco) was purchased from Thermo Fisher Scientific. Tris buffer (1.0M, pH=8.8) was purchased from 1st Base Pte Ltd, Singapore.

3.2.2 Preparation of dopamine-functionalized albumin (BCD)

A modified protocol based on our previous publications[68] was used for preparation of dopamine-functionalized albumin (BCD) coating material. First, 5mL CA solution (0.2g/mL) with 1×PBS as solvent was added to a three-neck flask fitted with one N₂ inlet, after which the pH was adjusted to 6.0 by 6M NaOH solution. Then 0.3g EDC and 0.3g NHS were added. 1.75g BSA was dissolved in 10mL 1×PBS and added to the flask drop by drop. The mixture reacted for 6h under 37°C and pH 5.5-6.0. The products were dialyzed against deionized (DI) water for 2 days for purification and freeze-dried afterwards (BT48, Millrock Technology Inc, USA). The resultant purified product was intermediate product named BCA. Then 1g BCA, 1g dopamine hydrochloride, 1g EDC and 1g NHS were dissolved in 100mL 1×PBS and added to a Schlenk tube. pH of the mixture was adjusted to around 6.0. Three cycles of freeze-thaw were applied to completely remove oxygen to avoid oxidation of dopamine. The mixture reacted under N₂ protection and 37°C. Finally, the products were dialyzed against DI water for 2 days and freeze-dried to harvest final product. The final product was abbreviated as BCD originated from reactants bovine albumin-citric acid-dopamine. Chemical structures of BCA and

BCD were characterized by ^1H NMR (Bruker Avance II 300MHz NMR) with D_2O (Sigma Aldrich, USA) as solvent.

3.2.3 Fabrication of LhCG and dLhCG

LhCG[13–16] and dLhCG[17–19] fabricated based on our published protocol were used as model xenografts. Porcine chondrocytes were seeded in alginate hydrogel with gelatin microspheres as micropores forming agent and grown in high glucose Dulbecco's Modified Eagle Medium (DMEM). Chondrocytes grew, proliferated in hydrogel and secreted ECM, forming 3D micro-tissues. Afterwards, alginate template was removed by rinsing in sodium citrate solution (55mM in 0.15M NaCl) to harvest LhCG. Decellularized LhCG by successive physical, chemical and enzymatic methods was named dLhCG.

3.2.4 Characterization of BCD coating

First of all, in order to prove the feasibility of BCD coating formation, 20mg/mL BCD in Tris buffer (10mM, pH 8.5) was coated onto surface of coverslip (CellPath Ltd, UK) by drip coating and drying under room temperature overnight. Water contact angle was detected on a Kruss DSA25 Contact Angle Analyzer with a 5 μL DI water drop. The final contact angle result was averaged from five samples' values.

dLhCG and LhCG were rinsed in 20mg/mL BCD overnight with Tris buffer (10mM, pH8.5) and culture medium as solvent, respectively to obtain BCD coated

dLhCG (BCD-dLhCG) and BCD coated LhCG (BCD-LhCG). DI water and pure culture medium were applied afterwards respectively to wash BCD-dLhCG and BCD-LhCG. For visualization of BCD coating on grafts, BCD was labeled with FITC (BCD-FITC) through chemical reaction. Briefly, the isothiocyanate group on FITC reacted with amino groups on BCA (BCA-FITC), after which the BCA-FITC went through an EDC/NHS coupling with dopamine hydrochloride according to the protocol mentioned above. UV-vis (UV-2450, Thermo Fisher USA) was used to ensure the successful conjugation and estimate fluorophore to protein ratio (F/P) for BCA-FITC and BCD-FITC according to a reported equation[138]:

$$\frac{F}{P} = \frac{A_{max} \times \epsilon_{prot}}{(A_{280} - cA_{max}) \times \epsilon_{dye}} \quad (\text{equation 1})$$

where A_{max} is the absorbance value at around 500nm in this study; A_{280} is the absorbance value of albumin at around 280; c is a correction factor for adjustment of absorption at 280nm contributed by FITC ($c=0.29$ referring to [138]); ϵ_{prot} ($\epsilon_{prot}=43,645\text{M}^{-1}\text{cm}^{-1}$ referring to [139]) is the molar extinction coefficient for bovine serum albumin in this study and estimated from amino acid sequence; ϵ_{dye} ($\epsilon_{dye}=63,096\text{M}^{-1}\text{cm}^{-1}$ referring to [138]) is the molar extinction coefficient of FITC in this study.

BCD-FITC was subsequently coated onto dLhCG and LhCG based on the above-mentioned methods, then fixed in 4% paraformaldehyde (Sigma Aldrich, USA), embedded in paraffin (Surgipath® Paraplast®, Leica, USA), sectioned on a slicer (RM2255, Leica, USA) and observed under a fluorescent microscope

(Olympus IX71S1F-3, Japan).

The surface elemental information about carbon (C), oxygen (O), nitrogen (N), phosphorous (P) and sulfur (S) was detected by X-ray photoelectron spectroscopy (XPS) on a Kratos AXIS Supra spectrometer with a 40eV bandpass energy. The high-resolution C spectra were fitted by Origin 9.0. Before XPS measurement, dLhCG and BCD-dLhCG were freeze-dried, while LhCG and BCD-LhCG were dried in a critical point dryer (CPD) (K850, Quorum Technologies Inc., England) after fixation by 2.5v/v% glutaraldehyde (Sigma Aldrich, USA) and gradient dehydration in ethanol.

Stability of BCD coating on LhCG and dLhCG was measured by a BCG Albumin Assay Kit (Sigma Aldrich, USA) via testing BCD content on per milligram dried graft. BCD-dLhCG was digested by collagen II solution after rinsing in DI water for 1d, 7d, 14d and 21d, respectively. BCD-LhCG went through freeze-thaw cycles for three times, followed by digested in collagen II solution. BCD content in the digested samples were tested. dLhCG and LhCG were also digested followed the same protocol and tested by the same assay kit at corresponding time points as background.

3.2.5 Morphology of BCD-LhCG and BCD-dLhCG

Morphology of dLhCG, BCD-dLhCG, LhCG and BCD-LhCG was observed by scanning electronic microscopy (SEM) (JEOL JSM 6700F, Japan). dLhCG and BCD-dLhCG were dried in a freeze dryer, while LhCG and BCD-LhCG were dried

by CPD as mentioned above. Platinum was coated on samples by sputtering for 150s at 10mA (JFC-1600, JEOL Asia Pte. Ltd., Japan) before SEM observation.

3.2.6 Cytotoxicity of dLhCG and BCD-dLhCG

Before implanted *in vivo*, cytotoxicity of BCD-dLhCG and dLhCG was measured by a WST-1 Assay Kit (Sigma Aldrich, USA). Chondrocytes were cultured in wells of 96 well plate (2500 cells per well) and incubated with dLhCG and BCD-dLhCG respectively for 1d, 4d and 7d. The culture medium at certain time points was collected and incubated together with WST-1 for 3h. The detection and calculation were carried out according to the manufacturer's protocol.

3.2.7 In vivo immunocompatibility study in a rat omentum implantation model

All animal experiment protocols have been approved by Institutional Animal Care and Use Committee (IACUC), Nanyang Technological University, Singapore according to the local law.

A rat omentum implantation model was applied to study *in vivo* immune and inflammatory performance of LhCG, BCD-LhCG, dLhCG and BCD-dLhCG. Forty-eight Sprague Dawley female rats (n=4, 250-300g) were divided into six groups. Besides the four groups mentioned above, natural porcine cartilage discs with a diameter of ~0.5cm were decellularized followed the same protocol as LhCG was decellularized and chosen as a positive control. Natural autologous cartilage slices were collected from rat ear of the negative control group and implanted to

omentum of the same individual. First, an incision of around 2cm was cut on the rats' abdomen. Each rat was embedded with two grafts (~0.5cm in diameter) of the same group, after which the incision was closed by 4-0 Nylon sutures and stainless-steel clips. Two time points, day 1 and day 14, were chosen for the collection and characterization of grafts. Specifically, the implanted two grafts and surrounding tissues were collected from omentum, one of which was fixed in 4% paraformaldehyde for staining, the other of which was kept in liquid nitrogen for gene expression evaluation.

3.2.8 Pathological analysis

A pathologist who was blinded to the experimental groups carried out the pathological analysis by evaluating infiltrated inflammatory cells conditions according to a five-point grading scheme[119–122] based on hematoxylin and eosin (H&E) (Sigma Aldrich, USA) staining results. The grafts and surrounding tissues were fixed in 4% paraformaldehyde, embedded, sectioned and stained with H&E following a standard protocol from manufacturer.

3.2.9 Immunohistochemistry analysis

The sectioned samples were stained with MPO (Myeloperoxidase), CD68 and CD3 for detection of neutrophils, macrophages and T cells, respectively by immunohistochemistry (IHC) staining with UltraVisionTM Quanto Detection System HRP DAB kit (Thermo Fisher Scientific, UK). The details of antibodies

were listed as follows: Anti-MPO (Abcam, ab9535), Anti-CD68 (Abcam, ab31630) and Anti-CD3 (Abcam, ab16669).

3.2.10 Gene expression

The gene expression of inflammation related cytokines including monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) was quantified by quantitative real-time polymerase chain reaction (qRT-PCR) and semi-quantified by agarose gel electrophoresis.

First of all, total RNA was extracted by TRIzol[®] reagent (Life Technology, USA), after which RNA concentration was detected by NanoDrop 2000c (ThermoFisher Scientific, USA). Then cDNA was synthesized and performed reverse transcription with reagents purchased from Promega. qRT-PCR was carried out on a CFX Connect Real-Time PCR System (BIO-RAD, USA). Primers, purchased from Integrated DNA Technologies Singapore, were listed in [Table S3.1](#). *GAPDH* gene was used as a housekeeping gene for normalization of target genes. A comparative $2^{-\Delta\Delta C_T}$ method was applied for the calculation.

Furthermore, cDNA was amplified with Taq DNA polymerase (NEW ENGLAND BioLabs, England) and visualized by agarose gel electrophoresis in Tris-Borate-EDTA Buffer (Vivantis Technologies, Malaysia) after mixing with SYBR[®] Safe DNA gel stain (Invitrogen, USA).

3.3 Results

3.3.1 Characterization of BCD macromolecules and BCD coating

Preparation of BCD was illustrated in Figure 3.2a. First of all, BCA prepolymer was prepared by grafting CA onto BSA through EDC/NHS coupling for introduction of extra carboxyl for additional reaction sites. Afterwards, dopamine was grafted onto BCA by EDC/NHS coupling to synthesize BCD, so that no free dopamine will be introduced. Chemical structures of BCA and BCD were characterized by ^1H NMR and shown in Figure 3.2b. Peaks at around 3.5ppm and 1.1ppm on ^1H NMR spectrum of BCA belong to $-\text{COCH}_2-$ and $-\text{CH}_2\text{COOH}$ respectively on the grafted citric acid, confirming the successful modification by citric acid. Moreover, the appearance of signal at around 3.2ppm on ^1H NMR spectrum of BCD belonged to $-\text{NH}-\text{CH}_2-\text{CH}_2-$ on grafted dopamine and confirmed the successful introduction of dopamine.

Figure 3.2 (a) Preparation of BCD and (b) ^1H NMR spectra of BSA, BCA and BCD[137].

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BCD was first coated onto coverslip to evaluate its coating formation feasibility

and wettability. Water contact angles of coverslip surface with and without BCD coating were measured. It is shown in Figure 3.3 that water contact angle increased to $60.9^{\circ} \pm 0.6^{\circ}$ from around 0° , indicating there formed a uniform BCD coating on superhydrophilic coverslip surface. Afterwards, BCD was coated onto LhCG and dLhCG for further investigation, the schematic illustration of which was shown in Figure 3.1.

Figure 3.3 Water contact angles of coverslip with and without BCD coating[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

3.3.2 Characterization of BCD coating on grafts

In order to visualize BCD coating on grafts, FITC was grafted onto BCD for labeling (Figure 3.4a). The absorbance peak at around 500nm on UV-vis spectrum belongs to BCA-FITC, while that at around 277nm belongs to unlabeled BCA (Figure 3.4b). Similarly, two absorbance peaks at 287nm and 507nm shown in Figure 3.4c belong to unlabeled BCD and BCD-FITC, respectively. The presence of absorbance peak at around 507nm confirmed the existence of BCD-FITC. F/P of BCA-FITC (~2.2) and BCD-FITC (~1.5) was estimated based on equation 1.

Subsequently BCD-FITC was coated onto dLhCG and LhCG and observed under fluorescent microscope. BCD was uniformly coated on surface of dLhCG (Figure 3.4d) and preferred to adhere to cell membrane compared to ECM on LhCG (Figure 3.4e).

Figure 3.4 (a) Preparation of BCD-FITC, UV-vis spectrum of (b) BCA-FITC and (c) BCD-FITC, fluorescent image of (d) BCD-dLhCG and (e) BCD-LhCG[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

XPS was conducted to further detect the chemical information on graft surfaces within 10nm. Albumin was rich in cysteine (Cys), which could not be detected in

collagen. In this case, albumin was more abundant in S than collagen.

Figure 3.5 (a)XPS spectra of dLhCG, BCD-dLhCG, LhCG and BCD-LhCG, P 2p and S 2p spectra were zoomed in and shown aside, XPS spectrum of C 1s for (b) dLhCG, (c) BCD-dLhCG, (d) LhCG and (e) BCD-LhCG[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

According to [Table 3.1](#), S content of dLhCG increased from 3.1% to 4.1% after coated with BCD, confirming successful coating of BCD onto dLhCG. Similarly,

there was an increase in S content of BCD-LhCG (4.7%) compared to LhCG (3.4%), confirming successful coating of BCD onto LhCG. dLhCG was decellularized and rarely contained phosphorus which was one of the main elements constituting cell membrane. Therefore, LhCG (3.8%) contained more P than dLhCG (1.3%). However, due to the existence of BCD coating on the surface, there was not much difference in P content of BCD-dLhCG (1.3%), BCD-LhCG (1.9%) compared to dLhCG (1.3%). Moreover, P signal at around 132eV was nearly invisible in XPS spectrum of BCD-dLhCG (Figure 3.5a). Besides, the high resolution of C 1s spectra were fitted with peaks at around 284.7eV (C-C, C-H), 286.1eV (C-N, C-S, C-O), 287.8eV (C=O) and 288.5eV (-O-C=O) and shown in Figure 3.5b-e.

Table 3.1 Surface elemental composition of dLhCG, BCD-dLhCG, LhCG and BCD-LhCG[137].

	C (%)				O (%)	N (%)	P (%)	S (%)
	C-C/C-H	C-N/C-S/C-O	C=O	-O-C=O				
dLhCG	18.0	13.1	5.1	2.6	36.5	20.3	1.3	3.1
BCD-dLhCG	15.4	12.6	5.9	2.8	34.8	23.1	1.3	4.1
LhCG	19.4	11.5	8.4	0.8	29.7	23.0	3.8	3.4
BCD-LhCG	17.6	13.1	6.9	2.5	29.4	23.9	1.9	4.7

3.3.3 Stability of BCD coating on grafts

Stability of BCD coating should be taken into consideration to ensure existence of disguise coating before cells migrate in and proliferate. Bromocresol green could specifically bind with albumin and form a colored complex whose intensity at 620nm is proportional to albumin concentration. Durability was measured based on

this reaction by a BCG Albumin Assay Kit. As shown in Figure 3.6a and 3.6b, BCD content decreased on day 7 compared to day 1 result, but remained almost the same from the 7th day on.

Figure 3.6 BCD content of (a) BCD-dLhCG and (b) BCD-LhCG after rinsing in DI water for 1d, 7d, 14d and 21d, respectively[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

3.3.4 Morphology of grafts

Figure 3.7 SEM images of (a) dLhCG, (b) LhCG, (c) BCD-dLhCG and (d) BCD-LhCG[137].

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Morphology of dLhCG, BCD-dLhCG, LhCG and BCD-LhCG was observed under SEM. As shown in Figure 3.7a, there was a porous structure observed in dLhCG with pore size in hundreds of microns. BCD coating did not damage the porous structure of dLhCG and was visible under SEM (Figure 3.7c). Abundant assembled chondrocytes could be observed in LhCG and BCD-LhCG under SEM (Figure 3.7b and 3.7d). Therefore, LhCG with and without BCD coating was more densely packed than dLhCG and BCD-dLhCG.

3.3.5 *In vivo* rat omentum implantation for evaluation of immunocompatibility

Before *in vivo* implantation, cytotoxicity of dLhCG and BCD-dLhCG was evaluated on chondrocytes by WST-1. dLhCG and BCD-dLhCG showed no cytotoxicity with cell viability of $89.4\% \pm 2.7\%$ and $92.5\% \pm 14.2\%$ after 7 days incubation respectively (Figure 3.8). And BCD coating did not interfere biocompatibility of dLhCG with comparable cell viability on day 1, day 4 and day 7.

Figure 3.8 Cytotoxicity of dLhCG with and without BCD coating for 1d, 4d and 7d, respectively[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

Embedded grafts and surrounding tissues were collected on day 1 and day 14 for evaluation of acute immune and inflammatory responses. Photos of collected grafts were shown in [Figure 3.9](#). Obvious blood coagulation was observed surrounding LhCG graft collected on day 14, indicating a possible severe inflammation. Pathological analysis, IHC staining and PCR were further carried out for assessment on cell level, protein level and gene level, respectively. First of all, there was no hyperacute immune and inflammatory responses with all rats surviving.

Figure 3.9 Photos of samples collected from sacrificed rats, scale bar=0.5cm[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

3.3.6 Pathological analysis

Three kinds of inflammatory cells including neutrophils which are characterized by multi-lobed nucleus, macrophages which are characterized by small oval or indented nuclei with relatively luxuriant cytoplasm and lymphocytes which are characterized by round dark nuclei with relative scarce cytoplasm were quantified by a pathologist who was blinded to the experimental design and groups and plotted in [Figure 3.10a-3.10c](#) according to a five-point grading scheme, where

no inflammatory cells showing in any field scored 0, scattered sparse inflammatory cells showing in high power field scored 1, inflammatory cells showing in at least one low power field scored 2, abundant inflammatory cells showing in every low power field scored 3, badly inflammatory cells infiltration visible in low power field scored 4 ([Figure 3.10d](#)).

Neutrophils were the first inflammatory cells to migrate out of blood stream to the inflammation site. As shown in [Figure 3.10a](#), on day 1 post-surgery, there showed the most serious neutrophils infiltration in LhCG and dLhCG, followed by PC which were mild in neutrophils infiltration. Minimal to mild neutrophils infiltration was observed in BCD-LhCG. By contrast, there were minimal neutrophils in BCD-dLhCG and negative control RE. As the first inflammatory cells migrating in, neutrophils had a short lifespan. As a result, 14 days later, there showed few neutrophils in all grafts. Macrophages and lymphocytes were later recruited to the implantation site. According to [Figure 3.10b](#), on day 1 post-surgery, moderate macrophages were present in LhCG, which showed the most severe macrophages infiltration among all grafts. PC came next with mild to moderate macrophages infiltration. Mild macrophages were observed in dLhCG. Minimal to mild macrophages could still be detected in BCD-LhCG, while macrophages infiltration was nearly negligible in BCD-dLhCG as well as RE. On day 14 post-implantation, macrophages infiltration levels almost remained as it was on day 1 except for a more intensively macrophages invasion ranging from mild to moderate observed in dLhCG. As shown in [Figure 3.10c](#), on day 1 post-implantation,

lymphocytes infiltration was not severe in all grafts with mild infiltration in PC, dLhCG and LhCG, minimal to mild in BCD-LhCG and minimal in BCD-dLhCG and RE. 14 days later, LhCG, PC and dLhCG were moderately infiltrated by lymphocytes, while that in BCD-LhCG, BCD-dLhCG and RE remained minimal.

Figure 3.10 Pathological analysis based on quantitative assessment of inflammatory cells including (a) neutrophils, (b) macrophages, (c) lymphocytes on day 1 and day 14 and (d) the 5-point grading system (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

3.3.7 IHC staining

IHC staining of MPO, CD68 and CD3 was conducted for identification of neutrophils, macrophages and T cells respectively and where they were located, and evaluate immune and inflammatory responses on a protein level. IHC staining

images for LhCG, BCD-LhCG, dLhCG and BCD-dLhCG were shown in [Figure 3.11](#), and those of positive control PC ([Figure 3.12](#)) as well as negative control RE ([Figure 3.13](#)) were provided in [Supplementary data](#).

Figure 3.11 H&E and IHC staining images for (a) day 1 and (b) day 14 grafts, scale bar=200 μ m[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

Figure 3.12 H&E and IHC staining images of PC on day 1 and day 14 post-implantation, respectively[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

Figure 3.13 H&E and IHC staining images of RE on day 1 and day 14 post-implantation, respectively[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

On day 1 post implantation (Figure 3.11a), MPO, a peroxidase enzyme abundantly expressed in neutrophils, was mainly distributed on the surface of LhCG with some inside LhCG. Abundant MPO was observed all over the whole dLhCG graft. By comparison, only minimal MPO was found on the surface of BCD-LhCG and nearly no MPO was stained in BCD-dLhCG. CD68 is expressed in macrophages. CD68 was mainly found on the surface of LhCG and all over dLhCG. Sparse CD68 could be observed in BCD-LhCG, while only minimal CD68 was detected near the surface of BCD-dLhCG. CD3 is a symbol for T cells. There was

CD3 distributed both on the surface and inside LhCG. CD3 was also observed inside dLhCG. CD3 was present on the surface of BCD-LhCG. And only minimal CD3 was sparsely present in BCD-dLhCG. On day 14 post implantation (Figure 3.11b), MPO could still be found in some areas of LhCG and dLhCG, while no obvious MPO was detected in BCD-LhCG and BCD-dLhCG. CD68 was intensively distributed in LhCG and uniformly distributed in dLhCG. Nearly no CD68 was present in BCD-LhCG and BCD-dLhCG. Moreover, intensive stained CD3 could be observed inside LhCG and dLhCG. By contrast, CD3 was nearly invisible in BCD-LhCG and BCD-dLhCG.

3.3.8 PCR

qRT-PCR and agarose gel electrophoresis were carried out for evaluation of inflammatory and immune responses quantitatively and qualitatively on a gene level. Expression of inflammation-related genes involving *MCP-1*, *IL-6* and *IL-1 β* was measured. On day 1 post implantation, As shown in Figure 3.14a, LhCG expressed the most *MCP-1*, followed by PC and dLhCG. *MCP-1* expression was still detectable in BCD-LhCG and slightly visible on agarose gel. *MCP-1* was negligible in BCD-dLhCG and RE. *IL-6* showed the highest expression in PC, followed by dLhCG. There was also an obvious *IL-6* expression detected in LhCG (Figure 3.14b). PC showed the highest value in *IL-1 β* expression, followed by dLhCG and LhCG. And *IL-1 β* expression was nearly invisible in BCD-LhCG, BCD-dLhCG and RE on agarose gel (Figure 3.14c).

Figure 3.14 Gene expression of (a) *MCP-1*, (b) *IL-6*, (c) *IL-1β* for day 1 grafts and (e) *MCP-1*, (f) *IL-6*, (g) *IL-1β* for day 14 grafts, and gel electrophoresis of *GAPDH*, *MCP-1*, *IL-6* and *IL-1β* for (d) day1 grafts and (h) day14 grafts[137]. Reproduced with permission from © The Royal Society of Chemistry 2020.

On day 14 post implantation, dLhCG showed the highest *MCP-1* expression, followed by LhCG. *MCP-1* expression in PC was relatively lower than LhCG and dLhCG but still obvious. By contrast, nearly no *MCP-1* was detected in BCD-LhCG, BCD-dLhCG or RE (Figure 3.14e). *IL-6* expression could be detected in LhCG, dLhCG and PC, while that in BCD-LhCG, RE and BCD-dLhCG was invisible (Figure 3.14h) with low expression value (Figure 3.14f). As for *IL-1β* (Figure 3.14g), LhCG showed the highest expression value with PC and dLhCG followed. Moreover, there was slight *IL-1β* expressed in BCD-LhCG which was confirmed on agarose gel. However, *IL-1β* expression in RE and BCD-dLhCG was not detected.

3.4 Discussion

In this study, a facile approach for attenuation of inflammatory and immune responses of xenografts by coating albumin on the surface was introduced. LhCG

and dLhCG which were derived from porcine source ECM based on our previously published protocol were chosen as the model xenografts. First of all, in order to improve adhesion of albumin on xenografts, albumin was modified by dopamine, a mussel-inspired adhesive via EDC/NHS coupling (Figure 3.2a). The chemical structures of intermediate product BCA and final product BCD were measured by ^1H NMR (Figure 3.2b). The appearance of signals at 3.5ppm of $-\text{COCH}_2-$ and 1.1ppm of $-\text{CH}_2\text{COOH}$ confirmed the successful introduction of citric acid on BSA. Moreover, the appearance of signal at 3.2ppm of $-\text{NH}-\text{CH}_2-\text{CH}_2-$ confirmed the successful modification of BCA by dopamine. Subsequently, the feasibility of BCD coating formation was tested on a coverslip and confirmed with an increase in water contact angle of coverslip surface from 0° to $60.9^\circ \pm 0.6^\circ$ after coated with BCD (Figure 3.3).

Characterization of BCD coating on grafts were conducted by fluorescent microscopy and XPS. First of all, BCD was labeled with FITC by chemical reaction. Briefly, isothiocyanate group on FITC reacted with amino groups on BCA to obtain intermediate polymer BCA-FITC. Then BCD-FITC was synthesized through an EDC/NHS coupling between BCA-FITC and dopamine (Figure 3.4a). The successful labeling was confirmed by a UV-vis spectrometer. The absorption at around 280nm and 500nm belong to absorption of protein and bound FITC respectively. The ratio of fluorescein to final protein product (F/P) could be estimated by measuring absorbance at the two wavelengths. F/P ratio is determined as the moles of FITC to moles of protein. F/P ratios of BCA-FITC and BCD-FITC

were 2.2 and 1.5 respectively and calculated based on absorbance at around 280nm and 500nm (Figure 3.4b and 3.4c) according to equation 1. BCD-FITC was further coated onto dLhCG and LhCG, after which the samples were sectioned and observed under a fluorescent microscope. As shown in the fluorescent images, BCD was uniformly coated onto the surface of dLhCG (Figure 3.4d), while BCD was inclined to adhere to cell membrane of chondrocytes in LhCG (Figure 3.4e). The preference of BCD to cell surface compared to matrix surface in LhCG was probably caused by the mobility of cell membrane. BCD was attached to cell membrane while moving along with the cell membrane flow. Furthermore, XPS was carried out for analysis of surface chemistry. dLhCG and LhCG are porcine derived ECM based grafts, containing mainly collagen and glycosaminoglycan[13,18]. Collagen has abundant glycine residues which accounts for about 1/3 and proline residues but no tryptophan or cysteine[140], while albumin is relatively rich in cysteine[37]. In this case, albumin has more Sulphur than collagen. According to Table 3.1, Sulphur content of dLhCG surface increased to 4.1% from 3.1% after coated with BCD, while that of LhCG surface increased to 4.7% from 3.4% after coated with BCD, indicating the successful coating of BCD onto graft surface. In addition, it is worth noting that Phosphorus accounted for 1.9% on BCD-LhCG surface, much lower than that on LhCG surface (3.8%) and comparative to that of dLhCG (1.3%) and BCD-dLhCG (1.3%). dLhCG is constituted mainly by collagen and glycosaminoglycan. BCD is a dopamine modified albumin. Cell membrane has a phospholipid bilayer structure. In this case,

cell membrane was the primary source of Phosphorus, contributing to the higher Phosphorus content in LhCG than dLhCG and BCD-dLhCG. What's more, the lower Phosphorus content on BCD-LhCG surface than LhCG surface was in consistent with the fluorescent microscopy result that BCD was inclined to adhere to cell membrane in LhCG (Figure 3.4e).

Only when BCD coating survives at least until cells migrate in and proliferate, will the BCD coating work for immune evasion. In this case, durability of BCD coating on xenografts should be paid attention to. As shown in Figure 3.6a and 3.6b, there was a decrease of BCD content on day 7, which might be caused by removal of the nonspecific absorption of BCD on dLhCG and LhCG. However, after day 7, BCD content on both LhCG and dLhCG became quite stable with no obvious decrease observed as time went by. The BCD coating was stable for at least 21 days on dLhCG and LhCG, which guaranteed its use as a disguise for alleviation of acute immune and inflammatory responses.

Morphologies of dLhCG, BCD-dLhCG, LhCG and BCD-LhCG were measured under SEM. A porous structure was observed in dLhCG (Figure 3.7a), while chondrocytes assembled in a dried LhCG graft, resulting in a relative densely packed structure than dLhCG (Figure 3.7b). BCD-dLhCG kept the porous structure with BCD visible on the surface (Figure 3.7c). It is reported that physical properties like pore size[141] or stiffness[142] could affect immune and inflammatory behavior and resolution. Pore sizes of dLhCG and BCD-dLhCG are in tens of

microns which are large enough for inflammatory cells to pass and infiltrate. Therefore, the effect of pore size herein on immune and inflammatory responses could be eliminated. Similar to LhCG, clusters of chondrocytes were observed in BCD-LhCG (Figure 3.7d).

After characterization of BCD coating on grafts *in vitro*, *in vivo* evaluation of inflammatory and immune responses was measured in a rat omentum implantation model. Great omentum refers to layers of apron-like peritoneum surrounding abdominal organs, which are sensitive to foreign bodies. It will be more evident for the grafts not to elicit inflammatory and immune responses if they survive in omentum. First of all, a cytotoxicity test was conducted on dLhCG and BCD-dLhCG before they were embedded into omentum. As shown in Figure 3.8, there was a slight decrease in cell viability as time went by but still over 85% chondrocytes viable in both groups on day 7. And there was no obvious difference in cell viability between dLhCG group and BCD-dLhCG group.

First of all, no hyperacute immune response occurred with all rats alive. And it is worth noting that LhCG graft collected on day 14 was covered by blood coagulation, implying a possible severe inflammation (Figure 3.9). Then pathological analysis, IHC staining and PCR were applied for a further assessment of inflammatory and immune responses on cell, protein and gene level respectively.

Immune and inflammatory responses are highly coordinated processes involving inflammatory cells. Neutrophils are the first white blood cells to respond

to inflammation activated by foreign bodies, release factors to attract monocytes and present antigens to activate T cells[143]. Macrophages mainly carry out phagocytosis and produce growth factors and cytokines such as some pro-inflammatory cytokines like IL-6 and IL-1 β [144]. T cells are activated by antigen-receptor ligation as well as signals from antigen-presenting cells and recruited mainly to destroy cells which have been infected with antigens from transplanted foreign bodies. Performances on acute immune and inflammatory responses were evaluated in this study on day 1 and day 14 since acute immune and inflammatory responses reacted and deteriorated rapidly and would progress into persistent chronic responses[136].

On day 1 post implantation, LhCG induced the most severe immune and inflammatory responses that were comparative to that of the positive control PC. LhCG showed mild to moderate neutrophils infiltration (Figure 3.10a) observed mainly on the surface of LhCG and surrounding tissues (Figure 3.11a), moderate macrophages infiltration (Figure 3.10b) with macrophages intensively distributing in the surrounding tissues and sparsely distributing inside graft (Figure 3.11a), and mild lymphocytes infiltration (Figure 3.10c) with T cells distributing intensively on the surface of LhCG and sparsely distributing inside graft (Figure 3.11a). There was an obvious inflammation induced by dLhCG. dLhCG was infiltrated mildly by macrophages and lymphocytes and more seriously by neutrophils (Figure 3.10), indicating dLhCG was undergoing an early phase of acute immune and inflammatory responses. Moreover, inflammatory cells were found all over dLhCG

graft, especially neutrophils and macrophages (Figure 3.11a). Both LhCG and dLhCG elicit relatively severe immune and inflammatory responses because of their xenogeneic source proteins in ECM. The more serious inflammation in LhCG than dLhCG was possibly due to extra antigens present on porcine chondrocytes in LhCG leading to recognition of foreign bodies. Furthermore, inflammatory cells were observed mainly on the surface of LhCG and its surrounding tissues, while inflammatory cells infiltrated in dLhCG distributed mainly inside graft. It is because the porous structure, which was confirmed by SEM (Figure 3.7a), provided dLhCG with larger contact surface area with tissues and more space for cells to migrate in. By contrast, inflammation in the relatively densely packed LhCG was observed mostly on the surface and surrounding tissues on day 1 post implantation. However, immune and inflammatory responses were alleviated after coating xenografts with BCD. There were minimal to mild neutrophils and lymphocytes observed only on the surface and a few macrophages found inside BCD-LhCG. The slight inflammation in BCD-LhCG was probably caused by part of antigens in ECM exposed. According to *in vitro* fluorescent images (Figure 3.4e), there was a preference of BCD coating to adhere to cell membranes, causing some part of ECM exposed to tissues. Moreover, there was nearly no inflammation in BCD-dLhCG with minimal inflammatory cells infiltrated which was comparative to performance of negative control RE.

On day 14 post implantation, as the first inflammatory cells to migrate in with short lifespan, number of infiltrated neutrophils reduced to minimal in all grafts.

LhCG exhibited the most serious inflammation with mild to moderate macrophages infiltrated in LhCG (Figure 3.10b) especially at the interface of graft and surrounding tissues (Figure 3.11b), and moderate lymphocytes infiltrated (Figure 3.10c) both inside graft and on the surface of surrounding tissues (Figure 3.11b). dLhCG also induced obvious immune and inflammatory responses which were comparative to that of positive control PC. Mild to moderate macrophages and lymphocytes infiltration (Figure 3.10b and 3.10c) were observed inside dLhCG (Figure 3.11b). What's more, BCD was proved to work as a stealth coating for xenografts to evade from immune recognition with inflammatory cells nearly invisible in BCD-LhCG and BCD-dLhCG grafts.

Immune and inflammatory responses on a gene level was evaluated followed by evaluation on cell and protein levels. Inflammation related genes expression including *MCP-1*, *IL-6* and *IL-1 β* was measured. Inflammation related cytokines including *MCP-1*, *IL-6* and *IL-1 β* are another significant indicator of immune and inflammatory responses. MCP-1 regulates activation of monocytes and macrophages with monocytes/macrophages as the main source of MCP-1[127]. IL-6 is a well-recognized pro-inflammatory cytokine, contributing to recruitment of neutrophils, monocytes and T cells and differentiation of monocytes to macrophages[128]. IL-1 β is another important pro-inflammatory cytokine affecting the pathway of inflammatory reaction[145]. *MCP-1* expression of grafts was roughly in consistent with the trend of macrophages infiltrated. LhCG showed the highest *MCP-1* expression on day 1 post implantation, followed by PC and then

dLhCG. *MCP-1* was expressed in BCD-LhCG whose signal was weak but still slightly visible on agarose gel. By contrast, *MCP-1* expression was negligible in BCD-dLhCG and RE. 14 days later, LhCG and dLhCG, similar to PC, showed much higher *MCP-1* expression compared to that of BCD-LhCG, BCD-dLhCG and RE. Moreover, it is reported that MCP-1 was secreted mainly by pro-inflammatory M1 macrophages instead of anti-inflammatory M2 macrophages[141], indicating gene expression value of MCP-1 could to some extent reflect M1 macrophages infiltration conditions. According to results in Figure 7a and 7e, LhCG showed high M1 macrophages recruitment on both day 1 and day 14 which were even higher than positive control PC. M1 macrophages infiltration in dLhCG became more severe on day 14 compared to day 1. There were M1 macrophages infiltrated in BCD-LhCG graft on day 1 but reduced level was observed after 14 days. For BCD-dLhCG and negative control RE, M1 macrophages remained low in recruitment on both day 1 and day 14 based on MCP-1 expression. Furthermore, PC, dLhCG and LhCG showed high expression value in genes encoding other two pro-inflammatory cytokines IL-6 and IL-1 β on both day 1 and day 14 post implantation. However, the expression of genes encoding pro-inflammatory cytokines was reduced or even undetectable after coating xenografts with BCD, which was comparative to the results of negative control RE and indicated BCD could work for immune evasion. However, it is worth noting that *MCP-1* expression on day 1 post implantation and *IL-1 β* expression on day 14 post implantation in BCD-LhCG were reduced compared to LhCG but still detectable, implying slight immune and inflammatory

responses were induced due to exposed antigens on porcine ECM possibly caused by uneven coverage of BCD on LhCG.

3.5 Summary

In this chapter, a facile method for immune evasion of xenografts was developed by coating albumin with passivation property. First of all, albumin was successfully modified by dopamine via EDC/NHS coupling for enhancement of adhesion on grafts. LhCG and dLhCG fabricated based on our previous published protocol were chosen as the xenograft models. Dopamine modified albumin (BCD) was uniformly coated onto the surface of dLhCG, while BCD was more inclined to adhere to cell membrane than ECM in LhCG according to fluorescent images. The increase in Sulphur of BCD-LhCG and BCD-dLhCG compared to LhCG and dLhCG respectively further confirmed successful coating of BCD on the grafts. Moreover, BCD coating remained stable on both LhCG and dLhCG for at least 21 days, despite a decrease on day 7 compared to day 1. Acute immune and inflammatory responses were evaluated on cell, protein and gene levels by pathological assessment, IHC staining and PCR, respectively. LhCG showed the most serious inflammation, followed by dLhCG. However, BCD coating was proved to effectively attenuate immune and inflammatory responses with reduced inflammatory cells infiltrated and lower gene expression encoding inflammation related cytokines. Slight immune and inflammatory responses were observed in BCD-LhCG due to the possible exposed antigens on ECM, while inflammation was

negligible in BCD-dLhCG. In summary, albumin coating works for alleviation of acute immune and inflammatory responses induced by LhCG and dLhCG, which could be further applied to other xenografts. In the long run, future investigations will focus on regeneration of stabilized albumin coated engineered xenografts.

Supplementary data

Table S3.1 Primer sequences for qRT-PCR and PCR

Gene name	PCR	Sequence (5'→3')		Product size (dp)
<i>MCP-1</i>	qRT-PCR	Forward primer	TGA ACT TGA CCC ATA AAT CTG AAG	121
		Reverse primer	AAG GCA TCA CAT TCC AAA TCA C	
	PCR	Forward primer	ACC TGC TGC TAC TCA TTC AC	325
		Reverse primer	CTA CAG AAG TGC TTG AGG TG	
<i>IL-1β</i>	qRT-PCR	Forward primer	GAG TCT GCA CAG TTC CCC AA	89
		Reverse primer	ATG TCC CGA CCA TTG CTG TT	
	PCR	Forward primer	GAC CTG TTC TTT GAG GCT GAC	578
		Reverse primer	TCC ATC TTC TTC TTT GGG TAT TGT T	
<i>IL-6</i>	qRT-PCR	Forward primer	TGC CCT TCA GGA ACA GCT ATG	102
		Reverse primer	TGA AGT AGG GAA GGC AGT GG	
	PCR	Forward primer	CTC TGA ATG ACT CTG GCT TTG	390
		Reverse primer	CTT GGG ACT GAT GTT GTT GAC	
<i>GAPDH</i>	qRT-PCR	Forward primer	CTCCCATTCCTCCACCTTTG	185
		Reverse primer	CTTGCTCTCAGTATCCTTGC	
	PCR	Forward primer	TGC CAT CAA CGA CCC CTT CA	569
		Reverse primer	TGA CCT TGC CCA CAG CCT TG	

Chapter 4 Dopamine modified natural biological coating on ECM based grafts for enhanced host-graft interfacing affinity

4.1 Introduction

In the previous two chapters, we mainly focused on natural biological coatings for enhanced immunocompatibility of xenografts. Particularly, stabilized albumin was coated on LhCG and dLhCG as a “stealth” coating to attenuate immune and inflammatory responses induced by xenografts. In order to improve adhesion of albumin on grafts, dopamine, a mussel inspired adhesive, was applied for modification of albumin. It is reported that dopamine would form chelating bonds with metal ions in mild basic environment and reinforce its adhesion property[146]. Therefore, we considered establishing an adhesive extracellular matrix (ECM) based graft, decellularized living hyaline cartilage graft (dLhCG) particularly in this study, by combining dopamine functionalized coating and introducing ferric ions. The adhesive ECM based graft was aimed at solving the fixation failure between host tissues and grafts, another issue that need to be addressed for clinical use.

As is mentioned in chapter 1, displacement or even fall-off of grafts after implantation would cause failure of implants. In this case, the interfacing affinity between grafts and host tissues need to be taken into consideration before clinical translation. The current existing fixation methods such as sutures or staples are not suitable for ECM based grafts considering its porous structure. Therefore, we

consider preparing an adhesive ECM based graft by coating a prepolymer based on dopamine first and introducing ferric ions later *in situ*.

Figure 4.1 Schematic illustration of the technique design.

In this chapter, two kinds of adhesive prepolymer based on dopamine with chondroitin sulfate (CS) and serum albumin (SA) as backbone were coated on

dLhCG and chelated with ferric ions *in situ* for enhancement of interfacing affinity of dLhCG in cartilage defects. The basic idea illustrating this technique was shown in [Figure 4.1](#). A polymer backbone was needed to eliminate the possible toxicity of dopamine monomer caused to human and avoid formation of a rigid crosslink structure. CS, a cartilage derived sulfate glycosaminoglycan (GAG), was applied as the main chain with no impurities introduced. Moreover, another kind of natural biological materials SA, a commonly used biocompatible protein, was also applied as backbone for comparison. The adhesive dLhCG was evaluated from aspects including morphology, cytotoxicity, adhesion strength and chondrocytes migration.

4.2 Materials and methods

4.2.1 Materials

All the chemicals were purchased from Sigma Aldrich unless otherwise mentioned. 1×phosphate buffered saline (PBS) was purchased from Gibco. Tris buffer (1.0M, pH=8.8) was purchased from 1st Base Pte Ltd, Singapore.

4.2.2 Preparation of dopamine modified chondroitin sulfate (CSD) and dopamine modified albumin (BCD)

Dopamine modified chondroitin sulfate (CSD) was prepared based on an EDC/NHS coupling reaction[147]. Firstly, chondroitin sulfate sodium salt (CS) was dissolved in 1×PBS and transferred to a Schlenk tube. Then EDC and NHS were added and activated for 1h under N₂ protection and 37°C with pH 6.0. Dopamine

hydrochloride in 1×PBS was subsequently added followed by three cycles of freeze-thaw to eliminate oxygen in the system to avoid oxidation of dopamine during the reaction. The mixture reacted under N₂ protection and 37°C with pH 6.0 for 6h. The final product CSD was collected and dialyzed against deionized (DI) water for 2 days followed by freeze-drying (BT48, Millrock Technology Inc, USA).

Dopamine modified albumin (BCD) was also prepared based on an EDC/NHS coupling reaction[68]. Firstly, 1g citric acid (CA) was dissolved in 1×PBS (0.2g/mL) and added to a three-neck flask followed by addition of 0.3g EDC and 0.3g NHS. 1.75g bovine serum albumin (BSA) in 10mL 1×PBS was added afterwards dropwise. The mixture reacted under N₂ protection and 37°C with pH 6.0 for 6h. Prepolymer BCA was collected after dialysis and freeze-dry of the reacted mixture. Subsequently, BCA (1g), dopamine hydrochloride (1g), EDC (1g) and NHS (1g) were dissolved in 1×PBS (100mL) and transferred to a Schlenk tube, followed by three cycles of freeze-thaw to eliminate oxygen in the system to avoid oxidation of dopamine during the reaction. The mixture reacted under N₂ protection and 37°C with pH 6.0. The final product BCD was collected after dialysis against DI water for 2 days and lyophilization.

Chemical structures of the involved products above were characterized by Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) (PerkinElmer Spectrum One) and Proton Nuclear Magnetic Resonance (¹H NMR) (Bruker Avance II 300MHz NMR) with deuterium oxide (D₂O) as solvent.

4.2.3 Fabrication of decellularized living hyaline cartilage graft (dLhCG)

dLhCG was fabricated according to our previous publications[17–19]. Porcine chondrocytes were collected from porcine cartilage and embedded in alginate hydrogel together with gelatin microspheres. Chondrocytes grew and proliferated in the 3D hydrogel, secreting ECM for graft establishment. Removal of alginate hydrogel was carried out with the help of sodium citrate solution. Afterwards, LhCG was collected and went through physical, chemical and enzymatic decellularization methods for fabrication of dLhCG.

4.2.4 Fabrication of CSD or BCD coated dLhCG and chelation with Fe^{3+}

dLhCG was rinsed in CSD (200mg/mL) or BCD (50mg/mL) solution in Tris buffer (10mM, pH 8.5) at room temperature (RT) for 1h, one group of which was rinsed in 150 μ L solution without wash afterwards (C/B-dLhCG-no wash) while the other group was rinsed in 500 μ L solution and washed three times by DI water after coating (C/B-dLhCG-wash). The weights of dLhCG both before and after coating were recorded to calculate the mass of CSD or BCD. CSD or BCD coated dLhCG was freeze-dried and kept in 4°C for further use.

C/B-dLhCG-wash or C/B-dLhCG-no wash swelled in Tris buffer (10mM, pH 8.5) first, after which $FeCl_3$ of certain concentration was added for chelating of CSD/BCD with Fe^{3+} and preparation of chelated C/B-dLhCG-wash (CFe/BFe-dLhCG-wash) or C/B-dLhCG-no wash (CFe/BFe-dLhCG-no wash).

4.2.5 Water contact angle

CSD or BCD was dissolved in Tris buffer (10mM, pH 8.5), coated on porcine cartilage surface and dried at room temperature for 2h. Water contact angles of CSD or BCD coated porcine cartilage surfaces as well as uncoated surface were tested afterwards on a Kruss DSA25 Contact Angle Analyzer with a 5 μ L DI water drop for evaluating feasibility of CSD or BCD to form coating on dLhCG.

4.2.6 Morphology of grtafts

Morphology of grafts was observed under scanning electron microscopy (SEM, JEOL JSM 6700F, Japan). All samples were freeze-dried and coated with platinum by sputtering (JFC-1600, JEOL Asia Pte. Ltd., Japan) for 150s at 10mA before observation.

4.2.7 Lap shear strength test

First of all, the adhesion of two slices of cartilage with CSD or BCD as adhesive was measured for comparison as well as for optimization of CSD or BCD concentration, Fe³⁺ concentration and incubation time. CSD or BCD in Tris buffer of certain concentration (500mg/mL, 200mg/mL, 100mg/mL and 50mg/mL) was mixed with FeCl₃ Tris buffer solution of certain concentration (200mM, 100Mm, 50mM and 0mM) *in situ* on one slice of cartilage, after which the two slices of cartilage were glued together. Subsequently, the adhesive was dried *in situ* in fume hood for 1h. Then the samples were incubated in a 37°C water bath for 15min, 1h

and 3h, respectively.

The adhesion between CFe-dLhCG or BFe-dLhCG and cartilage was measured by a lap shear strength test. First, two slices of cartilage with a dimension of around 2mm×8mm×20mm were collected from commercially available porcine bones. The exposed surface of cartilage was shaved by a knife to mimic cartilage surface of osteoarthritis patients and used for adhesion. A hole with a diameter of 6mm was punched on one slice of cartilage and filled with dLhCG or dLhCG coated with adhesive macromolecules. C/B-dLhCG were infiltrated with Tris buffer (10mM, pH 8.5) *in situ* first followed by FeCl₃ in Tris buffer. Then the other slice of cartilage without hole was adhered to the one embedded with CFe/BFe-dLhCG. Afterwards, the adhesive was dried *in situ* in fume hood for 1h. Then the samples were incubated in a 37°C water bath for 3h. C/B-dLhCG, dLhCG infiltrated with only Tris buffer or FeCl₃ in Tris buffer was also tested as controls.

The lap shear strength test was conducted on a tensile meter (5543 Tensile Meter) and illustrated in Figure 4.2.

Figure 4.2 Schematic illustration of lap shear strength test between cartilage and graft.

4.2.8 *In vitro* cytotoxicity

In vitro cytotoxicity of CFe-dLhCG and BFe-dLhCG on chondrocytes was evaluated by a WST-1 Assay Kit (Sigma Aldrich, Singapore). First of all, chondrocytes were seeded in 96 well plates (2500 cells/well) and incubated in 37°C incubator in contact with CFe-dLhCG and BFe-dLhCG for 1d, 4d and 7d, respectively. 10µL WST-1 was added to the 100µL culture medium at corresponding time point and incubated together with chondrocytes for 3h in a 37°C CO₂ incubator. The absorbance of incubated mixture was read and calculated on a microplate reader (Thermo Electron Corporation, USA) based on the manufacturer's protocol. Cytotoxicity of dLhCG and C/B-dLhCG was also evaluated as controls.

4.2.9 Migration of chondrocytes in grafts

In order to evaluate chondrocytes migration from cartilage to grafts, an *in vitro* model by cartilage ring embedded with grafts was developed (Figure 4.3). First of all, a disc of cartilage (8mm in diameter, ~2mm in thickness) was punched from commercially available porcine bones, after which the rough surface of the cartilage was polished by a knife to eliminate other cells. Cartilage rings were made by punching and removing a cartilage disc with 6mm in diameter in the center of the 8mm-diameter cartilage disc. The cartilage rings (1mm in width, ~2mm in thickness) embedded with grafts were incubated with high glucose Dulbecco's Modified Eagle's Medium (DMEM) in a 24 well plate, the well bottom of which was coated with agarose, in a 37°C CO₂ incubator and collected on day 15 and day 30

respectively.

Figure 4.3 Schematic illustration of cell migration experiments design.

The collected cartilage rings embedded with grafts were fixed in 4w/v% paraformaldehyde, dehydrated and embedded in paraffin (Surgipath® Paraplast®, Leica, USA). Then the samples were sectioned to 10µm thick on a slicer (RM2255, Leica, USA). The slides were stained with 4',6-diamidino-2-phenylindole (DAPI) and observed under a fluorescent microscope (Olympus IX71S1F-3, Japan).

4.3 Results

4.3.1 Characterization of chemical structures of CSD and BCD

CS was modified by dopamine via an EDC/NHS coupling (Figure 4.4a). The appearance of peak at around 6.7ppm on ^1H NMR spectrum of CSD which belongs to H on benzene ring of dopamine confirmed the successful addition of dopamine to CS (Figure 4.4b). Moreover, the two peaks at around 2.8ppm and 2.3ppm on ^1H NMR spectrum of CSD belong to $-\text{CH}_2-$ of dopamine, further proving successful synthesis of CSD. The synthesis of BCD and ^1H NMR spectra were demonstrated

in Figure 3.2 in chapter 3.

Figure 4.4 (a) Synthesis of CSD, (b) ¹H NMR spectra of CS, dopamine and CSD, respectively.

ATR-FTIR was also carried out for determination of chemical structures of CS, CSD, BSA and BCD. The appearance of peak at around 1549cm^{-1} on ATR-FTIR spectrum of CSD confirmed the existence of benzene ring belonging to dopamine on CSD (Figure 4.5a). However, there was no obvious difference in ATR-FTIR spectrum between BSA and BCD since BSA is a protein with benzene ring from units like tyrosine (Tyr) and phenylalanine (Phe). The peak at around 1648cm^{-1} on ATR-FTIR spectra of BSA and BCD belongs to amide I of protein, while that at around 1285cm^{-1} and 1393cm^{-1} belong to amide III (Figure 4.5b).

Figure 4.5 ATR-FTIR spectra of (a) CS, CSD and (b) BSA, BCD, respectively.

In order to evaluate the feasibility of CSD or BCD to form a coating on dLhCG, cartilage was applied as the substrate for a wettability measurement. dLhCG was derived from chondrocytes, composed mainly of chondrocytes secreted ECM which is rich in collagen and designed to reconstruct cartilage structure. Therefore, cartilage herein could mimic dLhCG for the water contact angle test. The decrease of water contact angle of cartilage surface from $77.9^{\circ} \pm 2.2^{\circ}$ to $32.8^{\circ} \pm 3.5^{\circ}$ after coating CSD confirmed the existence and successful adhesion of CSD on cartilage (Figure 4.6). Similarly, the decrease of water contact angle of cartilage surface from $77.9^{\circ} \pm 2.2^{\circ}$ to $60.2^{\circ} \pm 5.8^{\circ}$ after coating BCD confirmed the existence and successful adhesion of BCD on cartilage (Figure 4.6).

Figure 4.6 Water contact angle of cartilage surface and CSD or BCD coated cartilage surfaces.

4.3.2 Morphology of grafts

Chondrocytes migration into grafts after implantation is of vital importance for cartilage repair and regeneration. Therefore, whether the porous structure of dLhCG

would be blocked or not after coating with CSD or BCD needs to be investigated. Furthermore, both C/B-dLhCG-wash and C/B-dLhCG-no wash were observed under SEM.

As shown in Figure 4.7b and 4.7f, dLhCG had a porous structure with a pore size of hundreds of microns. CSD macromolecules also had a porous structure (Figure 4.7a and 4.7e). After coated with CSD, dLhCG remained porous with decreased pore sizes (around tens of microns). Specifically, a honeycomb-like structure was detected on surface of C-dLhCG-no wash with a pore size of around tens of microns.

Figure 4.7 SEM images of (a, e) CSD, (b, f) dLhCG, (c, g) C-dLhCG-wash and (d, h) C-dLhCG-no wash under $\times 300$, $\times 600$ magnification, respectively.

BCD showed a sheet-like structure under SEM (Figure 4.8a and 4.8e). BCD was observed twined on dLhCG for B-dLhCG-wash (Figure 4.8c and 4.8g) and accumulating on surface of dLhCG in form of sheets for B-dLhCG-no wash (Figure 4.8d and 4.8h). It is worth noting that B-dLhCG-wash changed the least in pore size in comparison with dLhCG.

Figure 4.8 SEM images of (a, e) BCD, (b, f) dLhCG, (c, g) B-dLhCG-wash and (d, h) B-dLhCG-no wash under $\times 300$, $\times 600$ magnification, respectively.

4.3.3 Lap shear strength

The adhesion between grafts and cartilage was measured by lap shear test, before which factors including CSD or BCD concentration, Fe^{3+} concentration and incubation time in 37°C water bath were optimized by lap shear test between two slices of cartilage.

For determination of CSD or BCD concentration, Fe^{3+} concentration and incubation time was fixed at 50mM and 3h, respectively. It is shown in [Figure 4.9a](#) that there was a decrease in lap shear strength when BCD concentration decreased from 500mg/mL to 200mg/mL. However, the lap shear strength increased to $69.9 \pm 11.1 \text{ kPa}$ and $78.3 \pm 10.0 \text{ kPa}$ respectively after BCD concentration further decreased to 100mg/mL and 50mg/mL. The highest lap shear strength ($163.3 \pm 9.1 \text{ kPa}$) appeared as CSD concentration reached 200mg/mL. CSD of higher concentration (500mg/mL) and lower concentration (100mg/mL and 50mg/mL) resulted in a much lower lap shear strength with $38.4 \pm 3.9 \text{ kPa}$ at 500mg/mL and

9.4±0.3kPa, 8.3±0.3kPa at 100mg/mL and 50mg/mL (Figure 4.9d). Therefore, 50mg/mL BCD and 200mg/mL CSD were applied for further optimization.

Figure 4.9 Lap shear strength between two slices of cartilage glued by BCD adhesive with different (a) BCD concentration, (b) Fe^{3+} concentration and (c) incubation time or CSD adhesive with different (d) CSD concentration, (e) Fe^{3+} concentration and (f) incubation time.

For determination of Fe^{3+} concentration, 50mg/mL BCD and 200mg/mL CSD were used with incubation time fixed at 3h. For 50mg/mL BCD, there was an increase in lap shear strength when Fe^{3+} concentration decreased from 200mM to

100mM, after which a downward trend was detected as Fe^{3+} concentration further decreased from 100mM to 0, indicating 100mM as the optimized Fe^{3+} concentration with a lap shear strength of $136.2 \pm 14.8 \text{ kPa}$ (Figure 4.9b). As shown in Figure 4.9e, lap shear strength increased as Fe^{3+} concentration decreased from 200mM to 50mM with a peak value of $163.3 \pm 9.1 \text{ kPa}$ at 50mM. Then CSD lost adhesion after Fe^{3+} concentration reached as low as 10mM. Moreover, 50mg/mL BCD by itself without chelating showed weak adhesion between cartilage ($3.7 \pm 1.8 \text{ kPa}$), while 200mg/mL CSD without chelating did not show potential as adhesive by itself.

Therefore, 200mg/mL CSD chelating with 50mM FeCl_3 and 50mg/mL BCD chelating with 100mM FeCl_3 were chosen for optimization of incubation time. Both showed the strongest adhesion after 3h incubation in 37°C water bath (Figure 4.9c and 4.9f). Lap shear strength became higher as incubation time prolonged for BCD. For CSD, little difference was detected in lap shear strength after 15min- or 1h-incubation.

As a result, 200mg/mL CSD chelating with 50mM FeCl_3 and 50mg/mL BCD chelating with 100mM FeCl_3 with incubation time of 3h were decided as the optimized working conditions for adhesion.

With optimal working concentrations and incubation time determined, we conducted lap shear strength test between grafts and cartilage based on the model illustrated in Figure 4.2. Lap shear strengths of dLhCG swelling solely in Tris buffer or FeCl_3 solution of certain concentration (50mM for CSD related groups and

100mM for BCD related groups) and C/B-dLhCG were also measured as controls. Both CFe-dLhCG and BFe-dLhCG showed an enhanced lap shear strength with cartilage compared to dLhCG (Figure 4.10). Specifically, the lap shear strength between CFe-dLhCG-wash and cartilage reached 72.6 ± 8.3 kPa, while that of CFe-dLhCG-no wash with cartilage was 70.9 ± 16.6 kPa (Figure 4.10a). Similarly, the lap shear strength between BFe-dLhCG-wash and cartilage reached 55.2 ± 0.8 kPa, while that of BFe-dLhCG-no wash with cartilage was 62.3 ± 9.4 kPa (Figure 4.10b). Moreover, B-dLhCG exhibited a slightly higher lap shear strength with cartilage (3.5 ± 0.3 kPa) than dLhCG (0.5 ± 0.1 kPa), further confirming BCD's adhesion by nature. However, it is notable that no obvious difference was detected between samples that was washed and not washed before freeze-dry during fabrication, which indicates covering dLhCG with accumulated CSD or BCD does not make any difference compared to coating dLhCG with CSD or BCD through adherence by dopamine. What's worse, the accumulated CSD or BCD on dLhCG would result in a reduced pore size smaller than the washed samples according to SEM images (Figure 4.7 and 4.8). Therefore, CFe/BFe-dLhCG-wash was selected for further investigations on cells. C/B-dLhCG and CFe/BFe-dLhCG in the following two sections 4.3.4 and 4.3.5 refers in particular to C/B-dLhCG-wash and CFe/BFe-dLhCG-wash, respectively.

Figure 4.10 Lap shear strength between cartilage and (a) dLhCG, Fe-dLhCG, C-dLhCG, CFe-dLhCG-wash and CFe-dLhCG-no wash or (b) dLhCG, Fe-dLhCG, B-dLhCG, BFe-dLhCG-wash and BFe-dLhCG-no wash, respectively.

4.3.4 *In vitro* cytotoxicity

In order to figure out effects of CSD or BCD concentration and ferric ions' concentration on chondrocytes viability, *in vitro* cytotoxicity was measured by WST-1 based on Fe^{3+} concentration and CSD/BCD concentration. First of all, chondrocytes incubated together with dLhCG showed viability of $103.0 \pm 6.3\%$, $106.1 \pm 24.4\%$ and $106.2 \pm 10.4\%$ on day 1, day 4 and day 7 respectively. The effect of Fe^{3+} concentration on cytotoxicity involving 200mM, 50mM and 10mM was investigated and shown in Figure 4.11. BCD and CSD involved herein was fixed at 200mg/mL. According to the results, the introduction of CSD or BCD did not sacrifice much cytocompatibility of dLhCG. And there was comparable cell viability between C-dLhCG and B-dLhCG in general. However, when C/B-dLhCG chelated with 200mM FeCl_3 , chondrocytes viability was remarkably reduced. Moreover, there is an increase of chondrocytes viability with the decrease of FeCl_3 concentration for BFe-dLhCG. As for CFe-dLhCG, the cytotoxicity increased as

FeCl₃ concentration increased on day 1. But there was not obvious difference between 50mM FeCl₃ and 10mM FeCl₃, which performed better in cytocompatibility than 200mM on day 4 and day 7.

Figure 4.11 WST-1 results based on different Fe³⁺ concentration on (a) day 1, (b) day 4 and (c) day 7.

The effect of various CSD or BCD concentration on cytotoxicity was shown in [Figure 4.12](#). FeCl₃ concentration involved herein was fixed at 50mM. For C-dLhCG and CFe-dLhCG, a rough trend of lower CSD concentration accompanying with better cytocompatibility was observed. As for B-dLhCG and BFe-dLhCG, BCD concentration did not conduct obvious influence on chondrocytes viability on day

1, while BCD concentration of 50mg/mL showed mildly better performance than that of 100mg/mL and 200mg/mL on day 4 and day 7 in general.

Figure 4.12 WST-1 results based on different CSD or BCD concentration on (a) day1, (b) day4 and (c) day7.

4.3.5 Migration of chondrocytes in grafts

Cell migration in grafts is of significance for tissue repair and regeneration. Therefore, whether CSD or BCD coating and the chelating structure with Fe^{3+} would interfere chondrocytes migration or not should be taken into consideration and worth investigation. Chondrocytes migration was carried out based on a model

illustrated in Figure 4.3, the results of which were shown in Figure 4.13.

Figure 4.13 Cell migration by cartilage to (a, b) dLhCG, (c, d) C-dLhCG, (e, f) CFe-dLhCG, (g, h) B-dLhCG and (i, j) BFe-dLhCG on day15 and day 30, respectively. The white dash line divides cartilage and grafts.

As shown in [Figure 4.13a](#), migration of chondrocytes from cartilage to dLhCG mainly occurred on the surface of dLhCG after 15 days. By comparison, on day 15, few chondrocytes moved into C-dLhCG ([Figure 4.13c](#)) and B-dLhCG ([Figure 4.13g](#)) from cartilage. There were more chondrocytes infiltrated in CFe-dLhCG ([Figure 4.13e](#)) and BFe-dLhCG ([Figure 4.13i](#)) after 15 days, which were chelated with Fe^{3+} compared with corresponding two groups without chelation.

On day 30, nearly all chondrocytes migrate in dLhCG out of cartilage ([Figure 4.13b](#)). Cell migration was also obvious in B-dLhCG ([Figure 4.13h](#)), while cells migrated in C-dLhCG ([Figure 4.13d](#)) was much less than B-dLhCG on day 30 but increased slightly compared to C-dLhCG on day 15. Chondrocytes infiltration performed better in C-dLhCG after chelating with Fe^{3+} ([Figure 4.13d](#)). However, nearly no more chondrocytes moved into BFe-dLhCG from day 15 to day 30 ([Figure 4.13j](#)).

In summary, B-dLhCG showed the best performance in chondrocytes migration, but dramatically decreased in the capability of accommodating chondrocytes after chelating with Fe^{3+} . Although C-dLhCG exhibited no advantages in accommodation of chondrocytes compared to B-dLhCG, chondrocytes infiltration in CFe-dLhCG was much more significant than both in C-dLhCG and in BFe-dLhCG.

4.4 Discussion

In this chapter, the other problem of tissue engineering to be addressed, the

interfacing affinity of grafts on host tissues which referred in particular to cartilage herein, was paid attention to and investigated. TISSEEL, a fibrin glue developed by Baxter, is widely used as a commercially available bioglue in surgery. TISSEEL consists of two main components, one of which is human fibrinogen as clottable protein and the other of which is human thrombin and could realize immediate sealing by a principle of clotting[99]. Although TISSEEL is nowadays a dominant sealant in market with advantages in fast working and strong adhesion between tissues for wound closing, its resultant coagulating clot without a porous structure might block grafts pores when applied in tissue engineering for fixation of grafts, which leads to failure in cell migration and final failure of grafts and makes it insufficient for tissue engineering. As shown in SEM images in [Figure 4.14](#), the porous structure of dLhCG after coated with TISSEEL was destroyed. Moreover, TISSEEL with fibrinogen and thrombin as main components is human blood product, the safety and contamination of which are still under consideration. Therefore, an adhesive to enhance adhesion between grafts and tissues with interpenetrating crosslinking structure by chemical reactions or physical interactions with good biocompatibility and less impurities needs to be developed.

Two bioadhesives developed by our group, CSD and BCD, were introduced in this chapter and coated onto dLhCG to enhance the adhesion between cartilage and dLhCG, a tissue-engineered cartilage graft developed by our group. The adhesion was achieved after addition of FeCl_3 and by the chelation between catechol on dopamine and Fe^{3+} . CS, a cartilage derived sulfate GAG, or BSA, a common

biocompatible plasma protein, were selected as backbone for the adhesives, respectively. Dopamine, a mussel-inspired adhesive molecule, was introduced as functional units to carry out adhesive property by chelating with Fe^{3+} under alkaline environment. CS is an ideal backbone of adhesive aimed at cartilage tissue engineering since CS is cartilage derived which means no impurities will be introduced when CS is used. BCD was also investigated considering its good performance as sealant[68].

Figure 4.14 SEM images of TISSEEL-dLhCG under (a) x300 and (b) x600 magnification, respectively.

First of all, CSD and BCD, whose chemical structures were characterized by ^1H NMR and ATR-FTIR, were synthesized by EDC/NHS coupling. The appearance of peak at around 6.7ppm on ^1H NMR spectrum of CSD (Figure 4.4b) and the peak at around 1549cm^{-1} on ATR-FTIR spectrum of CSD (Figure 4.5a) confirmed the existence of benzene ring belonging to dopamine on CSD. Moreover, the two peaks at around 2.8ppm and 2.3ppm on ^1H NMR spectrum of CSD belonging to $-\text{CH}_2-$ of dopamine also proved the successful synthesis of CSD. Chemical structures of BCD could be referred to in chapter 3 (Figure 3.2).

CSD and BCD were designed to coat on dLhCG first by polymerization of dopamine under alkaline environment. Before coating CSD or BCD on dLhCG, the feasibility of CSD or BCD to retain on surface of dLhCG as a coating was investigated with cartilage surface as a mimic of dLhCG surface. dLhCG, a chondrocytes-derived cartilage graft composed mainly of chondrocytes secreted ECM, was developed as a graft to restore cartilage components and structure for cartilage tissue repair. However, its porous structure made it difficult for surface analysis such as wettability test. Therefore, porcine cartilage surface, which shares similar source and structure with dLhCG, was chosen as a mimic of dLhCG surface for water contact angle test. The decrease of water contact angle of cartilage surface from $77.9^{\circ} \pm 2.2^{\circ}$ to $32.8^{\circ} \pm 3.5^{\circ}$ and $60.2^{\circ} \pm 5.8^{\circ}$ after coating CSD and BCD respectively confirmed the existence and successful adhesion of CSD and BCD on cartilage (Figure 4.6). Furthermore, the successful coating of BCD on dLhCG has also been proved by fluorescent microscopy for detection of FITC labeled BCD and illustrated in chapter 3 (Figure 3.4d).

Whether CSD or BCD coating will block porous structures of dLhCG or not is the first issue that should be taken into consideration. After determining chemical structures of CSD and BCD and their feasibility to form coatings on dLhCG, we carried out SEM observation for morphology characterization. It is observed CSD had a porous structure while BCD had a sheet structure. Pore size of dLhCG decreased to tens of microns from hundreds of microns with CSD coatings, especially C-dLhCG-no wash. C-dLhCG-no wash was observed with a

honeycomb-like structure (Figure 4.7d and 4.7h). BCD was detected twining on dLhCG from SEM images of B-dLhCG-wash (Figure 4.8c and 4.8g) and accumulating on dLhCG sheets by sheets from SEM images of B-dLhCG-no wash (Figure 4.8d and 4.8h). Decrease of pore size in B-dLhCG-wash, B-dLhCG-no wash was not as obvious as that of C-dLhCG-wash, C-dLhCG-no wash.

Adhesive strength between grafts and cartilage was investigated in form of lap shear strength, before which the working concentration of CSD or BCD and Fe^{3+} as well as incubation time in 37°C water bath were optimized. An optimization was necessary in that the adhesion was mainly achieved by chelating between Fe^{3+} with catechol groups on dopamine, the chelation structure was reported to be one ferric ion bond with three catechol groups[146]. An optimal combination of CSD or BCD concentration and Fe^{3+} concentration should be found. For BCD concentration, adhesion became generally stronger as BCD concentration decreased from 500mg/mL to 50mg/mL (Figure 4.9a), thus 50mg/mL BCD with a highest lap shear strength of $78.3 \pm 10.0 \text{ kPa}$ was chosen as the working concentration, after which a Fe^{3+} concentration of 100mM was decided (Figure 4.9b). For CSD concentration, the strongest adhesion ($163.3 \pm 9.1 \text{ kPa}$) appeared when 200mg/mL CSD was applied (Figure 4.9d) with Fe^{3+} concentration of 50mM (Figure 4.9e). Moreover, the adhesive strength was enhanced with an increased incubation time (Figure 4.9c and 4.9f). Finally, an incubation time of 3h was chosen.

The optimized working concentration and incubation time were applied further

in measurement of adhesive strength between dLhCG and cartilage. dLhCG with both CSD coating and Fe^{3+} chelating showed enhanced adhesive strength of over 70kPa with CFe-dLhCG-wash of $72.6 \pm 8.3 \text{ kPa}$ and CFe-dLhCG-no wash of $70.9 \pm 16.6 \text{ kPa}$. Similarly, both BFe-dLhCG-wash ($55.2 \pm 0.8 \text{ kPa}$) and BFe-dLhCG-no wash ($62.3 \pm 9.4 \text{ kPa}$) also showed enhanced adhesive strength. However, no obvious difference was detected between the two kinds of process methods (wash and no wash), indicating covering dLhCG with accumulated CSD or BCD by external force would not contribute to better adhesive performances. It might be due to the failure of contact and function of excess CSD or BCD with the interfaces as effective component. Therefore, CFe-dLhCG-wash and BFe-dLhCG-wash were chosen for further investigations with cells. And CFe/BFe-dLhCG mentioned in [Figure 4.11-Figure 4.13](#) referred in particular to CFe/BFe-dLhCG-wash. In addition, lap shear strength between dLhCG and cartilage with TISSEEL as adhesive was also measured as control with a lap shear strength value of $80.9 \pm 14.6 \text{ kPa}$.

In vitro cytotoxicity on chondrocytes was studied before cell migration test to find out influences of CSD or BCD concentration and Fe^{3+} concentration on chondrocytes viability. According to [Figure 4.11](#), higher cell viability was observed with lower FeCl_3 concentration from 200mM to 10mM in BFe-dLhCG-wash. B-dLhCG showed relatively higher cytotoxicity than BFe-dLhCG-wash with 10mM Fe^{3+} on day 1 and day 4, which might be caused by release of BCD solution to the medium in the first few days considering the small sheets-like structure of BCD ([Figure 4.8a](#) and [4.8e](#)). There was generally an increase in chondrocytes viability

with a decreased FeCl_3 concentration from 200mM to 0 in CFe-dLhCG-wash. 50mM FeCl_3 concentration samples and 10mM FeCl_3 concentration samples showed similar cell viability on day 4 and day 7 for CFe-dLhCG-wash. As shown in [Figure 4.12](#), chondrocytes viability was slightly weakened by CSD of higher concentration but was nearly not affected by BCD concentration in general. Therefore, for CFe/BFe-dLhCG-wash, ferric ion's concentration plays a more significant role than CSD or BCD concentration in cytotoxicity.

During tissue repair, cells migrate from surrounding tissues to grafts, proliferate and grow in grafts to realize tissue regeneration. Thus, whether the coating and the chelating structure would hinder chondrocytes migration or not worth investigating. Herein, samples were embedded in cartilage rings, which served as chondrocytes source, and incubated in high glucose DMEM. As can be seen in [Figure 4.13](#), chondrocytes migration was not obvious or mainly appeared on grafts surface initially after 15 days incubation. And only several chondrocytes could be detected in C-dLhCG-wash and B-dLhCG-wash on day 15, which might be due to the reduced pore size of C/B-dLhCG-wash. CFe/BFe-dLhCG-wash accommodated comparable number of chondrocytes with dLhCG, which performed better than C/B-dLhCG-wash, indicating the interpenetrating structure achieved by chelation had improved porous structure than dLhCG covered with CSD or BCD on surfaces.

30 days later, cell migration to dLhCG was the most significant with almost all

chondrocytes moving from cartilage to dLhCG. B-dLhCG-wash came next with abundant chondrocytes infiltrated. On the contrary, scarce chondrocytes migrated to C-dLhCG-wash. The reason why there was a sharp difference between B-dLhCG-wash and C-dLhCG-wash might lie in the narrowed pore sizes after coating. It is shown in SEM images that pore size of C-dLhCG-wash decreased to tens of microns, while that of B-dLhCG-wash remained hundreds of microns though reduction still existed compared to dLhCG. However, when it comes to samples with Fe^{3+} chelation, no more chondrocytes appeared in BFe-dLhCG-wash from day 15 to day 30, while CFe-dLhCG-wash accommodated moderate chondrocytes on day 30. On the other hand, cell migration became more significant in C-dLhCG-wash after chelating with ferric ions, which indicates CFe-dLhCG-wash with interpenetrating network after chelation has improved pore size compared to C-dLhCG-wash. B-dLhCG-wash showed a nice performance in chondrocytes migration but dramatically reduced in its capability of accommodating chondrocytes after chelating with ferric ions. It was concluded from *in vitro* cytotoxicity characterization that Fe^{3+} concentration plays a more crucial part for cell viability. The interpenetrating network structure was realized by a reversible chelation of catechol groups on dopamine with ferric ions under alkaline environment, which would inevitably partly collapse during the 30-day incubation. The released Fe^{3+} , especially those on the interface between graft and cartilage rings, was detrimental for chondrocytes viability. BFe-dLhCG-wash, chelated with 100mM Fe^{3+} , might lead to a worse growing habitat for chondrocytes, resulting in

death of some of the chondrocytes. CFe-dLhCG-wash, with 50mM Fe^{3+} applied during fixation with cartilage, might produce less free ferric ions during incubation and showed advantages in cell migration.

4.5 Summary

In this chapter, CSD and BCD were coated onto dLhCG as adhesives for further fixation with cartilage with addition of ferric ions. CS, a cartilage derived sulfated GAG, was chosen as the main backbone, which would introduce no impurities for cartilage tissue engineering. BSA, a universal biocompatible plasma protein, was also used as main backbone for comparison. Dopamine was introduced on CS and BSA, which would polymerize with existence of oxygen and the catechol groups on which could chelate with ferric ions under alkaline environment. CSD and BCD were attached to dLhCG first mainly via self-polymerization of dopamine. Then CSD or BCD coated dLhCG was fixed in defects of cartilage by addition of Tris buffer (10mM, pH 8.5) and ferric ions to form interpenetrating network for adhesion. Both CFe-dLhCG (up to 70kPa) and BFe-dLhCG (up to 55kPa) showed enhanced adhesive strength with cartilage compared to dLhCG. But CFe-dLhCG performed better in chondrocytes migration with moderate chondrocytes infiltrated after 30 days, while BFe-dLhCG only accommodated a few scattered chondrocytes on day 30. In summary, CSD is superior to commercially available adhesives for grafts fixation in retaining porous structures, introducing no impurities and more suitable as an adhesive coating on dLhCG than BCD in stronger adhesion with cartilage at

optimized working concentration and better performance in chondrocytes migration.

Chapter 5 Conclusions and future work

5.1 Conclusions

In the above three chapters, two current existing issues in tissue engineering field, immunocompatibility and fixation of grafts, were paid attention to for clinical translation of extracellular matrix (ECM) based implants. Natural biological coating was a common idea to address the above-mentioned problems. In order to attenuate immune and inflammatory responses elicited by xenografts, the function of autologous cell membrane coating and stabilized albumin coating was investigated. The basic idea of applying autologous cell membrane coating on xenografts was taking advantage of its embedded protein in cell membrane which contributes to the disguise of xenografts and recognition as self by the host, while that of stabilized albumin coating was making use of its passivation nature to avoid adsorption of other proteins and further coagulation. Furthermore, dopamine was introduced in order to enhance adhesion of albumin on grafts. Inspired by this dopamine modified albumin coating on grafts, we considered developing dopamine based self-adhesive ECM based grafts to solve the fixation problem. In this thesis, a dopamine based self-adhesive decellularized living hyaline cartilage graft (dLhCG) was engineered to enhance interfacing affinity between cartilage and dLhCG.

Coating is a facile method for modification of grafts, especially ECM based

grafts. In chapter 2 and chapter 3, by coated with natural biological materials, LhCG and dLhCG were functionalized with membrane proteins for self-recognition or passivation surface against endogenous proteins *in vivo*. On one hand, considering cellular and acellular antigenicity of ECM based xenografts, autologous cell membrane, a disguise that could cover the antigens and replace with self-recognition membrane proteins, was coated on LhCG and dLhCG for suppression or alleviation of immune and inflammatory responses. LhCG and dLhCG coated with autologous red blood cell (RBC) membrane showed better performance in immunocompatibility with less inflammatory cells infiltrated and less inflammation related gene expression. On the other hand, protein adsorption on implants in the host was the initiation of coagulation and further immune and inflammatory responses. From this perspective, a stabilized albumin coating was applied for passivation of xenograft surface. Albumin is a popular anti-fouling material which could repel adhesion of other proteins nonspecifically. LhCG and dLhCG with passivating dopamine modified bovine serum albumin (BCD) coating showed enhanced immunocompatibility with less inflammatory cells infiltrated and lower value in inflammation-related gene expression.

It is worth noting that in order to improve the adhesion of albumin on grafts, dopamine was introduced. It is reported that polydopamine could bind with metal ions for chelating bonds. In this case, by coating dopamine derived adhesive macromolecules, ECM based grafts are capable of adhering to surrounding host tissues after addition of ferric ions *in situ* during implantation. In chapter 4, an

adhesive dLhCG was developed for enhanced interfacing affinity with cartilage by coating dopamine-based chondroitin sulfate (CS) (CSD) or serum albumin (SA) (BCD). A macromolecule backbone was needed to avoid formation of rigid crosslinking system as well as toxicity caused to host by small molecules of dopamine. In chapter 4, CS, a cartilage derived sulfated glycosaminoglycan (GAG), was chosen as a backbone with no impurities introduced. SA was also chosen as a comparison. Both CSD and BCD showed potential as adhesive coating for fixation of ECM based grafts such as dLhCG with enhanced adhesive strength, in which CFe-dLhCG reached $72.6 \pm 8.3 \text{ kPa}$ and BFe-dLhCG was $55.2 \pm 0.8 \text{ kPa}$. But CSD is superior and suitable as adhesive coating for fixation of ECM based grafts since CFe-dLhCG-wash showed better performance in both adhesive strength at optimized working concentration and chondrocytes migration with no impurities introduced and lower ferric ions concentration applied.

5.2 Future work

Natural biological materials including RBC membrane and BCD have been studied on ECM based grafts as functional coatings with LhCG and dLhCG as model grafts for enhanced immunocompatibility. However, more efforts need to be made towards clinical translation. First of all, the immune evasion coatings were stable enough to carry out their functions and proved not to damage the structure of LhCG or dLhCG. However, in general, considering the ECM surface, which cells might prefer to adhere to, was covered, a cartilage implantation experiment *in vivo*

needs to be carried out to evaluate if the tissue regeneration will be affected. Therefore, an *in vivo* osteoarthritis (OA) rat model needs to be established. Specifically, Sprague-Dawley male rats (32-week old) will be anesthetized by Ketamine (75mg/kg)/Xylazine (10mg/kg) mixture before surgery. Then a parapatellar incision will be made on the right knee after sterilization treatment on skin. The right knee joint will be exposed, after which the joint capsule will be opened by transecting the anterior cruciate ligament with microscissors. The incisions will be closed by Nylon-4 suture and clip after treatment. Afterwards, tissue regeneration with autologous cell membrane or stabilized albumin coated ECM based grafts can be studied based on the *in vivo* OA rat model.

Natural biological materials including CSD and BCD have been investigated as adhesive coatings for enhanced interfacing affinity of ECM based grafts with surrounding host tissues. The optimization of adhesive strength, how materials and additives concentration affect cell viability and *in vitro* cell migration have been studied. However, considering the difference of internal environment and existence of immune system, tissue regeneration investigation by an *in vivo* OA rat model which is illustrated above is also necessary.

In addition, in chapter 2, surface area of dLhCG and LhCG could be measured by Brunauer-Emmett-Teller (BET) surface area analysis after which coverage ratio of RBC membrane coating could be calculated combined with ICP-MS or fluorescent intensity results.

Furthermore, in chapter 2, an optimized whole blood volume of 0.6mL on dLhCG and LhCG with a diameter of ~5mm was decided according to tracking of effective cell membrane amount on grafts with gradient whole blood volume applied. However, in chapter 3, optimization of BCD concentration used for coating has not been studied in detail. Therefore, a gradient concentration of BCD-FITC could be coated on dLhCG and LhCG, after which BCD-dLhCG and BCD-LhCG can be fixed and sectioned. The fluorescence of the sectioned sample could be observed under fluorescent microscope and measured by Image J. In addition, the influence of gradient BCD on chondrocytes viability can be studied by WST-1 Assay Kit. The above two characterization will narrow down the choice of BCD concentration.

In chapter 2 and chapter 3, in addition to IHC staining, quantitative analysis of MPO, CD68 and CD3 on grafts collected from sacrificed rats needs to be studied by enzyme-linked immunosorbent assay (ELISA).

Moreover, in chapter 4, BFe-dLhCG showed poor performance in cell migration which might be caused by higher ferric ions concentration. Therefore, the effect of ferric ions concentration on chondrocytes migration on day 15 and day 30 should be further investigated.

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