Surface modification to improve biointegration of materials in a core-skirt keratoprosthesis

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2017

Riau, A. K. (2017). Surface modification to improve biointegration of materials in a core-skirt keratoprosthesis. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/71955>

<https://doi.org/10.32657/10356/71955>

NANYANG
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UNIVERSITY

**SURFACE MODIFICATION TO IMPROVE
BIOINTEGRATION OF MATERIALS IN A CORE-SKIRT
KERATOPROSTHESIS**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2017

**SURFACE MODIFICATION TO IMPROVE
BIOINTEGRATION OF MATERIALS IN A CORE-SKIRT
KERATOPROSTHESIS**

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

2017

Abstract

Corneal disease is a major leading cause of blindness worldwide. The majority of the reversible causes could benefit from corneal transplantation. However, transplantation-grade donor corneas are often difficult to procure. In addition, organ transplantation is frequently plagued by clinical, ethical and logistical issues. Prognosis of corneal transplantation is also poor in most advanced corneal diseases. In these cases, implantation of an artificial cornea becomes the next viable option. Core-skirt keratoprosthesis (KPro) is a type of artificial cornea and the most widely used ocular prosthetic device. This KPro construct consists of a transparent optical core which is usually made of a polymer, such as poly(methyl methacrylate) (PMMA), surrounded by a skirt material which is usually a biological material that promotes biointegration, such as the donor corneal tissue.

Various studies in the literature have highlighted a common problem associated with core-skirt KPros, which is the poor interfacial bonding and biointegration between the two materials (PMMA and biological material). Surface modification is an attractive technique that can be employed to improve the interactions and bonding strength between the two dissimilar materials without significantly altering the design of the KPro. The main objective of this thesis, therefore, was to apply surface modification on PMMA to enhance its interactions and adhesion to the skirt material. Collagen type I hydrogel was selected as the substitute skirt material to eliminate the use of human donor corneas.

Calcium phosphate (CaP) coating deposited via simulated body fluid incubation on dopamine activated PMMA sheets (referred to as d-CaP) was able to produce better adhesion with collagen hydrogel compared to untreated PMMA and PMMA treated with other surface modifications, such as oxygen plasma irradiation, 3-APTES coating, and CaP coating on plasma activated substrate. The d-CaP coating was biocompatible and appeared to promote biointegration. The d-CaP coating, however, could easily undergo delamination which reduced its potential to be used to modify KPro optical cylinders. In addition, the coating exhibited a calcium deficient

bone-like apatite with a significantly lower Ca/P ratio compared to pure HAp.

A novel dip coating method, which was simple to execute for immobilizing HAp nanoparticles on PMMA was introduced in this thesis to address problems associated with the d-CaP coating. One time dip in 20% (w/v) HAp nanoparticles in 5% (w/v) PMMA/chloroform for 60 seconds, followed by surface plasma irradiation (referred to as HAp-coated PMMA) resulted in a homogenous coating on PMMA sheets, offering high hydrophilicity, resistance to delamination and preservation of Ca/P ratio of pure HAp. These advantages resulted in 2.5 times improvement in adhesion strength (over 28 days in artificial tear fluid) between collagen hydrogel and HAp-coated PMMA than between hydrogel and d-CaP, and an order of magnitude improvement compared to untreated PMMA. Corneal stromal fibroblasts were able to form good adhesion and proliferate on the HAp-coated PMMA.

The dip coating technique could be extended to immobilize HAp nanoparticles on PMMA cylinders, bringing the studies closer to the clinical application of the coating for KPro optical cylinders. However, minor modification in the dip coating method (12 times 5-second-dips instead of 1 time 60-second-dip) was necessary to immobilize the nanoparticles on PMMA cylinders of 3 mm in diameter due to the curved surface and smaller surface area compared to PMMA sheets.

Corneal infections can result in poor biointegration of a KPro and vice versa. An advantage of substituting donor cornea with collagen hydrogel as the skirt material was presented here, whereby an antibiotic (vancomycin) was loaded in the hydrogel (referred to as VH) and was shown to be bactericidal effective in vitro for up to 7 days. When implanted in the rabbit corneas, which were infected with *Staphylococcus aureus*, the VH-implanted corneas were clear and non-edematous and showed a reduction of log 2.5 in bacteria compared to the blank hydrogel-implanted corneas.

Acknowledgements

The completion of my PhD study and this thesis would not have been possible without the help and support of many individuals. Unfortunately, it is not possible to mention each and every individual who helped me along the way. I would like to thank everyone who was involved scientifically and became my friends during the journey.

First and foremost, I would like to thank Prof. Subbu Venkatraman for the special opportunity that he gave me to undertake a PhD degree under his supervision in the School of Materials Science and Engineering, Nanyang Technological University. Prof. Venkatraman provided tremendous help, support and encouragement throughout the entire process of completing my study. His involvement and advice in all the studies that comprise this thesis were invaluable. I also appreciate the great freedom that he gave me to make my own decision in a large part of this research.

I would like to acknowledge the fundamental contribution to this research by A/Prof. Jodhbir Mehta, who is an ophthalmologist in Singapore National Eye Centre and the leader of Tissue Engineering and Stem Cell Research Group, Singapore Eye Research Institute (SERI). His immense knowledge and experience in the field of clinical and translational corneal research provided the fundamental aspect of all studies that shape this thesis. By addressing the immediate clinical need, it made the experiments that I carried out much more meaningful and closer to resolving a healthcare problem.

I am also particularly grateful to Prof. Bo Liedberg for his invaluable knowledge and guidance related to material surface functionalization and fundamentals of surface chemistry. Prof. Liedberg's expertise in surface chemistry and advice were important pieces of a puzzle necessary to completing this thesis. I would like to thank him for allowing me to use the facility in his lab in the Centre for Biomimetic and Sensor Science, Nanyang Technological University.

This research would not have been possible without advice and support from many people at different institutions. I am grateful to: Dr. Debasish Mondal for introducing and teaching me the fundamentals of materials science, a field which I had no previous exposure to, and sharing the know-how of collagen hydrogel casting; Dr. Alagappan Palaniappan for his patience in optimizing and teaching me the quartz crystal microbalance technique; Dr. Gary Yam and Ms. Melina Setiawan for their help and sharing their knowledge in cell culture of corneal stromal fibroblasts; Mr. Patrick Lai for teaching me how to operate an AFM; Mr. Wilson Lim for teaching me how to operate a FTIR spectroscope, a water contact angle goniometer and a TGA instrument; Dr. Thet Tun Aung for being a friend and fellow PhD student, who was kind enough to help me with some of the microbiology studies that I conducted; and Prof. Roger Beuerman for giving me permission to conduct the microbiology studies in his lab and especially for being the person who recruited me to work in SERI and gave me the initial glimpse into the world of science and engineering.

I would also like to thank SingHealth Foundation for the Talent Development Fund Award that provided partial funding support for my tuition fee. The fund was a big financial relief and had tremendously helped me in my initial decision to pursue a PhD degree.

Last but not least, I would like to thank my parents, my brother, and especially to my wife, for their encouragement and unwavering support of my academic pursuits. They were and will always be the rock in my life. There were many ups and downs that I had to go through and which my family had to either directly or indirectly endure with me throughout this journey, but their continued support kept me going during the difficult times.

Awards, presentations and patents

The following are awards that I obtained, as well as presentations and patent that arose from my PhD studies:

Awards

- Singapore General Hospital 21st Annual Scientific Meeting, Young Investigator's Award, 3rd place
- World Biomaterials Congress 2016, Trainee Award
- SingHealth, Publish! Award (Medical Research Category) 2016

Presentations

- Riau AK, Mondal D, Yam GH, Liedberg B, Venkatraman SS, Mehta JS. Biointegration of collagen hydrogel, as donor corneal substitute, on PMMA for Boston keratoprosthesis. Poster presentation in ARVO 2015 Annual Meeting, Denver, CO, USA on 5 May 2015.
- Riau AK, Mondal D, Murugan E, Aung TT, Liedberg B, Venkatraman SS, Mehta JS. Vancomycin loaded collagen-based artificial cornea for prevention of early post-operative ocular infections. Oral presentation in SGH 21st Annual Scientific Meeting, Singapore on 10 April 2015.
- Riau AK, Mondal D, Aung TT, Beuerman RW, Liedberg B, Venkatraman SS, Mehta JS. Performance of collagen-based corneal implant with anti-infective capability in rabbit model of infectious keratitis. Oral presentation in WBC2016, Montreal, Canada on 19 May 2016.

Patent

- Patent no: 10201606175P
Title of invention: Dipcoating of hydroxyapatite and titanium oxide nanoparticles onto poly(methyl methacrylate) substrate to improve biointegration of core-skirt keratoprosthesis.
Inventors: Subbu S. Venkatraman, Jodhbir S. Mehta, Bo Liedberg, Andri K. Riau, Debasish Mondal.

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Abbreviations

11-MUA	11-mercaptoundecanoic acid
3-APTES	(3-aminopropyl)triethoxysilane
AFM	atomic force microscopy
ALDH	aldehyde dehydrogenase
ALK	anterior lamellar keratoplasty
AS-OCT	anterior segment-optical coherence tomography
ATF	artificial tear fluid
ATR-FTIR	attenuated total reflectance-Fourier transform infrared spectroscopy
BH	blank hydrogel
CaP	calcium phosphate
CD	circular dichroism
CK	cytokeratin
DAPI	4',6-diamidino-2-phyllindole
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EdU	5-ethynyl-2'-deoxyuridine
EDX	energy dispersive X-ray spectroscopy
ECM	extracellular matrix
EK	endothelial keratoplasty
EthD-1	ethidium homodimer-1
GPC	glypican
HAp	hydroxyapatite
IACUC	Institutional Animal Care and Use Committee
IPN	interpenetrating network
IR	infrared
IVCM	in vivo confocal microscopy
KPro	keratoprosthesis
MIC	minimum inhibitory concentration
MPC	2-methacryloyloxyethyl phosphorylcholine
mRNA	messenger ribonucleic acid
MRW	mean residual weight
NAG	N-acetylglucosamine

NAM	N-acetylmuramic acid
NHS	N-hydroxysuccinimide
OCP	ocular cicatricial pemphigoid
OOKP	osteo-odonto keratoprosthesis
PAA	poly(acrylic acid)
PBS	phosphate buffered saline
PCL	poly(ϵ -caprolactone)
PdI	polydispersity index
PEG	polyethylene glycol
PET	polyethyleneterephthalate
PK	penetrating keratoplasty
PLGA	poly(lactic-co-glycolic acid)
PMMA	poly(methyl methacrylate)
pHEMA	poly(2-hydroxyethyl methacrylate)
PTFE	polytetrafluoroethylene
PVA	polyvinyl alcohol
PVP	polyvinylpyrrolidone
QCM	quartz crystal microbalance
RI	refractive index
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SAM	self-assembled monolayer
SBF	simulated body fluid
sccm	standard cubic centimeters per minute
SD	standard deviation
SEM	scanning electron microscopy
SJS	Stevens-Johnson syndrome
SLC	solute carrier
SMILE	small incision lenticule extraction
TiO ₂	titanium oxide
TSA	tryptic soy agar
UVO	ultraviolet oxidation
VH	vancomycin-loaded hydrogel
ZO	zonula occludens

Chapter 1

Introduction

This chapter introduces the background of my study, which is motivated by known clinical problems associated with the use of artificial cornea or keratoprosthesis (KPro) with core-skirt design concept. Motivation behind the use of artificial cornea will be discussed first, followed by statement of problems blighted by the current core-skirt KPro design, and definition and advantages of core-skirt design of a KPro. Hypothesis, objectives, specific goals and scope of the study designated to address the shortcoming of current KPro will be elaborated before the outline of this thesis is finally discussed.

1.1 Motivation behind development of artificial cornea

Corneal disease is a major leading cause of blindness worldwide, second only to cataract [1]. Globally, corneal disease is estimated to be responsible for blindness in 4.9 million individuals, which is equivalent to 12% of the total 39 million who are reportedly blind [1,2]. Corneal blindness can be caused by genetic mutation, degeneration, dystrophy, infection or inflammation of corneal tissue. The majority of these cases could potentially benefit from corneal transplantation. Corneal transplantation is currently the most effective treatment method when the disease has detrimentally affected the corneal structure and clarity. In the United States, there were 42642 corneal transplantations performed in 2010 compared to 12623 solid-organ (kidney, liver, lung and heart) transplantations in 2008 [3]. In the UK, 3565 corneal transplantations were performed in 2010 and 2011 compared to 3360 kidney and liver transplantations [3]. These facts render the cornea the most transplanted tissue, even when compared to kidney and liver transplantation combined. It will come as no surprise that the high demand of transplantation-grade donor corneas may outstrip their supply in the near future. Besides the increasingly limited supply of donor materials, organ transplantation is frequently plagued by clinical, ethical and logistical limitations, for example the lack of infrastructure necessary for a transplantation service in many countries due to limitations in storage facilities, proper operating theaters, trained surgeons, or cultural and religious issues [4]. There is also a potential risk of disease transmission through organ transplantation, even after stringent screenings of the donors have been performed [5]. In addition, immunological rejection leading to graft failure and the side effects in response to necessary life-long use of immunosuppressant are commonly encountered by organ transplanted patients [6,7].

Besides the aforementioned limitations associated with the donor corneal transplantation, the prognosis of the procedure is poor in most advanced corneal diseases, especially in patients, who have suffered from multiple graft failures [8], or who suffer from tear deficiency, chemical burns, glaucoma, severe vascularization or autoimmune disease [9]. In these cases, implantation of an artificial cornea becomes the next best treatment option. Core-skirt keratoprosthesis (KPro) or collar-button

KPro is a type of artificial cornea and has been the most widely used KPro design concept in the world. Boston KPro and osteo-odonto keratoprosthesis (OOKP) are examples of commercially available artificial cornea that follows the core-skirt design concept. This KPro construct consists of a transparent optical core which is generally made of polymer, such as poly(methyl methacrylate) (PMMA) or poly(2-hydroxyethyl methacrylate) (pHEMA), surrounded by a skirt material which is usually a biological material, such as the donor corneal tissue (see illustration in **Figure 1.1**). The core-skirt construct has been the most successful KPro design owing to the presence of thermoplastic optical core that provides clear window to the patient's vision and at the same time, provides resistance to biodegradability of the device, while the surrounding donor biological tissue allows for host tissue integration [10].

Figure 1.1. Illustrations of cross-sectional and top view of core-skirt or collar-button design concept of a KPro. A core-skirt KPro consists of a transparent optical core, normally made of PMMA, which is surrounded by a skirt material, which can be a human donor cornea or a polymer.

1.2 Problem statement

There are several core-skirt KPros that have been used clinically with varying surgical complexity and retention rate, such as Boston KPro, AlphaCOR, OOKP and BioKPro III to name a few. The Boston KPro is currently the most commonly used

core-skirt KPro worldwide. The Boston KPro was first implanted in a patient in 1974 and received FDA approval in 1992 [11]. The KPro (**Figure 1.2, 1.3A and 1.3B**) is assembled by four components, from the most anterior to the most posterior order:

- (1) The front part or the PMMA optical core.
- (2) The donor corneal tissue, which is centrally trephined to insert the optical cylinder.
- (3) The back plate, which was originally made of PMMA but is now titanium, with holes to facilitate nutrient flow from the aqueous humor to the corneal graft.
- (4) The titanium locking ring to secure the whole assembly in place.

Figure 1.2. Illustration of Boston KPro and its specifications. The device is made of four components. The centrally trephined donor corneal graft is sandwiched between a PMMA optical core (the front part) and a titanium back plate. The whole assembly is secured by a titanium locking ring. This figure was adopted from Gomaa and colleagues' work with minor modifications [10].

Although the Boston KPro has been widely used and does provide good visual recovery [13], there are some shortcomings associated with its current design and in fact, with the design of most KPro devices that adopt the core-skirt constructional concept:

- (1) Poor interfacial adhesion between the PMMA optical cylinder and donor biological material results in epithelial downgrowth, detachment of the graft material from PMMA surface, and aqueous humor leakage (**Figure 1.4**).

- Perpetual eyelid blinking, resulting in shear stress on the device, causes the optical cylinder to become loose and eventually extruded [14,15].
- (2) Poor interfacial adhesion and healing between the PMMA optical cylinder and donor biological tissue, providing an opportunity for bacterial infection and inflammatory cell invasion [16,17].
 - (3) Although PMMA is biocompatible to many cell types, including the corneal stromal cells, cellular adhesion and subsequent biointegration on its surface is generally poor [18,19]. The poor biointegration exaggerates the already weak bonding between the PMMA and biological tissue [20].
 - (4) The existing usage of donor biological material in the device assembly contradicts the concerns for global donor corneal shortage and potential immunological rejection.

Figure 1.3. Appearance of Boston KPro implanted in a patient's eye. (A) Slit lamp photograph of the Boston KPro. (B) Anterior segment-optical coherence tomograph (AS-OCT) showing cross-sectional view of Boston KPro in vivo. OC = PMMA optical cylinder. CG = donor corneal graft. BP = back plate. GHJ = graft-host junction. HC = host cornea. This figure was obtained from reference [12].

It appears that the problems of the current core-skirt KPro are mainly compounded by the poor interfacial seal between the two materials (PMMA and biological tissue) that have dissimilar surface properties and are not compatible to each other. Surface modification is an attractive technique that can be employed to improve the interactions and bonding between the two dissimilar materials without significantly altering the bulk properties of both materials. Therefore, the clinical advantage of applying surface modification in addressing this problem is an improved performance of the device without the need to change the primary components and design of the

original KPro. Developing a medical device with new biomaterials can be a tedious process that would take more than a decade to reach the clinical trial stage.

Figure 1.4. Detachment of corneal graft from PMMA surface viewed by AS-OCT. The AS-OCT shows a KPro patient with a gap (G) in between the stem of the PMMA optical cylinder (OC) and donor corneal graft (DC) due to poor interfacial bonding. This figure was obtained from reference [12].

1.3 Hypothesis of study

I hypothesize that appropriate surface modification on PMMA can improve the resistance to interfacial shear adhesion between PMMA and donor corneal substitute material (collagen type I hydrogel) exceeding the maximum shear stress of 130 mN/cm^2 generated by the eyelid wiping process and also enhance the biointegration of corneal stromal cells on PMMA, and that incorporation of antibiotic in the collagen hydrogel can improve the early-postoperative protection against bacterial invasion of a core-skirt KPro construct.

1.4 Objectives and goals of study

The objective of my study was to deploy surface modification techniques on PMMA to enhance its interactions and adhesion to the skirt material. Without such surface modification, tissue integration as well as delamination of components are problematic for this design. In my KPro model and also in an attempt to eliminate the use of human donor cornea, collagen type I hydrogel was used as the skirt material. The surface modification was performed on PMMA due to its relatively unreactive and hydrophobic surface (making it difficult to be bonded to another material) rather

than on the collagen hydrogel, which is hydrophilic in nature and contains many reactive functional groups ($-NH_2$, $-COOH$ and $-SH$). Since long-term integration of an implant is be influenced by many events, including cellular biointegration [21,22], the objective of study was also centered on the improvement of adhesion and biointegration of corneal stromal fibroblasts on the surface modified PMMA. More specifically, the goals of the study were:

- (1) To perform PMMA surface modification techniques that have previously been shown in to improve adhesion of PMMA to soft biomaterial or tissue. These methods include plasma treatment [23], calcium phosphate (CaP) coating via simulated body fluid (SBF) incubation [22], and aminosilane coating [24]. The resistance to shear adhesion between the surface modified PMMA and collagen type I hydrogel was assessed. In addition, their effects of the surface modifications on proliferation, adhesion and viability of corneal stromal fibroblasts were also studied. For the ease of characterization and cell seeding, PMMA sheets were used to achieve this goal. Once CaP coating was identified as the best method that produced the highest shearing resistance between collagen hydrogel and surface modified PMMA, failure analysis was performed in order to determine the potential weaknesses of the coating.
- (2) Immobilization of hydroxyapatite (HAp) nanoparticles on PMMA via dip coating was then carried out as a surface modification method to address the weaknesses of CaP coating in goal (1). The resulting shear adhesion strength of collagen hydrogel on the improved surface coated PMMA and the effects of the HAp-coated PMMA on the adhesion and survivability of corneal stromal fibroblasts were assessed.
- (3) The dip coating technique that had been optimized in goal (2) was then employed to coat the surface of PMMA cylinders. Goal (3) would take the study a step closer to the application of the coating technique on actual KPro optical cylinder that is currently in use clinically.
- (4) Corneal infections often caused by *Staphylococcus aureus* could result in poor biointegration and various complications of a KPro and vice versa, whereby poor integration between PMMA and biological material provides an opportunity for bacterial infiltration [25,26]. An antibiotic-loaded skirt material can potentially minimize the incidence of corneal infections and improve the biointegration of the KPro. By substituting donor cornea with collagen

hydrogel as a KPro skirt material, vancomycin was able to be incorporated into the hydrogel, and was shown to be bactericidal effective in vitro and in a rabbit experimental model of bacterial keratitis.

1.5 Scope of study

There are not many studies in the literature that have investigated techniques to improve the interfacial bonding and biointegration of KPro PMMA optics. The importance of the initial adhesion of the tissue to the PMMA optic is often overlooked. Long-term retention of a KPro in the eye is subject to many events including cellular infiltration. Because the migration rate of corneal stromal cells is slow [27], bonding between the two dissimilar materials have to occur and remain stable first before cells arrive near the interface to lay a more recognizable matrix to mediate the biointegration process further. My study covered the employment of surface modification on PMMA, particularly the optimization of the novel method to immobilize HAp nanoparticles on PMMA surface via non-thermal dip coating technique, with an aim to improve its bonding with donor tissue substitute (collagen hydrogel) to more than the peak stress stress that can be generated by the eyelid wiping process (130 mN/cm^2) [28], as well as to enhance corneal stromal cell adhesion, proliferation and survivability. Although the sheer stress generated directly on the corneal surface or KPro should be significantly less due to the presence of tear film lubrication in between the eyelid and corneal or KPro surface, 130 mN/cm^2 was set as the threshold of improvement. In a bigger picture, surface modification of PMMA was selected as a method to improve biointegration of the core-skirt KPro to circumvent the need to change the primary components and design of the original KPro. Surface modification was also favoured because the resulting coating does not cause obstruction of light, that has to penetrate through the center of the PMMA cylinder for vision (**Figure 1.5**). Moreover, developing a medical device with new biomaterials can be a tedious process that would delay its delivery for clinical use and the process could take more than a decade to reach the clinical trial stage [29].

Figure 1.5. Schematic of ideal surface coating outcome for a KPro optical cylinder. In order to allow light to penetrate through the center of the optic to allow for vision, the coating process ideally should result in thin coating that wraps the PMMA cylinder. Both ends of the cylinder can be capped to prevent potential obstruction of light path by the coating or nanoparticles.

Early corneal infections due to iatrogenic infections or post-operative infections caused by opportune bacterial invasion on recovering epithelial barrier can occur after implantation of a KPro and sometimes necessitate the removal of the device. Incorporating and sustaining the release of an antibiotic in the skirt material substitute (collagen hydrogel) could overcome this problem. The aim was to produce a collagen-based skirt material that could release an antibiotic for at least 7 days to provide protection during recovery of the epithelial barrier. Drug delivery from collagen-based vehicles, including vancomycin-soaked collagen shield, has been attempted previously, but the drug release could not be sustained for more than a day [30-32]. A small portion of my study covered the anti-infective capability of vancomycin-loaded crosslinked collagen hydrogel.

1.6 Thesis outline

This thesis addresses the specific aims outlined in section 1.4 and is organized according the following format:

Chapter 1 provides the background of research, statement of problem that is intended to be solved in my studies, and hypothesis, objectives, specific goals and scope of the thesis designed to address the known clinical problems associated with a core-skirt KPro construct.

Chapter 2 provides a review of prior art, covering the basic anatomy and physiology of the cornea, various general corneal transplantation techniques, some examples of corneal disease, history and evolution of KPro, introduction of the three main biomaterials used in the studies (PMMA, HAp and collagen), usage of surface modification in bonding soft to hard materials and improving cellular biointegration. Prior art covering previous attempts on PMMA surface modification, HAp coating on thermoplastics, and blinking motion that acts on ocular surface and KPro is discussed to provide an insight into the challenges to be overcome in the thesis. Introduction to bacterial infections which can affect the survival and biointegration of a KPro, and discussion about treatment and collagen-based corneal drug delivery system are also provided. Finally, research gaps intended to be addressed in the thesis are discussed.

Chapter 3 addresses the topic in goal (1) and describes several surface modification techniques on PMMA sheets, such as oxygen plasma treatment, SBF-mediated HAp coating and aminosilane coating. Characterization of the surface coatings was performed by water contact angle assessment, scanning electron microscopy (SEM), atomic force microscopy (AFM), energy dispersive X-ray spectroscopy (EDX) and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). Shear adhesion test was then carried out on a tensile tester (with modified load cell fixture) to assess the interfacial bonding strength between PMMA and collagen hydrogel. Finally, primary corneal stromal fibroblasts were seeded on the surface modified PMMA sheets. The cell viability, proliferation, adhesion and morphology were analyzed by live/dead cell assay, Click-IT EdU assay, vinculin and paxillin expression, and SEM, respectively.

Chapter 4 addresses the topic in goal (2) and (3). First, some shortcomings associated with SBF-mediated HAp coating on PMMA sheets described in Chapter 3 were demonstrated. Following that, with a primary aim to address the limitations of the previous HAp coating method, a novel immobilization technique of HAp

nanoparticles by dipcoating was introduced. Characterization of the resulting dipcoated PMMA surfaces was performed by SEM, AFM and ATR-FTIR. Shear adhesion test was then carried out on a tensile tester to assess the interfacial bonding strength between PMMA and collagen hydrogel. Primary corneal stromal fibroblasts were then seeded on the surface modified PMMA. The cell viability, proliferation, adhesion and morphology were analyzed by live/dead cell assay, Click-IT EdU assay, vinculin expression and SEM, respectively.

Chapter 5 introduces an advantage of substitution of donor cornea with collagen hydrogel as the skirt material of a core-skirt KPro, whereby an antibiotic was loaded in the hydrogel and was shown to be bactericidal effective in vitro for up to 7 days. After the release profile of the antibiotic from the hydrogel was determined, and biomechanical and optical properties examined, the drug-loaded hydrogel was implanted within the corneal stroma after a tissue with similar thickness was extracted from the rabbit cornea. A rabbit model of infectious keratitis was then created by injection of *Staphylococcus aureus* in the stroma to assess the bactericidal efficacy of the antibiotic-loaded hydrogel. In vivo corneal imaging techniques, such as slit lamp photography and AS-OCT, were performed during the follow up period. Inflammatory reaction of infected cornea was finally investigated by performing histology and immunohistochemical staining of CD18 following animal sacrifice.

Chapter 6 contains summary drawn from the studies in this thesis and discussion about the hypothesis, new hypothesis generated from the studies, and future direction of the research.

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Chapter 2

Literature review

This chapter contains a review of prior art, which covers the basic anatomy and physiology of the cornea and introduction of various corneal transplantation techniques. Introduction of advanced corneal diseases that benefit from KPro implantation over conventional corneal transplantation will then be discussed. To understand the inception of core-skirt KPro design concept, history and evolution of KPro will be reviewed. Examples of core-skirt KPro that had been developed and used clinically over decades will be given. The three primary biomaterials used throughout the studies (PMMA, collagen and hydroxyapatite) and various surface modification techniques on these materials will be reviewed. Shearing force affecting ocular surface and possibly the implanted KPro will be briefly reviewed. Finally, previous studies in the literature on occurrence of bacterial infections in KPro patients, their treatment and collagen-based drug delivery system will be discussed. At the end of the chapter, literature gaps to be addressed in this thesis will be given.

2.1 Anatomy and physiology of cornea

The cornea is a clear and transparent window at the front of the eye. The cornea offers 75% of the refractive power of the human eye (the lens is responsible for the remainder refractive power), allowing light to be transmitted through and focused onto the retina. As well as a photo-protector from UV radiation [1], the cornea acts as a thick and viscoelastic physical barrier that protects the posterior segment of the eye from various external insults, which may be physical, chemical or microbial. The complex multilayered structure of the cornea allows it to elicit its functions as described above. The cornea is a highly organized group of cells and proteins arranged into 5 distinct layers, from the most anterior to the most posterior order (**Figure 2.1**):

- (1) *Epithelium* is the anterior-most surface consists of 5-7 layers of cells, which are connected by desmosomes and anchored to the underlying basement membrane by hemidesmosomes [2]. The epithelium acts as a semi-permeable barrier function, which provides a smooth ocular surface that absorbs oxygen and nutrients from tear film, but creates a barrier to the water flow into the corneal stroma from tear film [3]. The epithelium is inter-penetrated with tiny nerve terminals that provide corneal sensitivity to pain when scratched or rubbed [4].
- (2) *Bowman's membrane* is a thin acellular layer that serves as the basement membrane for the overlying epithelial cells. It is a transparent sheet of tissue made of randomly oriented collagen fibers [5].
- (3) *Stroma* accounts for 90% of the corneal thickness, which consists of hydrated extracellular matrix (ECM) (78% water, 16% collagen, 6% glycosaminoglycans and other minor proteins) [6]. The stroma is interspersed with fibroblast-like cells called keratocytes. Stromal collagen consists primarily of type I (70%) and type V (20%) collagen that contribute to the strength and viscoelasticity of the cornea. The highly ordered orthogonal arrangement and spacing of the collagen fibers are responsible for producing the corneal transparency [5,7]. The highly ordered structure is maintained by optimal stromal deturgescence (balanced hydration in the cornea) due to the presence of water-absorbing glycosaminoglycans and water extrusion via the actively pumping monolayer of endothelial cells [6,8].

Figure 2.1. Illustration of cross-sectional view of human corneal tissue. The main physical function of each anatomical layer and the common cell or membrane markers that are used to identify specific layer are described in the illustration. CK: cytokeratin. ALDH: aldehyde dehydrogenase. ZO: zonula occludens. GPC: glypican. SLC: solute carrier.

- (4) *Descemet's membrane* is a thin but strong acellular sheet of tissue that serves as a protective layer against injuries and infection [9]. Descemet's membrane is primarily composed of collagen types IV and VIII, and is synthesized by the endothelial cells [9,10].
- (5) *Endothelium* is the posterior-most layer of the cornea. Endothelial cells are important in maintaining optimal stromal deturgescence [8]. In physiological conditions, fluid can leak from the anterior chamber into the stroma. Excessive water in the stroma can cause the tissue to swell and become hazy. The endothelial cells function to pump the excess fluid out of the stroma [11], maintaining a perfect balance of corneal hydration.

Corneal transparency is obviously essential to the vision. Any structural aberration, be it due to physical, chemical, genetic or microbial insult, that occurs in any one of the layers could upset the homeostasis of the cornea and impair the vision of a person when the cornea becomes diseased and opaque. No drugs are currently available and able to fully restore the corneal microstructure that has been damaged. In most cases of corneal disease, the only effective way to restore the vision is by corneal transplantation.

2.2 Corneal transplantation in brief

Full-thickness corneal transplantation was first performed in a human eye as early as 1905 by Dr. Eduard Zirm [12]. The full-thickness corneal replacement has been coined as penetrating keratoplasty (PK). From the 1970s, PK had been the commonest corneal transplant procedure performed worldwide when full-thickness diseased host corneal tissue was excised and replaced with a full-thickness healthy donor tissue (**Figure 2.2A**). Since then, PK has been considered as the ‘gold standard’ of corneal transplantation procedure [13,14].

Although PK is still a popular corneal transplantation technique, outcomes can be affected by intraocular complications, prolonged visual recovery, induced high astigmatism and poor graft survival rate [15]. Because the cornea is a multi-layered tissue, disease may affect only individual layers of the cornea. Hence, selective corneal thickness replacement is now a more desirable procedure, whereby only the diseased corneal layers are removed and at the same time, the unaffected layers are retained. If the disease affects the anterior corneal stromal layers, anterior lamellar keratoplasty (ALK) (**Figure 2.2B** and **2.2C**) can be performed to replace the diseased stromal tissue without the removal of the host’s unaffected corneal endothelium, thus eliminating the risk of endothelial graft rejection. Endothelial keratoplasty (EK) (**Figure 2.2D** and **2.2E**), on the other hand, is performed to replace the dysfunctional corneal endothelial cells.

Although corneal transplantation is effective in restoring the vision of many patients with mild to moderately severe corneal disease, the prognosis of the procedure

in treating advanced corneal diseases is poor. The poor prognosis is due to the failure of the donor graft to sustain normal corneal functions and also due to the excessive inflammatory condition in the hostile corneal microenvironment in patients with an advanced disease [16,17]. These patients would usually undergo multiple corneal transplantation procedures, carrying a high risk of graft rejection. Implantation of a KPro then becomes the next treatment option for this population of patients or for patients that are deemed not suitable for a keratoplasty due to certain circumstances, such as in children, pre-existing retinal disease and glaucoma, and poor ocular surface conditions [18,19].

Figure 2.2. Different corneal transplantation or keratoplasty techniques. (A) Full-thickness corneal replacement or PK. (B) Partial anterior stroma replacement or superficial ALK. (C) Full stromal thickness replacement or deep ALK. (D) Replacement of thin posterior stroma, Descemet's membrane and endothelium technique or Descemet's stripping EK. (E) Replacement of Descemet's membrane and endothelial layer technique or Descemet's membrane EK. Deeper color parts indicate donor corneal tissue. Lighter color parts show host corneal tissue. This figure was adopted from reference [14] with minor modifications.

2.3 Corneal diseases that benefit from KPro implantation

The most favourable indication of KPro implantation is still the multiple graft failure cases, but the device can technically be used to treat any corneal diseases as long as the patients do not also suffer from retinal or optic nerve disease. However, KPro

has currently only been used as primary treatment for chemical burns [20], aniridia [21], herpetic keratitis [22], bullous keratopathy [23], trachoma (**Figure 2.3A**) [24], and autoimmune ocular diseases, such as Stevens-Johnson syndrome (SJS) (**Figure 2.3B**) and ocular cicatricial pemphigoid (OCP) [25,26]. Patients with autoimmune diseases are normally difficult to treat and expected to present a less encouraging prognosis [19].

Figure 2.3. Examples of severe corneal disease. (A) Excessive corneal scarring and vascularization in a trachoma patient. (B) Fully keratinized ocular surface in a SJS patient. (C) Bilateral congenital glaucoma in a children patient. These images were obtained from reference [24], [25] and [28].

Because children have a more robust immune response, which typically results in prolonged visual recovery and excessive neovascularization, the prognosis of PK in pediatric patients is generally poorer than in adults [27]. The KPro has been regularly used in and successfully treated children with congenital corneal diseases, e.g. Peter's anomaly, congenital glaucoma (**Figure 2.3C**) and dermoid tumor [27,28]. The PMMA core is impervious to vascularization and resistant to biodegradability; therefore, providing unobstructed visual axis promptly.

2.4 History and evolution of KPro

Attempts made to replace an opaque or diseased cornea with KPro dated back to more than two centuries ago [29,30]. However, due to the lack of understanding of

materials science and physiology of the cornea at the time, many materials and tested implant devices were found to be non-biocompatible to the corneal tissue. However, an incidental discovery of corneal tolerance to plexiglass fragments (PMMA) from fighter plane canopies in many pilots' eyes during the World War II reinvigorated and offered a new direction for KPro development [31]. Earlier KPro used plastic as full thickness corneal replacement but the device implantation resulted in corneal melting [32]. Subsequent studies revealed that the plastic material formed a barrier that inhibited the circulation of water and nutrients in the cornea, leading to unbalanced hydration and corneal melting [33,34]. Stone and Herbert deduced that inlay implant (partial thickness corneal replacement) could be better tolerated than full thickness implants [32].

The aforementioned findings have led to the inception of core-skirt design concept of KPro, whereby the KPro has to function not only to restore the visual axis (via a clear plastic optic) promptly, but also to integrate with host tissue, impart partial corneal functions, as well as withstand hostile corneal microenvironment in patients with severe corneal disease. The design concept has been adopted by a number of commercially available devices that are in clinical use today, namely the Boston KPro, OOKP and AlphaCor. In brief, the Boston KPro consists of a PMMA optical cylinder supported by a donor corneal tissue. The OOKP, performed only at a few eye centers worldwide due to its complex multi-step surgical procedures, consists of a patient's incisor tooth that is integrated into the oral mucosal membrane [18]. The mucosal membrane integrated tooth is then used to support a PMMA optical core [18]. The AlphaCor, which is now rarely used due to complications, is manufactured from pHEMA [35]. The device comprises a clear dense central optical core and an opaque spongy skirt, which has higher water concentration than that of the core [35]. Out of the three devices, the Boston KPro is the most widely used artificial cornea worldwide, owing to its high retention rate [36,37], relative ease to perform procedure, and low cost to manufacture.

There are other less known core-skirt KPros, which vary mainly in the primary material of the skirt. These KPros have been used in only a few countries for selected few patients. However, biointegration of these KPros are generally poor, resulting in low device retention rate [38]. **Table 2.1** summarizes these KPros, Boston KPro,

OOKP and AlphaCor with respect to the materials used to construct the core and skirt portions of the implant devices.

Table 2.1. Various core-skirt KPros in the literature.

KPro type	Optical core material	Skirt material	Reference
OOKP	PMMA	Autologous tooth integrated in oral mucosal membrane	[18], [39]
Cardona KPro	PMMA	Polytetrafluoroethylene (PTFE) reinforced with Dacron	[40]
Boston KPro	PMMA	Donor corneal tissue	[41]
AlphaCor	pHEMA	Porous pHEMA	[42]
BioKPro III	PDMS coated polyvinylpyrrolidone (PVP)	PTFE	[43]
Seoul KPro	PMMA	PTFE	[44]
Pintucci KPro	PMMA	Dacron	[45]

2.5 Biomaterials to construct KPro

The term “biomaterial”, which is intended to be used to construct a medical device, could be defined as “a non-viable material intended to interact with biological systems” [46]. More specifically, it refers to synthetic materials that interact with natural (either modified or not) or biological materials. Ideally, biomaterials have to be able to exist in contact with tissues of the body without causing an unacceptable level of harm to the body. This mutual acceptance or co-existence of biomaterials and tissue interactions is what is now termed as biocompatibility [46]. For long-term survival of a medical device in the body, the biomaterials also have to be bioactive, meaning the materials should elicit a specific biological response at the interface which results in the formation of a bond between the tissue and the biomaterials [47]. A combination of good biocompatibility and bioactivity of the biomaterials that comprise a medical

device generally results in biointegration of the device to the host tissue [48]. Improving the interactions between PMMA (synthetic material) and collagen (natural material) in a modified core-skirt KPro is the focus of this thesis; hence, these two biomaterials are reviewed below. Hydroxyapatite, which will be used as the coating layer to mediate the bonding between PMMA and collagen, will also be briefly reviewed.

2.5.1 Collagen

Collagen is the most widely used naturally occurring material in the biomedical field. The collagen proteins are a family of highly characteristic fibrous proteins that typically provide elasticity and tensile strength to matrices, as well as support cells and tissues. Collagens are ubiquitous in the body of all multi-cellular animals and account for approximately 30% of body proteins [49]. Collagen is of particular structural importance to the cornea. It accounts for approximately 70% dry weight of the cornea [50].

Figure 2.4. Illustration showing hierarchy of fibrillary collagen. This figure was obtained from reference [51].

Collagens (**Figure 2.4**) are typically a protein complex with basic units, that consist of triple α -helices (tropocollagen) in which the three polypeptide chains are wound around each other like a rope forming into microfibrils. A group of microfibrils

then forms a fibril. Finally, aggregation of collagen fibrils forms a collagen fiber by their.

In cells, similar to the synthesis of many proteins, synthesis of collagen proteins begins following the translation of their specific messenger ribonucleic acid (mRNA) sequence. Following their synthesis, the three polypeptide α -chains align along the common hydrophobic areas. The formation of disulfide bonds at the C-terminals causes the α -chains to intertwine and form a triple helix (procollagen). The procollagen molecule is then transported out of the cell via exocytosis. In the ECM, the both ends of procollagen are hydrolyzed by peptidase to form a tropocollagen. Lateral association of tropocollagen molecules, that are staggered lengthwise in a three dimensional space, becomes the building block of collagen fibers. The assembly is of a combination of hydrophobic interactions and crosslinking of lysine and hydroxylysine. The crosslinking is an important feature as it adds considerable strength to the growing fiber. The process of collagen synthesis is summarized in **Figure 2.5**.

Figure 2.5. Illustration of collagen synthesis process in mammalian cells. This figure was obtained from reference [52].

Collagen can also be extracted *ex vivo* from native tissues, such as skin, tendons and ligaments, from animal sources by pepsin treatment [53,54]. The resulting collagen molecules can be dissolved in acidic solutions and reconstituted into fibrils that can be fashioned into different shapes and sizes to mimic tissue structure, such as skin and cornea. To complement collagen extracted from animals, significant advances have also been made in the production of human recombinant collagens [55]. However, the production yield of the recombinant protein is usually small. Efforts have been made to improve the production of human collagen in a recombinant system, such as yeast, plants and insect cells [56,57]. These recombinant systems can reproduce full-length collagen fibrils with the same amino acid sequence as native human collagen. By co-expressing prolyl hydroxylase genes with the collagen genes, recombinant collagens as stable as the naturally occurring material can now be produced.

2.5.1.1 Collagen as artificial cornea

Recombinant human collagen type III has been fabricated into an artificial cornea and is currently in a clinical trial phase 2 study [58]. These acellular, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) crosslinked constructs were able to induce the growth of epithelial cells over the implants, corneal nerve regeneration and support stromal cells migration into the implants, integrating the implants stably into the patients' eye (**Figure 2.6**) [58,59]. Another advantage of the collagen-based implants is the obviation of any immunosuppression beyond a short course of prophylactic steroids, in comparison to the donor cornea patients who require administration of steroids *ad infinitum*. The patients had an average corrected visual acuity of 20/54 and gained more than 5 Snellen lines of vision on an eye chart after 4 years. The post-operative visual acuity could potentially be improved with the use of more robust materials for better shape retention or a different suture retention method that does not impede epithelial re-growth into the center of the cornea.

The fabrication of interpenetrating network (IPN) hydrogel containing collagen and 2-methacryloyloxyethyl phosphorylcholine (MPC) was aimed to create a more

Figure 2.6. In vivo confocal microscopy (IVCM) images of non-operated, and collagen artificial cornea and donor cornea implanted patients' eye. Collagen hydrogel implanted eye showed comparable outcomes to donor cornea implanted eye. Corneal epithelial cells, stromal keratocytes and nerves had fully regenerated at 24 months post-operatively. This figure was obtained from reference [59] with minor modifications.

robust material [60]. The collagen-MPC hydrogels were biomechanically stronger and also more stable against both enzymatic and UV degradation. Full-thickness collagen-MPC hydrogels implanted onto guinea pig corneas showed ingrowth of endogenous epithelial and stromal cells with active corneal sensory nerves at 8 months post-operatively [61]. More recently, these composite hydrogels were grafted into three

patients with severe corneal pathologies, who were at a high risk for donor corneal graft rejection [62]. At 9-12 months post-operation, the implants provided relief from pain, discomfort, and restored corneal integrity by promoting endogenous regeneration of corneal tissues and improved vision in the patients.

2.5.2 Poly(methyl methacrylate)

PMMA is a thermoplastic acrylic resin formed by polymerization of methyl methacrylate monomers (**Figure 2.7**). It is one of the most widely used optical materials due to its relatively cheap cost, and ease of molding and fabrication. It has been the material of choice to construct various artificial corneas owing to its good impact strength and favourable optical properties. PMMA can transmit up to 92% of visible light and produces a refractive index of 1.49 [63]. The material has tensile strength of 61-70 MPa and impact strength of 12.3 J/m [63].

Figure 2.7. Skeletal structure of PMMA.

2.5.2.1 PMMA in construction of artificial cornea

Early generations of artificial cornea were one material, button-like piece which was made from hydrophobic polymer, such as PMMA, PTFE, Dacron or nylon [45,64,65]. The artificial cornea design has evolved from one material piece to the more accepted design of core-skirt configuration as described previously in section 1.1. Among these polymers, PMMA remains as the most extensively used material for the core of the core-skirt KPro due to its remarkable transparency. In addition, PMMA is impermeable to vascularization and stromal fibroblasts (stromal repair cells) and does not promote epithelialization; all of which could impede the vision of a patient [66,67]. PMMA is also resistant to biodegradation and is able to provide clear vision

to patients almost immediately after implantation of a KPro, regardless of inflammation and vascularization induced by the operation.

The use of PMMA to construct a KPro, however, has been plagued by a number of problems, such as the relatively unreactive surface functional groups and low hydrophilicity which contribute to its poor interactions with soft material (donor corneal tissue). These unfavourable surface properties also reduce the opportunity for cell attachment and long-term biointegration of the KPro into the host's cornea. These factors often compromise the longevity of the prosthetic device in the eye. Due to the shear stress created by the eyelid wiper, the graft material often becomes detached from the PMMA.

2.5.2.1.1 Shear stress generated on ocular surface and KPro by blinking motion

Friction occurs when two surfaces are in contact and move relative to each other. On the ocular surface, during a complete blinking motion, the lower and upper eyelids move with respect to the corneal surface. The tear film lubrication on the corneal surface alleviates the friction that occurs to prevent damage to the lid wiper and/or the corneal surface [68]. The residual sheer stress generated by the lid wiper can be assumed to also act on the KPro that is implanted in a patient's eye. The true shear stress created between the cornea and eyelid in vivo has not been reported before. The measurement is difficult to standardize due to inter-individual difference in the tear film viscosity [69,70], lid wiper pressure [71], lid wiper geometry [72], blinking velocity [73], surface roughness [74], and thickness of lubricant [75]. The shear stress created by blinking motion currently can only be resolved by mathematical models.

When resolving the “tribology of blinking motion,” the classical Stribeck curve (**Figure 2.8**) becomes obsolete, because of the additional presence of the glycocalyx and the mucin brush-like structure on the ocular surface and lid wiper [74,76]. The high coefficient of friction expected in the low-velocity regime due to the solid contacts does not occur because of the presence of these unique anatomical structures.

In addition, the tribological model of the eye may be influenced by physical parameters, such as surface roughness and texture, lid wiper pressure, and tear film composition.

Figure 2.8. Classical Stribeck curve showing lubricant thickness variation. 1 = boundary lubrication. 2 = mixed lubrication. 3 = hydrodynamic or complete fluid film lubrication. Red dashed line indicates the increasing lubricant thickness between sliding partners. Black dashed line indicates brush lubrication. This figure was adapted from reference [77].

It is assumed that a complete blinking motion quickly transitions from brush lubrication to complete fluid film lubrication at increasing blink velocity [78]. A recent mathematical model demonstrated that complete fluid film lubrication between a silicone hydrogel and upper lid wiper occurred shortly after the beginning of a blink [73]. With the almost instant transition to the complete fluid film lubrication, the possibility of direct and significant wear between the sliding partners can be prevented. At blink velocity between 100 and 200 mm/s, the resulting shear stress was demonstrated to be between 80 and 130 mN/cm², respectively [73]. The blink velocity in human patients, measured with a high-speed imaging system, was reported to be between 60-180 mm/s [79]. It can be assumed that the sheer stress generated directly on the corneal surface or KPro should be significantly less than 80-130 mN/cm² due to the tear film lubrication. Nevertheless, the adhesion between the PMMA core and skirt material of a KPro should ideally be stronger than the maximum theoretical shear stress generated by the blinking motion.

2.5.2.2 Alternative polymers to replace PMMA central optic

Due to some of the aforementioned weaknesses of PMMA, many studies have attempted to find an alternative material as the central optic of a core-skirt KPro but the results have fallen short of being translated to clinical use. Poly-HEMA appears to be a promising material to replace PMMA due to its transparency and tunable mechanical properties. AlphaCor, which is a pHEMA-based artificial cornea, was able to provide clear vision to patients in short term [42], but had a low survival rate in long term due to the incompatibility of the polymer with human corneal stromal cells [80-82]. PDMS, another transparent and oxygen permeable polymer, has been used in BioKPro artificial cornea [43], but because of its incompatibility with corneal stromal cells [83], the device has unacceptably poor survival rate [43]. Poly(4-methyl-1-pentene) has also been explored as an alternative material without further breakthrough [84].

Biocompatible and transparent polymers, such as poly(lactic-co-glycolic acid) (PLGA) [85], polyethylene glycol (PEG)/poly(acrylic acid) (PAA) [86], polyvinyl alcohol (PVA) [87], and poly(etherurethane urea) (PEUU) [88], have all been suggested as synthetic corneal scaffold. However, these polymers have relatively high degradation rate and can be easily penetrated by vascular tissue and corneal haze. These factors render many polymers unsuitable to be used as central optic of a KPro, which has to withstand the reducing environment of severe corneal diseases and has to be impenetrable to water and any cells in order to provide clear and unobstructed vision almost immediately after implantation to the patients.

Currently, PMMA still remains the most effective thermoplastic polymer as KPro central optic. The current trend of advancements of the core-skirt KPro design focuses on modification of the surface properties of the PMMA to improve its biointegration into the surrounding skirt material. Attempts at PMMA surface modification that have been reported in the literature will be discussed in the later section of this chapter.

2.5.3 Hydroxyapatite

Apatite is a mineral compound that contains calcium ions (Ca^{2+}) and orthophosphates (PO_4^{3-}). Apatite is of biological origin and an important inorganic component of hard tissue in vertebrate, such as bones, teeth and tendons in mammals [89,90]. HAp (**Figure 2.9**) is often referred to as an apatite with high OH^- ion concentration. The chemical formula of HAp is $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$, but is usually written as $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ to describe the two molecules that make up one HAp crystal unit cell. Pure HAp with Ca/P ratio of 1.67 never occurs in biological systems where small quantities of other elements, such as Na^+ and Mg^{2+} ions, are often substituted into the apatite crystal structure. This calcium-deficient apatite is the predominant structural component of enamel, dentine and bone mineral [91]. The chemical similarities of hexagonal HAp to bone and teeth apatite have propelled its use as a coating on orthopaedic and dental implants [92,93] and as a substitute material of various biomedical implants [94,95].

Figure 2.9. Crystal structure of hydroxyapatite. Blue = columnar calcium. Yellow = screw axis calcium. Orange = phosphorus. Green = oxygen. This figure was obtained from reference [96].

HAp and other derivatives of apatite with varying Ca/P ratios, precipitated from aqueous solution (**Table 2.2**), are biomaterials that have excellent biocompatibility [90,97]. They are non-toxic and not recognized as foreign materials

in the body, and most importantly exhibit bioactive behaviour that promotes biointegration into living tissue [90].

Table 2.2. Apatite derivatives that can be deposited from aqueous solution.

Compound	Chemical formula	Ca/P ratio
Hydroxyapatite	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	1.67
Calcium-deficient hydroxyapatite	$\text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5(\text{OH})$	1.5
Mono-calcium phosphate monohydrate	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	0.5
Dicalcium phosphate dehydrate	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	1.0
Octacalcium phosphate	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	1.33

2.5.3.1 Hydroxyapatite in construction of artificial cornea

HAp has never been used clinically in construction of any artificial cornea. However, several studies have explored the possibility to substitute the use of dental lamina with HAp for the construction of OOKP due to similarities between tooth/bone and HAp [98-100]. The OOKP, introduced over half a decade ago, is a form of KPro that involves removal of an autologous canine tooth and its surrounding bone from a patient (**Figure 2.10A**). Following this, the tooth is mechanically shaped to construct an osteo-odonto lamina (**Figure 2.10B**). A hole is then drilled through the dentine into which a PMMA optical cylinder is placed (**Figure 2.10C** and **2.10D**). The buccal mucosa is then integrated with the tooth by implanting the tooth-PMMA optical cylinder complex into the patient's mucosal lining for up to 4 months before finally being implanted into the diseased cornea.

It is obvious the existing OOKP involves an extremely complex and time consuming process, and possibly a traumatic experience to the patients. The procedure requires the patient's oral structures, such as the buccal mucosa and canine tooth, to be sacrificed. It is often limited by the availability of healthy tooth in adults or in

children, who have yet to develop adult dentition. In edentulous cases, where allografting is required, the patients have to rely on the goodwill of their relative. The use of an allograft also requires life-long administration of immunosuppressant. In addition, the dimensional limitations of the dental lamina restrict the size of the PMMA optical cylinder to 3.5-4 mm in diameter which results in restriction of the patient's visual field. These issues have led to an intense search for alternative to the use of patient's tooth and HAp appears to be the logical substitute material. Therefore, the material substitution could enhance the efficiency of the surgical procedure and increase the affordability and accessibility of the device worldwide.

Figure 2.10. Surgical procedure involved in an OOKP implantation. (A) The surgery first involves removal of a canine tooth and its surrounding bone. (B) The tooth is then mechanically shaped to construct an osteo-odonto lamina. (C) A hole is drilled through the dentine. (D) Finally a PMMA optical cylinder is placed in the hole. The traumatic experience is then followed by implantation of the dentine-optical cylinder complex in the buccal mucosal lining of the patient before being implanted in the diseased cornea. This figure was adapted from reference [101].

2.6 Surface modification of biomaterials

Interactions between tissue and biomaterial that constructed an artificial implant occur at the surface of the implant. Therefore, surface properties of a biomaterial play a critical role in the biointegration of an implant. Initial bonding between the implant and tissue is obviously necessary before the cells arrive near or onto the surface of the implant and lay a more recognizable foundation for the cells to grow, respond biologically and eventually biointegrate with to ensure long-term retention of the implantable device. When a biomaterial is implanted into the body and comes in contact with the tissue, proteins in the tissue are first absorbed onto the surface of the implanted material [102]. The protein adsorption event occurs well before cells migrate near or onto the surface. Migration of some cell types is extremely slow, e.g. corneal stromal keratocytes hardly migrate under normal circumstances [103]. Cells eventually arrive on the implant surface by detecting primarily the absorbed protein layer, rather than the surface of the biomaterial, and respond specifically to the proteins [104]. Cell attachment can occur when the cell is within a few nm of the biomaterial surface and is mediated by electrostatic forces. When atomic-level interactions with the cell membrane have been established, direct contact with the biomaterial surface can then occur [105]. Focal adhesion occurs later and is mediated by membrane integrins and cytoplasmic proteins that link the ECM to the cellular cytoskeleton [106]. Thus, the focal adhesions provide a channel for cross-talk between the ECM and the cell by regulating ECM protein assembly and remodelling and at the same time regulating cell adhesion, migration, proliferation, differentiation, and apoptosis [107].

The variety of materials intended for biomedical applications not only reflects the increasing need, but also the difficulty of the field with respect to the control of the interface between material and tissue. However, materials are never completely inert. Biocompatibility or the clinical success of an implantable device is highly dependent on the response of the tissue to the perturbation induced by the foreign material [108]. Any attempt to engineer biomaterials that have functional surfaces must take into the consideration the initial interactions between the implant surface and host tissue, and the ability of biological systems to recognize and respond to specific features of the

material surface subsequently. Therefore, implant surface coatings or modifications are of particular interest to influence biointegration.

Although the interaction mechanisms are not fully understood, tissue or cell interactions with biomaterial surface, and interactions between two different biomaterials can generally be attributed to and controlled by altering these physicochemical parameters of materials:

- (1) Surface wettability.
- (2) Surface charge or electrostatic interactions.
- (3) Surface functional groups.
- (4) Surface topography features and roughness.

2.6.1 Surface wettability

It is widely accepted that tissue/cell adhesion and subsequent biological activity are generally favourable on hydrophilic biomaterial surfaces [109-111]. Many polymers, i.e. PMMA and pHEMA, that are often used in medical applications are hydrophobic in their native state, and require surface modification or wetting procedures in order to support cell adhesion and growth [112]. According to Vogler's definition, hydrophobic surfaces are materials that exhibit water contact angles above 65° [105].

The water contact angle can be defined as the angle formed by the intersection of the liquid-vapor and liquid-solid interfaces. The measurement of the contact angle is obtained by applying a tangent line from the contact point along the interface of a sessile liquid droplet (**Figure 2.11A**). A large contact angle is usually observed when the liquid beads on the surface, while a small contact angle occurs when the liquid spreads on the surface. More specifically, a greater water contact angle indicates that wetting of the surface is not favourable whereby the fluid minimizes its contact area with the surface to form a more compact droplet profile. In contrast, a smaller contact angle means favourable wetting of the surface whereby the fluid spreads over a larger surface area. Complete surface wetting occurs when the droplet turns into a flat liquid puddle with contact angle of 0° .

The shape of a liquid droplet is largely determined by the surface tension of the liquid. In a droplet, each molecule in the bulk is pulled equally, resulting in zero net force in every direction by neighbouring molecules (**Figure 2.11B**). However, the

Figure 2.11. Illustrations of water contact angles and surface tension on smooth solid surface. (A) A small contact angle is observed when the surface is hydrophilic, while a large contact angle occurs when the liquid beads on hydrophobic surface. (B) Shape of water droplet on flat surface is determined by surface tension of the liquid.

molecules at the surface, that do not have neighbouring molecules to provide a balanced net force, are pulled inward by the molecules in the bulk. This results in the contact area contraction in order to maintain the lowest surface free energy. The force that contracts the contact area is called the surface tension and is responsible for the shape of the liquid droplet. Theoretically, the contact angle can be considered to be characteristic of a given solid-liquid system in a specific environment [113].

As postulated by Thomas Young [114], the contact angle of a liquid droplet on an ideal solid surface is defined by the mechanical equilibrium due to the action of three different interfacial tensions:

$$\gamma_{lv} \cos \theta_Y = \gamma_{sv} - \gamma_{sl}$$

where γ_{lv} , γ_{sv} , and γ_{sl} represent the liquid-vapor, solid-vapor, and solid-liquid interfacial tensions, respectively, and θ_Y is the contact angle.

2.6.2 Surface charge

The energy at a biomaterial surface can be defined by its charge density and the net charge polarity. Given that almost all interfaces are charged in aqueous solution [115], and that cell membranes are negatively charged, it is intuitive that electrostatic interactions will play a role in the biological response to biomaterials. It has been shown that cell membranes adhere closely to positively charged surfaces, whereas contact occurs only at distinct points and is usually weaker on near-neutral and negatively charged biomaterial surfaces [116,117]. These differences in attachment strength not only were attributed to electrostatic attraction and repulsion, respectively, but also due to differences in protein adsorption on respective surfaces that corresponded with distinct cell morphology and migration pattern [116]. A 220-kDa protein, which was thought to be fibronectin, was present primarily on positively charged surface [116,118]. Another cell adhesion protein in the 65- to 70-kDa range, suggestive of vitronectin was present on all surfaces [116]. Although interactions between negatively charged cell membranes and positively charged substrate are significant, surface wettability still provides a more dominant impetus for tissue/cell adhesion than surface charge [118]. Surface with positive or negative net charge may be hydrophilic, whereas surface with neutral charge is generally more hydrophobic.

2.6.3 Surface functional groups

Introduction or alteration of surface functional groups is perhaps the most important surface modification parameter that can be employed to enhance cell/tissue adhesion. Surface functional groups can be utilized to facilitate the addition of a crosslinker or an intermediate layer that binds tissue or biomaterial to the substrate, or to facilitate the addition of specific membrane ligands that enhance cell adhesion on the substrate. Chemical grafting, formation of self-assembled monolayer (SAM), and plasma irradiation techniques have been employed to create well-defined surfaces. Alteration of surface functional groups more often than not affects surface wettability and surface charge, and may also change surface topography of a biomaterial.

Chemical grafting can be applied to chemically immobilize specific compounds onto the biomaterial surface. The method involves covalent conjugation of either a monomer or protein to the surface to alter the surface chemistry. The process usually involves surface activation with reactive functional groups followed by grafting of specific functionality to the surface. Many functionalization methods fall into the chemical grafting category and differ only by the way in which the surface is activated, which includes UV radiation, chemical reactions, ozone exposure, or plasma treatment [119]. Physical adsorption techniques, such as dip coating method to lay a film with desired properties on the biomaterial surface, also fall into the chemical grafting category.

Creation of SAMs can provide a surface modification method that gives a more precise control of the density and conformation of a single or multiple functional groups on a surface than that by chemical grafting [120]. The general procedure to produce SAMs involves activation of the bulk material surface first and then graft polymerization of the molecules onto the activated surface. Formation of SAMs results in a surface with near thermodynamic equilibrium with closely packed and well-ordered functionalities on the material surface [106]. In addition, specific selection of terminal group provides a site for further functionalization of the SAMs-laid surface. However, formation of SAMs is usually more effective on silver- or gold-coated surfaces.

Another popular method of grafting functional groups onto biomaterials is plasma irradiation. Plasma, which is regarded as the fourth state of matter, contains highly energized atomic, ionic, radical, and molecular species. It is produced when gases are excited into energetic states by microwave, radio frequency, or electrons from a hot filament discharge [121]. The plasma irradiation technique can be performed with relatively inexpensive apparatus, but still provides an effective way to produce functionality to almost any biomaterial surface [122-124]. Plasma treatment is often used to activate a surface before chemical grafting is performed on the surface. In addition, plasma modification is able to uniformly functionalize surfaces regardless of shapes and sizes, allowing modification of films and sheets [123], micro- and nanoparticles [125], and artificial organ [126].

Plasma treatment of a surface can increase the surface wettability, introduce new functional groups, induce surface roughness, and result in chain scission and/or cross-linking. The final surface properties are a complex interplay between the types substrate treated with the plasma irradiation, cleaning process, gases used, plasma processing parameters, and storage time (aging). Generally, oxygen plasma treatment leads to the introduction of functional groups such as carboxylic acid groups, peroxide groups, and hydroxyl groups. Carboxylic groups may also be introduced by CO₂ or CO plasma treatment. CO₂ plasma treatment also produces hydroxyls, ketones, aldehydes, and esters [127]. Nitrogen or ammonia plasma treatment introduces primary, secondary, and tertiary amines, as well as amides. Polymers that are treated with argon or helium plasma will not directly lead to the incorporation of new functional groups, instead free radicals will be created on the surface [128]. The free radicals react with oxygen from the atmosphere [129]. It can be concluded that plasma treatment is not a highly selective surface modification technique because it does not result in one unique functional group.

2.6.4 Surface topography

Variations in surface texture or topography can affect tissue and cell adhesion. Surface topography affects tissue adhesion in a much simpler way than cell adhesion. Tissue generally adheres better on rough surfaces than smooth surfaces. Rough surface increases the contact area with tissue, providing more grip than a smooth surface and also increases friction to slippage. On the other hand, cells can react differently to certain surface topography and may not always adhere better on rougher material surface. For example, osteoblasts were found to attach well on rough titanium surface [130], but gingival fibroblasts and epithelial cells prefer to attach and grow on smooth titanium surface [131]. Many studies have been conducted to determine the effect of topography on cell behaviour, such as adhesion [132,133], morphology [134], migration [135], and differentiation [136].

The interactions of cells with substrates are mediated by subcellular microstructures, resulting in focal adhesions [137]. This phenomenon can induce distinct phenotypic expression by the cells, as changes in cell shape alter the

transduction of extracellular signals to the cell via integrin receptors [134]. Cells are sensitive to specific fluctuations in a material surface structure. Thus, the nature of cellular responses is influenced to the overall roughness of the surface.

2.7 Surface modifications of PMMA to improve biointegration of keratoprosthesis

There are not many studies in the literature that have investigated methods to improve the interfacial bonding and biointegration of KPro PMMA optics. Although individual studies employed different methods to measure the interfacial bonding strength, it was obvious that combination of changes in surface chemistry and increased surface hydrophilicity and roughness resulted from various surface modification techniques performed on the PMMA improved the adhesion of the substrate with collagen/tissue and also with corneal stromal cells. In an earlier study, Jeong and colleagues demonstrated a 7-time improvement in adhesion between PMMA disc (surface modified with dopamine and RGD peptide) and subcutaneous tissue after implantation of the disc subcutaneously in the dorsal lumbar area of the rat for 45 days [138]. In another study, Wang and colleagues applied d-CaP coating method on human KPro optic and applied a pull-out adhesion test to assess the force required to detach porcine corneal graft from the d-CaP coated KPro optic [139]. They found that it required 14.7 times more force to separate the graft from the surface modified PMMA optical cylinder than from the untreated cylinder after maintaining both constructs in organ culture for two weeks [139].

In a more recent study by Salvador-Culla and colleagues, they suggested that sandblasted titanium that was used as a sleeve around the KPro optic improved the adhesion strength (approximately 3 times) between the sleeve and porcine corneal graft [140]. The sleeve was shown to be biocompatible when implanted in the rabbit cornea for 10 months [140]. In a separate study, the sandblasted titanium substrates were shown to promote extracellular matrix deposition over time and enhance adhesion and proliferation of human stromal fibroblasts [141]. The resulting tissue adhesion strength on the surface modified titanium was, however, lower than that on the SBF-assembled CaP coated surface.

2.8 Hydroxyapatite coating on thermoplastic polymers

The most commonly found approach to coat hydroxyapatite on the polymeric surfaces reported in the literature is the deposition of bone-like apatite via incubation in SBF (pH 7.4) in 37°C. This technique has been applied on materials, such as PMMA [142-144], polystyrene [142,144], PDMS [142], polyethyleneterephthalate (PET) [145], poly(ϵ -caprolactone) (PCL) [146], and pHEMA [147]. However, the process can be long, requires refreshment of SBF with constant pH, and requires surface activation of the polymer in order to create a surface that promotes apatite nucleation (**Figure 2.12**). A study by Ryu and colleagues showed that titanium surface not activated with dopamine could not induce apatite nucleation [142]. In addition, the calcium and phosphate composition of the apatite layers varies depending on the types of the substrate and it often does not resemble the composition of HAp [148-150]. It has been shown that polymers with carboxyl groups are the most effective to induce apatite nucleation when immersed in SBF [151].

Figure 2.12. SEM images of apatite-like formation on dopamine-activated and non-activated surfaces. No apatite-like formation was observed on non-activated titanium (Ti) surface. On surface pre-treated with dopamine, aggregates of apatite-like material was seen 2 days after incubation in SBF. Long processing time (14 days) was required for full coverage of the coating even on the activated Ti surface. This figure was adapted from reference [142].

Other popular HAp coating techniques, such as plasma spraying [152,153], electrophoretic deposition [154,155], and magnetron sputtering [156,157], involve

high processing and annealing temperature ($>1000^{\circ}\text{C}$), which render them unsuitable to be employed for polymeric materials with relatively low melting temperature, such as the PMMA (110°C melting temperature).

2.9 Bacterial infections affect early biointegration of KPro

Bacterial infections or endophthalmitis in KPro is a devastating complication that can cause acute deterioration of vision. The reported infection rates in the literature vary, ranging from 3.2–17% of KPro patients' eyes [158-160]. The typical onset of endophthalmitis is the sudden experience of severe eye pain and redness with an acute reduction in vision (**Figure 2.13**) [161]. Bacterial infection is usually documented to occur approximately 10–18 months after KPro implantation [162]. However, the possibility of peri-operative or early post-operative infections cannot be completely ruled out. This is unfortunately true in less developed countries due to a combination of a few factors, such as poor medical infrastructure, microbiologically unsafe water and poor patients' compliance [163,164]. The circumstances are confounded in the fact that the majority of the global corneal blind patients that are in need of a KPro are in less developed countries. Another risk factor for the development of early infections is the pre-operative diagnosis that required a KPro, with most cases in eyes with conditions that are more susceptible to microbial infection, such as in Stevens–Johnson syndrome, mucous membrane pemphigoid, and ocular burns cases [161].

The occurrence of endophthalmitis early post-operatively can obviously affect the biointegration of the KPro and vice versa. The prognosis after endophthalmitis with a KPro is generally poor despite aggressive treatment with antibiotics. Progressive thinning of the donor graft usually developed causing the extrusion of the prosthetic device. Although some cases can be resolved with timely antibiotic treatment, approximately 53% of the infection cases require replacement of KPro [160]. Poor biointegration between PMMA and graft material can also increase the risk of bacterial infection. The potential space between the two materials creates an opportunity for microbial infiltration to gain access to the corneal stroma or the anterior chamber.

Figure 2.13. Photomicrograph of an infected patient's eye with KPro. Arrow indicates the development of acute bacterial keratitis. This figure was adapted from reference [165].

2.9.1 Bacterial flora and effective treatment

In order to prevent complications associated with bacterial infection, all KPro patients have to administer prophylactic topical antibiotics daily and indefinitely. During the earlier years of the KPro history, the treatment regimen included polymyxin-trimethoprim, ciprofloxacin or ofloxacin [166]. Because most cases of endophthalmitis after KPro implantation historically were from Gram-positive bacteria [164,167,168], topical vancomycin has been subsequently added to the prophylactic regimen. By summarizing the microbial culture results from 53 different studies, a review article by Robert and colleagues showed that 60% of the infection cases were caused by Gram-positive bacteria and 9% were caused Gram-negative bacteria [169]. The rest of the cases showed either negative cultures or due to fungal and mixed Gram-positive and Gram-negative bacterial infections.

Vancomycin is a branched glycopeptide produced by *Ammycolatopsis orientalis* bacterial species (**Figure 2.14**) [170]. Vancomycin acts by inhibiting cell wall formation in Gram-positive bacteria. The drug is, however, less effective in killing Gram-negative bacteria due to the different mechanism by which Gram-negative bacteria synthesize their cell walls and the various factors related to entering the outer membrane of Gram-negative organisms. The large molecule is able to form hydrogen bonds with the terminal D-Ala-D-Ala of the N-acetylglucosamine (NAG)/N-acetylmuramic acid (NAM) peptides in the Gram-positive bacterial cell wall (**Figure**

2.15, first and second panel from the left) [171]. This binding prevents cross-linking of the long polymer chains of NAG/NAM by occupying the docking site of DD-transpeptidase, the cell wall cross-linking enzyme (**Figure 2.15**, third and fourth panel from the left) [171].

Figure 2.14. Chemical structure of vancomycin.

Figure 2.15. Mechanism of vancomycin action in preventing cell wall synthesis of Gram-positive bacteria.

Similar to many drugs applied topically, the bioavailability of topical vancomycin is poor especially in the presence of an intact corneal epithelium [172].

Although in patients with advanced corneal disease, the corneal epithelial barrier is not always intact or is usually more 'leaky', the efficacy of topical antibiotic application is still limited by rapid tear fluid turnover and patients' compliance [173,174], which result in variations in drug administered concentration. To overcome these limitations, the ideal solution would be an antibiotic-eluting corneal prosthetic device capable of sustained and localized drug release. By substituting donor cornea with collagen hydrogel as a KPro skirt material, it may be possible to incorporate an antibiotic into the hydrogel.

2.9.2 Collagen-based corneal drug delivery system

Anti-infective collagen films or sheets of 0.01–0.5 mm thickness can be formed by air-drying aqueous mixture of collagen and antibiotic. Such collagen film was able to release pilocarpine for over 25 minutes, while the film dissolved without inducing any inflammatory response in rabbit eyes [175]. Longer release of pilocarpine of up to 15 days in vitro was achieved when the film was crosslinked with glutaraldehyde [176]. However, the authors did not assess the activity of the drug, which might be affected by the cross-linking process. Punch et al. [177] performed a study on films made from succinylated, soluble collagen loaded with insoluble antibiotics erythromycin estolate and found that the drug was able to be released with effective concentrations for more than 12 hours. Bloomfield et al. [178] compared delivery of gentamicin from succinylated collagen film with topical eyedrops, and subconjunctival antibiotic injection. The authors found that the collagen film resulted in higher drug levels in tear film, sclera and cornea. Succinylation appears to be advantageous in enhancing charge interaction between positive amino groups of the drug and the deprotonated carboxyl groups of succinylated collagen. Studies on drug-loaded collagen films have since been superseded by research on collagen shields in the literature.

Collagen shield is a dry contact lens-like films that have been immersed with antibiotic solution. Experiments performed in vitro demonstrated that collagen shields pre-soaked with a mixture of gentamicin and vancomycin released the majority of gentamicin within the first 30 min, while vancomycin was released over 6 hours [179].

In rabbit eyes, the release time was longer and the effective drug concentration was higher than the therapeutic range [179]. No adverse effects of the drugs and collagen shields were seen during the in vivo study [179]. In another study, Untermann et al. [180] demonstrated that collagen shields immersed in two drug concentrations of tobramycin had similar release profile (72 hours). Both concentrations produced significantly higher concentration of antibiotic in the cornea than subconjunctivally injected drug. However, toxic side effects were observed in the eyes treated with higher concentration dose [180]. Because of the typical short release time of the collagen shields, some authors concluded that a fortified topical antibiotic application pathway still leads to therapeutic drug concentration in the aqueous environment [181,182]. In addition to the short release time, the shields are usually not transparent, which reduces the potential use in the ophthalmology field.

2.10 Research gaps to be addressed

2.10.1 Gap 1: Improvement of surface modification method on PMMA and resulting adhesion between PMMA and collagen

Although a previous study has shown that the CaP-coated PMMA could improve the performance of a core-skirt KPro [139], the shear adhesion tests were performed using human donor corneas. To circumvent the use of human donor corneas, I substituted the human donor material with synthetic material (collagen type I hydrogel). I showed that CaP-coated PMMA could also improve the shear adhesion resistance to the collagen hydrogel exceeding the theoretical peak shear stress created by eyelid wiping motion (130 mN/cm^2) [73]. Although the sheer stress generated directly on the corneal surface or KPro should be significantly less due to the presence of tear film lubrication in between the eyelid and corneal or KPro surface, 130 mN/cm^2 was set as the threshold of improvement. Ultimately, the enhanced KPro with surface modified PMMA core and collagen hydrogel skirt could be aimed at reducing the heavy dependence on transplant-grade human donor corneas in the future and reducing various long-term complications associated with conventional KPro.

Although CaP coating on polymers via SBF incubation is a popular technique in the literature, there are drawbacks associated with the coating method, such as the long processing time, requirement of constant pH SBF, and resulting apatite-like layers containing impurities to name a few. There are other CaP coating techniques that have been described in the literature, but they involved high processing and/or annealing temperature ($>1000^{\circ}\text{C}$) which render them unsuitable for polymeric materials with relatively low melting temperature. I established a new non-thermal dip coating technique to immobilize HAp nanoparticles on PMMA. The procedure was simple and did not require complex equipment to perform. Moreover, the resulting coating could not be easily delaminated, still preserved the composition of pure HAp and was biocompatible to corneal stromal cells. The technique resulted in more than 2 folds improvement in adhesion between collagen and PMMA when compared to the previous technique utilizing SBF incubation.

2.10.2 Gap 2: Substitute KPro skirt material with anti-infective properties to reduce intra-operative infection risk

Corneal infections can occur during or early after implantation of a KPro and sometimes necessitate the removal of the device. The first week following KPro implantation is always crucial for epithelial cell recovery. The absence of epithelial barrier increases the susceptibility of the ocular surface to bacterial infection. An advantage of using a synthetic material as the skirt component of the KPro was the ability to incorporate an antibiotic which could sustain release the drug locally for a period of time. In my study, the aim was to produce a collagen-based skirt material that could release an antibiotic for at least 7 days to provide protection during recovery of the epithelial barrier. Previous idea that utilized drug releasing contact lens placed over a KPro may not be as effective due to poor drug penetration through the corneal epithelial barrier and rapid tear turnover rate [183]. Drug delivery from collagen-based vehicles, including vancomycin-soaked collagen shield, has been attempted previously, but the drug release could not be sustained for more than a day [176,177,179]. Many studies in the literature showed sustained release of drug in vitro; however, the activity of the drug released from the drug delivery vehicles was rarely shown even in vitro [184-186]. Activity of the drug may deteriorate during an

encapsulation process and when implanted in vivo. In my thesis, I showed that incorporation of vancomycin in collagen type I hydrogel produced a considerable improvement in sustaining drug release from a collagen-based system. Moreover, the efficacy of the released drug in preventing implantable device-associated *S. aureus* infections was also demonstrated in vivo.

2.11 References

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Chapter 3

Comparison of surface modification techniques on PMMA to improve the performance of core-skirt keratoprosthesis

In this chapter, several surface modification techniques on PMMA were explored, namely: oxygen plasma irradiation (plasma group); plasma treatment followed by calcium phosphate (CaP) coating (p-CaP); activation with dopamine and 11-MUA followed by CaP coating (d-CaP); and plasma irradiation followed by coating with (3-aminopropyl)triethoxy-silane (3-APTES). To create a KPro model, collagen type I hydrogel was constructed overlying the modified PMMA surfaces. All surface modifications produced improved shear adhesion strength compared to untreated PMMA. The p-CaP group showed the best interfacial adhesion with the hydrogel ($177 \pm 27 \text{ mN/cm}^2$), followed by d-CaP ($168 \pm 31 \text{ mN/cm}^2$), 3-APTES ($145 \pm 12 \text{ mN/cm}^2$) and plasma ($119 \pm 10 \text{ mN/cm}^2$). Longer-term stability of the adhesion was achieved by d-CaP, whereby after 14 and 28 days in PBS in 37°C , yielded $164 \pm 25 \text{ mN/cm}^2$ ($p=0.906$ compared to adhesion at day 1) and $131 \pm 20 \text{ mN/cm}^2$ ($p=0.053$), respectively. In contrast, significant reduction of adhesion strength was observed in p-CaP group over time ($p<0.001$). All surface coatings were biocompatible to human corneal stromal fibroblasts in vitro, except the 3-APTES group, which showed no live cells after 3 days. In contrast, cells on d-CaP surface showed good anchorage, evidenced by the expression of focal adhesion proteins and filopodia protrusions. In conclusion, d-CaP can not only improve and provide stability to the adhesion of collagen hydrogel on the PMMA surface, but also promote biointegration.

3.1 Background of study

The surface modification of corneal prosthetic devices has not been extensively studied in the literature, but has started to gain some attention due to increasing number of observations on the poor adhesion between the rigid prosthetic material (PMMA) and the soft corneal tissue. The weakest point of a core-skirt KPro is at the interface between the two inherently different materials in terms of their flexibility, and physical and chemical properties. The constant eyelid blinking motion and eyeball movement inevitably weaken the bonds between the hard and soft materials over time. Eventually, the interactions become diminished, creating an opening for epithelial downgrowth, bacterial infection and inflammatory cell invasion, which ultimately cause the device to extrude and fail [1,2].

Wang and colleagues had attempted to address the shortcoming elaborated in the preceding paragraph by coating the PMMA surface with HAp, which was achieved by incubation of dopamine and 11-mercaptoundecanoic acid (11-MUA)-activated polymer in 1.5x SBF for two weeks [3]. The idea of coating the PMMA surface with HAp stems from the ability of stromal fibroblasts to integrate with human tooth (which is predominantly composed of HAp) observed in the OOKP device [4]. Using in vitro mechanical testing, the authors showed that the force required to detach the corneal tissue from the HAp-coated PMMA rod was increased by 10 folds compared to non-coated PMMA [3]. Because this is the most relevant study in the literature, the described coating technique was used as the positive control group in the current study.

Plasma treatment is a convenient technique to modify the surface of polymers without affecting the bulk properties of the materials. With different gases, different functional groups can be introduced onto the surface of a polymer and at the same time the surface topography can also be changed [5,6]. In this study, oxygen plasma was applied in an attempt to incorporate $-C-O-$ groups onto the PMMA surface and increase the negative charges [6], as well as to produce rougher surface to benefit the adhesion to the corneal tissue, which is predominantly constructed by collagen type I [7]. In addition, changes in surface chemistry and topography induced by oxygen

plasma could become more favourable for further surface modifications, for example by grafting a crosslinker [8,9]. The crosslinker can then be covalently bonded to the collagen of the tissue.

An alkoxysilane, 3-APTES, is commonly used to attach proteins or soft materials on various types of hard material [10,11]. The $-NH_2$ terminal end of 3-APTES can subsequently be modified with another crosslinker, such as glutaraldehyde, to produce a reactive group that binds to materials with amine ends, such as collagen. The amine group can also react with carbodiimide-activated $-COOH$ end of collagen to form a covalent linkage. DiVito and colleagues had developed a composite hydrogel made of a mixture of 3-APTES and collagen type I, which was shown to be biocompatible in rabbit corneas *ex vivo* [12]. Therefore, 3-APTES can be a candidate molecule to modify the PMMA surface to improve its adhesion with corneal tissue.

In the current study, I used collagen type I hydrogel, instead of human corneal tissue, to study its adhesion on various modified PMMA surfaces as a KPro model. By using collagen hydrogel as donor corneal substitute, current heavy reliance on the donor corneas could be reduced and long-term complications normally associated with the use of donor corneas may be minimized. In a nutshell, this study attempted to show the potential improvements of interfacial adhesion between a surface modified rigid material and a soft material, and also to highlight a possibility to develop a modified KPro with PMMA core and collagen hydrogel skirt in the near future. The PMMA surface was modified using four different methods: 1) oxygen plasma irradiation; 2) surface activation with oxygen plasma, followed by CaP deposition; 3) surface functionalization with dopamine and 11-MUA, followed by CaP coating as described previously [3]; 4) surface activation with oxygen plasma, followed by 3-APTES coating. In order to assess the outcomes of the different coatings, the resulting interfacial adhesion strength, cytotoxicity of the surface coatings as well as the biointegration of human corneal stromal fibroblasts on the modified PMMA were investigated.

3.2 Materials and methods

3.2.1 Oxygen plasma functionalization of PMMA surface

PMMA sheets with 0.5 mm thickness were purchased from Goodfellow (Huntingdon, England). The sheet was cut into 2 x 3 cm pieces, washed in 70% ethanol for 15 minutes and rinsed extensively with distilled water. Oxygen plasma treatment was performed in a Covance multi-purpose plasma system (Femto Science, South Korea). The samples were treated with radio frequency oxygen plasma with 200W power for 2, 5 or 10 minutes. The PMMA sheets were placed between two parallel plate electrodes enclosed in the plasma reactor chamber. Air was removed with vacuum application for at least 30 minutes before the power was turned on. The pressure at the moment of plasma discharge was 0.2 Torr. The flow rate of oxygen was set at 20 standard cubic centimeters per minute (sccm). After the discharge stopped, oxygen gas flow was continued for a further 15 minutes to stabilize the resulting functional groups. This treatment method was referred to as *plasma* group.

3.2.2 Functionalization of PMMA surface with calcium phosphate

Two different methods were used to deposit CaP on the PMMA surface. In the first method, previously reported by Wang and colleagues [3], cleaned PMMA sheets (2 x 3 cm in size) was treated with 2 mg/ml dopamine (Sigma-Aldrich, St. Louis, MO) in 10 mM Tris buffer, pH 8.5 (Sigma-Aldrich) overnight at room temperature, followed by immersion in 50 μ M of 11-mercaptoundecanoic acid (11-MUA; Sigma-Aldrich) for 4 hours at room temperature, before being placed in 1.5x simulated body fluid (SBF) solution at 37°C for 14 days. The SBF was prepared according to the protocol described by Kokubo and colleagues [13]. In brief, the SBF solution contains 213.0 mol/m³ Na⁺, 7.5 mol/m³ K⁺, 2.3 mol/m³ Mg²⁺, 3.8 mol/m³ Ca²⁺, 221.7 mol/m³ Cl⁻, 6.3 mol/m³ HCO₃⁻, 1.5 mol/m³ HPO₄³⁻, and 0.8 mol/m³ SO₄²⁻. The SBF solution was refreshed daily. After 14 days, the sheets were washed extensively in distilled water and dried in a vacuum incubator at 37°C. This treatment was referred to as *d-CaP* group.

In the second CaP coating method, referred as *p-CaP* group, instead of coating with dopamine and 11-MUA, the PMMA surface was treated with oxygen plasma for 5 minutes. The oxygen plasma treatment on the PMMA was as described in section 3.2.1. Following plasma treatment, the sheets were incubated in 1.5x SBF at 37°C for 14 days and refreshed daily.

3.2.3 Functionalization of PMMA surface with 3-APTES

Cleaned PMMA sheets (2 x 3 cm in size) were first treated with oxygen plasma for 5 minutes (power setting and oxygen flow were as described in section 3.2.1). The PMMA sheets were immersed in 5% (v/v) 3-APTES (Sigma-Aldrich) in 70% ethanol overnight at room temperature. The sheets were then washed with 70% ethanol for 15 min, rinsed extensively with distilled water, dried in vacuum incubator at 37°C, and finally placed in 90°C incubator overnight to stabilize the coating. This treatment was referred to as *3-APTES* group.

3.2.4 Water contact angle analysis

The water contact angle was measured by a FTA200 contact angle goniometer (First Ten Angstroms, Inc., Portsmouth, VA) using a static sessile drop technique. At room temperature, distilled water was pumped out of a syringe at a rate of 5 µl/s and allowed to settle for 15 seconds on the samples before the image was taken. The contact angle was generated from the image with FTA32 software version 2.0 (build 276.2) (Portsmouth, VA).

3.2.5 Analysis of surface morphology and elemental composition

Surface morphology and roughness of functionalized PMMA sheets was observed with a Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA). Topographic images were captured in tapping mode employing monolithic silicon NCH-50 Point Probe (NanoWorld AG, Neuchatel, Switzerland). The scan size was set at 15 x 15 µm. The surface was scanned at a rate of 1 Hz with a resolution of 256 x 256 pixels. Three-dimensional topographic images and roughness (RMS value) data

were obtained using Nanoscope image processing software version 5.12r5 (Digital Instruments).

In addition to AFM, surface morphology of functionalized PMMA sheets was also analyzed by SEM. PMMA sheets (from d-CaP group), which had been subjected to shear adhesion test, were assessed with SEM to investigate adhesion failure analysis. In brief, the PMMA sheets were mounted on a stub secured by carbon adhesive tape. The sheets were sputter-coated with 10-nm-thick layer of gold and examined with a JSM-6300F microscope (JEOL, Tokyo, Japan) at accelerating voltage of 10 kV. Surface elemental composition was assessed by EDX attached to the microscope.

3.2.6 Analysis of surface functional groups by ATR-FTIR

Infrared (IR) spectra of the modified PMMA surfaces were collected in the range from 4000 to 650 cm^{-1} using a PerkinElmer Frontier FTIR spectrometer (Waltham, MA). The spectrometer was equipped with an ATR sampling universal accessory supplied with a top plate for ZnSe crystal. The plate was cleaned with 70% ethanol before acquisition of each spectrum. The PMMA sheet was placed on the plate and tightened down to the 40% gauge mark shown on the software for the instrument. Spectra were obtained with 16 scans and 4 cm^{-1} resolution.

To delineate the differences in resulting surface functional groups between PMMA treated with 2-, 5- or 10-minute oxygen plasma, spectral subtraction of the untreated PMMA from the plasma-treated PMMA in the 2400-4000 cm^{-1} region was performed. The subtraction factor was adjusted for each subtraction until the strong CH_3 peaks in the region of 2800-3100 cm^{-1} were cancelled out.

3.2.7 Casting of collagen hydrogel on functionalized PMMA surface

Casting of collagen hydrogel on the modified PMMA surfaces was performed immediately after surface modification. The collagen hydrogel was constructed as described previously [14]. Bovine atelocollagen type I was purchased from Koken

(Tokyo, Japan). Briefly, 0.5 ml aliquot of 10% (w/v) atelocollagen in acidic solution (pH 3.0) was loaded into a syringe mixing system (**Figure 3.1A**). The collagen solution was adjusted to pH 5.0 with 1N NaOH, followed by thorough mixing by pumping the syringes. Calculated amounts of 10% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma-Aldrich, St. Louis, MO) and 10% (w/v) N-hydroxysuccinimide (NHS; Sigma-Aldrich) were added to produce a 2:1 molar ratio of EDC to NHS and mixed with the collagen solution. Biomechanical properties of the resulting hydrogel were first measured to ascertain successful crosslinking/hydrogelation process (**Figure S1.1 and Table S1.1**). The mixed solution (0.1 ml) was dispensed onto the center of the PMMA sheet and flattened with a glass slide, resulting in a cylindrically shaped hydrogel (**Figure 3.1B**). Thickness of the hydrogels was consistently maintained by placing 0.5-mm-thick spacers under the glass side at the edges of the modified PMMA sheet. The hydrogel was cured in a humidified chamber at room temperature for 24 hours before being subjected to the shear bond strength test. Two groups that yielded the best adhesion to the PMMA and untreated PMMA were subjected for a longer term study, whereby the hydrogel-modified PMMA constructs were incubated in 0.01M phosphate buffered saline (PBS) at 37°C for 14 and 28 days, with PBS refreshed daily.

Figure 3.1. Syringe mixing system to cast collagen hydrogel. (A) Collagen solution was loaded in one syringe, which was coupled to a second, empty syringe through a T-shaped connector. In order, NaOH, NHS and EDC were injected through the septum in the connector marked by asterisk (*). Complete mixing was carried out by pumping the combined solutions between the two syringes. (B) Collagen hydrogel with 0.5 mm thickness (indicated by an arrow) was then casted on a surface modified PMMA sheet.

3.2.8 Shear adhesion strength test

Samples (hydrogel adhered on PMMA sheets; $n=5$ of each group) were secured at the base of a Chatillon mechanical tester (Largo, FL). The position of the sample was adjusted until the chisel-shaped fixture (attached to 10 N load cell) aligned at the center of the hydrogel at the bonding interface (**Figure 3.2**). The crosshead speed was set at 5 mm/min. The test was stopped and maximum load was recorded when the hydrogel was completely detached from the surface of PMMA sheets.

Figure 3.2. Shear adhesion strength test setup. A chisel-shaped fixture was attached to the load cell and would shear the bonding interface as the load cell travelled downwards. Maximum force needed to completely detach the collagen hydrogel from the PMMA surface was then recorded.

3.2.9 Culture and seeding of corneal stromal fibroblasts

Research grade cadaveric corneal tissues were purchased from Lions Eye Institute for Transplant and Research (Tampa, FL). They were preserved in Optisol-GS (Bausch&Lomb Surgical, Irvine, CA) and transported at 4°C to the laboratory. Central button (8 mm in diameter) was trephined and treated with dispase II (20 mg/ml, Roche, Basel, Switzerland) followed by gentle scraping to completely remove corneal epithelium and endothelium. The stromal tissue was trimmed into small pieces and digested with collagenase I (1 mg/ml, Worthington, Lakewood, NJ) in DMEM/F12 (Life Technologies, Carlsbad, CA) for 9-12 hours at 37°C. Cells were cultured in DMEM/F12 containing 10% fetal bovine serum (FBS; Life Technologies)

and 1% antibiotic/antimycotic (penicillin, streptomycin sulfate and amphotericin B; Life Technologies). At passage 3 to 5, the stromal fibroblasts with 1000 cells/cm² starting density was seeded on sterile modified PMMA sheets and cultured for 24 and 72 hours before further tests. Cells seeded on glass coverslips were considered as control.

3.2.10 Cell proliferation assay

Proliferation of corneal fibroblasts on modified PMMA surfaces was assessed using a 5-ethynyl-2'-deoxyuridine (EdU) assay kit (Life Technologies) according to the manufacturer's protocol. In brief, the cells were incubated in EdU (10 μ M) containing medium for 24h. They were then washed with PBS, fixed with 4% paraformaldehyde (Sigma), followed by blocking and permeabilization in 0.1% Triton X-100 (Sigma) in 3% bovine serum albumin (Sigma) at room temperature. Incorporated EdU was detected by Alexa Fluor 488 fluorescent-azide coupling Click-iT reaction [15]. Finally, the samples were mounted in Fluoroshield containing DAPI (4',6-diamidino-2-phenylindole; Santa Cruz Biotechnology, Santa Cruz, CA) and viewed under Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at 200X magnification. Total number of cells and EdU-positive cells were quantified from 3 random fields of each sample (n=3 of each group).

3.2.11 Cell viability assay

The cytotoxicity was analyzed using Live/Dead® Viability/Cytotoxicity assay kit (Life Technologies) according to the manufacturer's protocol. In brief, the cells were incubated with calcein AM and ethidium homodimer-1 (EthD-1) for 45 minutes, washed and mounted in Fluoroshield containing DAPI. Samples were viewed using a Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss) at 100X magnification. Live cells were stained green by calcein AM and dead cells in red by EthD-1. Both live and dead cells were quantified from 2 random fields on each sample (n=3 of each group), and the live/dead cell ratio was calculated. In addition, initial cell attachment efficiency was calculated by using the following formula:

Cell attachment efficiency (%)

$$= \frac{\text{Actual number of cells in a viewing field}}{\text{Theoretical number of cells expected to attach in a viewing field}} \times 100\%$$

where the theoretical number of cells expected to attach in a 100x viewing field (4.5 x 3.5 mm dimension) was equivalent to 157 after accounting for the 1000 cells/cm² seeding density.

3.2.12 Immunocytochemistry of cell attachment markers

Cells on glass coverslips and surface modified PMMA sheets were fixed with freshly-prepared neutral buffered 2% paraformaldehyde (Sigma-Aldrich). After quenching of free aldehyde with 50 mM ice-cold ammonium chloride and PBS washes, cells were permeabilized and blocked with PBS containing 0.425% saponin (Sigma-Aldrich), 1% bovine serum albumin (BSA; Sigma-Aldrich) and 2% normal goat serum (Life Technologies), followed by incubation of primary antibodies: mouse monoclonal anti-paxillin (Millipore, Billerica, MA) and mouse monoclonal anti-vinculin (Sigma-Aldrich). After PBS washes, cells were incubated with donkey anti-mouse IgG AlexaFluor 488-conjugated secondary antibody (Jackson ImmunoRes Lab, West Grove, PA) and AlexaFluor 568-conjugated phalloidin (Life Technologies). The samples were mounted in Fluoroshield containing DAPI and viewed under Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss).

3.2.13 Assessment of cell morphology

Cell morphology on functionalized PMMA sheets was observed by SEM. Cells were fixed in 2% glutaraldehyde (Sigma-Aldrich) in 0.1M sodium cacodylate, pH 7.4 (Millipore) overnight at 4°C. The samples were then washed twice in distilled water for 10 minutes each before being immersed in 1% osmium tetroxide (FMB, Singapore) for 2 hours at room temperature. Following washing in distilled water (twice for 10 minutes each), the samples were dehydrated in increasing concentrations of ethanol for 10 minutes each (from 25%, 50%, 75%, 95% to 100%, with 95% and 100% concentrations being performed twice). Critical point drying was then performed using Bal-Tec dryer (Balzers, Liechtenstein). Finally, the samples were mounted on stubs

secured by carbon adhesive tape, sputter coated with 10-nm thick gold and examined with a JSM-5600 microscope (JEOL).

3.2.14 Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). Statistical significance between groups was calculated by one-way ANOVA and post hoc Tukey comparison test. A value of $p < 0.05$ was considered to be statistically significant. All statistical analysis was performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL).

3.3 Results and Discussion

3.3.1 General consideration for choosing surface modification technique, shear adhesion test method, and hydrogel casting method

Surface hydroxyls resulted from oxygen plasma treatment (**Figure 3.3A**) can undergo condensation reaction with silanols of the 3-APTES (**Figure 3.3B**). The amine ends of 3-APTES coating are then subsequently covalently bonded to the carboxyls, which are abundantly present in the collagen, via carbodiimide chemistry (**Figure 3.3B**).

Dopamine, utilized in the d-CaP group, is a versatile underwater adhesive that can form on a variety of surfaces and further induce CaP deposition in SBF [16]. The addition of 11-MUA with carboxyl ends creates a negatively charged surface, which can enhance the dopamine-coated PMMA surface as nucleation site for CaP deposition (**Figure 3.3C**, left panel). Similar to functionalization with dopamine and 11-MUA, the activation of PMMA with oxygen plasma produces negatively charged surface via the creation of surface carboxyls and hydroxyls, which in turn acts as nucleation site for CaP (**Figure 3.3C**, right panel). Electrostatic bonding is proposed to be the main driving force for the interaction of CaP to collagen, while adsorption of functional binding groups of collagen by CaP and hydrogen bonding can be formed as secondary interaction forces (**Figure 3.3C**) [17,18]. In addition, the surface roughness of CaP-

coated PMMA also cannot be ruled out in improving its interfacial adhesion to collagen.

Figure 3.3. Illustrations of mechanism of various PMMA surface modifications. (A) Oxygen plasma treatment on PMMA introduces $-\text{OH}$, $-\text{COOH}$ and $=\text{O}$ functional groups. (B) 3-APTES coating on oxygen plasma activated PMMA surface and its subsequent interactions with the collagen hydrogel. (C) Apatite-like deposition on either dopamine+11-MUA functionalized surface (left panel) or oxygen plasma activated surface of PMMA (right panel) and subsequent interactions with the collagen hydrogel.

I had initially attempted to construct the collagen hydrogel, followed by bringing the hydrogel in contact with the modified PMMA in order to mimic the construction of the conventional KPro. However, the hydrogel could not form any adhesion with the underlying PMMA, regardless of the surface coatings. Adhesion

could only be achieved when the hydrogel was allowed to crosslink and settle overnight on the PMMA surface.

Figure 3.4. Hydrophilicity and roughness of oxygen plasma treated PMMA surface. (A) Water contact angle analysis of untreated PMMA and PMMA treated with oxygen plasma for 2, 5 and 10 minutes. (B) AFM revealed commensurate surface roughness with increasing plasma treatment time. (C) Bar graph showing the mean roughness (nm) of untreated and plasma treated PMMA surfaces. Height of error bars indicates standard deviation of means. * $p < 0.001$ in comparison to untreated PMMA. (D) Plasma 10 min resulted in opaque PMMA. In contrast, plasma 2 min and 5 min did not induce opaqueness to PMMA. Scale bar = 1 cm.

Several ASTM standardized adhesion tests, such as lap shear adhesion and peel adhesion tests, were considered and attempted. However, because the collagen casting method produced rather round hydrogels, trimming of sample into rectangular stripes and other minor sample manipulation steps (e.g. lifting and clamping of one edge of hydrogel to the load cell before the peel adhesion test) often detached or partially detached the sample from the PMMA sheets. As a consequence, these adhesion tests produced highly inconsistent results. The current adhesion test was

selected due to the similarity of study objective and sample size and shape to the occlusal adhesive tests in many orthodontic related studies [19-21]. The shear bond strength values were calculated from peak load at failure divided by the hydrogel surface area.

3.3.2 Optimization of plasma treatment to activate PMMA surface

I first investigated the effect of oxygen plasma treatment on the surface hydrophilicity of PMMA and determined the optimized plasma treatment time to activate the PMMA surface prior to further surface modifications. The surface hydrophilicity level of PMMA increased after a 2-minute plasma treatment, which yielded water contact angle of $43.4 \pm 5.8^\circ$ and the contact angle reduced further after a 5-minute treatment to $24.6 \pm 1.1^\circ$ (both $p < 0.001$ relative to untreated PMMA) (**Figure 3.4A**). Increasing the treatment time beyond 5 minutes produced a diminishing return in terms of hydrophilicity level, evident by higher water contact angle after 10-minute treatment ($42.0 \pm 1.5^\circ$) compared to that after 5-minute treatment (**Figure 3.4A**). This return was attributed to a substantial change in surface morphology (see AFM analysis below) rather than to a reduction in the amount of O-H functional groups that formed by increasing plasma treatment time beyond 5 minutes (see surface IR analysis in the following paragraph). On AFM, it was revealed that surface roughness increased with plasma treatment time, where the 2-min plasma treatment produced a roughness of 9.7 ± 1.0 nm ($p < 0.001$ relative to untreated PMMA) (**Figure 3.4B**), the 5-min plasma treatment produced a roughness of 18.7 ± 1.0 nm ($p < 0.001$ relative to untreated PMMA) (**Figure 3.4B**), and the 10-min plasma treatment increased the roughness to 31.7 ± 2.5 nm ($p < 0.001$ relative to untreated PMMA) (**Figure 3.4B**). This finding is consistent with a previous published study [22]. **Figure 3.4C** summarizes the average roughness of untreated PMMA and plasma-treated PMMA surfaces by AFM in a bar graph. Moreover, the 10-min plasma-treated PMMA appeared opaque due to prolonged surface plasma etching (**Figure 3.4D**).

Figure 3.5. Surface infrared spectra of oxygen plasma treated PMMA. Minute differences could be seen in the IR spectrum of untreated PMMA and plasma treated materials. (A) Differences in intensity of spectral peak at 3442 cm^{-1} , attributable to O-H functional group, could be seen. (B) Further analysis was performed by spectral subtraction of the untreated PMMA from the plasma treated PMMA. The subtraction process revealed that plasma 5 min and plasma 10 min produced similar spectral intensity, followed by plasma 2 min. (C) Intensity of spectral bands at 2951 cm^{-1} and 2995 cm^{-1} (C-H stretching vibration mode) was also slightly different between treatments. (D) Spectral bands between $1800\text{--}1600 \text{ cm}^{-1}$, corresponding to C=O functional groups.

I then investigated the surface IR spectra of the untreated PMMA and the PMMA treated with plasma for different lengths of time. Small differences in the

resulting surface IR spectra could be seen in certain spectral regions (**Figure 3.5**). First, there was a clear difference in the relative intensity of the peak between 3650-3350 cm^{-1} , attributable to O-H functional group (**Figure 3.5A**). The subtraction process revealed that plasma treatment for 2, 5 and 10 minutes produced a broad band peaking near 3400 cm^{-1} with highest intensities seen after 5 and 10 min plasma treatment (**Figure 3.5B**). This broad peak is characteristic of H_2O , suggesting that the amount of water attached to the plasma-treated PMMA increased with treatment time. A second spectral intensity difference was seen between 2951 and 2995 cm^{-1} (C-H stretching modes), where the untreated PMMA produced the highest peak intensity, followed by plasma 2 min, plasma 5 min and plasma 10 min (**Figure 3.5C**), suggesting that $-\text{CH}_3$ terminal functional groups were more abundant on untreated surface compared to the plasma treated PMMA. The reduction of peak intensities was in line with the formation of O-H groups due to partial oxidation of the PMMA surface. All plasma treatments resulted in higher band intensity at 1800-1600 cm^{-1} than that of untreated PMMA (**Figure 3.5D**), owing to the more abundant C=O groups (aldehyde and carboxylic acid) [23]. The FTIR results were corroborated by EDX analysis, showing a reduction of percentage of C atom and an elevation of percentage O atom with increasing plasma treatment time (**Table 3.1**). In order to provide PMMA surface with sufficient hydrophilicity and to avoid opaqueness due to increasing surface roughness, oxygen plasma treatment of 5 minutes was selected and performed to activate the PMMA surface before further surface modifications.

Table 3.1. Atomic composition on PMMA surface treated with different lengths of time of oxygen plasma.

	C	O
Untreated PMMA	$71.2 \pm 0.3\%$	$28.8 \pm 0.3\%$
Plasma 2 min	$70.9 \pm 0.6\%$	$29.1 \pm 0.6\%$
Plasma 5 min	$69.7 \pm 0.8\%$	$30.3 \pm 0.8\%$
Plasma 10 min	$69.1 \pm 0.3\%$	$30.9 \pm 0.3\%$

3.3.3 Surface hydrophilicity following surface modifications

The surface hydrophilicity of PMMA after various surface modifications is summarized in **Table 3.2**. The treatment that yielded the highest surface hydrophilicity (as measured by reduced water contact angle relative to untreated PMMA) was p-CaP group ($2.7 \pm 3.1^\circ$) and d-CaP group ($2.7 \pm 3.1^\circ$), followed by plasma group ($24.6 \pm 1.1^\circ$). One-way ANOVA with post-hoc Tukey test revealed that all surface modification techniques significantly reduced the water contact angle compared to untreated PMMA ($p < 0.001$), except the 3-APTES group. Treatment with 3-APTES slightly increased the water contact angle ($72.0 \pm 0.9^\circ$), but it was not significantly different than untreated PMMA ($68.4 \pm 3.0^\circ$) ($p = 0.286$). The hydrophobic character of aminosilane coating was consistent with a previous study [24], which could be attributed to the folding of amine terminal function to form hydrogen bonds with free silanol groups [25].

Table 3.2. Surface hydrophilicity of untreated PMMA and modified PMMA.

	Water contact angle	<i>p</i> value*
Untreated PMMA	$68.4 \pm 3.0^\circ$	
Plasma	$24.6 \pm 1.1^\circ$	2.07×10^{-12}
d-CaP	$2.7 \pm 3.1^\circ$	1.28×10^{-12}
p-CaP	$2.7 \pm 3.1^\circ$	1.28×10^{-12}
3-APTES	$72.0 \pm 0.9^\circ$	0.286

*Significant difference relative to untreated PMMA

$p < 0.05$ was considered to be statistically significant

3.3.4 Surface infrared spectra and elemental composition after surface modifications

The surface IR spectra of untreated PMMA and differentially modified PMMA surfaces are shown in **Figure 3.6**. Untreated PMMA showed distinct spectral peaks near $1100\text{--}1300\text{ cm}^{-1}$ and 1726 cm^{-1} , which were attributed to the presence of C-O and C=O stretching modes of the ester group, respectively. In addition, a spectral peak at

2951 cm^{-1} indicated the C-H stretching of PMMA. Spectral peaks of PMMA were not that prominent in the p-CaP and d-CaP groups due to the relatively thick coating layer, which almost completely masked the PMMA surface. A peak at 1027 cm^{-1} and a weak shoulder at 958 cm^{-1} in both groups were attributed to the P-O group of CaP. The peak at 1027 cm^{-1} in d-CaP group appeared to have slightly higher intensity than that in p-CaP group. Three peaks at 1611, 1488 and 1408 cm^{-1} , corresponding to the carbonate group of the CaP coating, could also be observed. In the 3-APTES group, spectral peaks appeared at 3294 and 3395 cm^{-1} , indicating the presence of the primary amine group of 3-APTES. Moreover, a peak at 1596 cm^{-1} confirmed the presence of primary amine groups (N-H bending mode) attributed to 3-APTES coating. A sharp peak at 1000 cm^{-1} indicated the presence of Si-O-Si on the aminosilane modified PMMA surface.

Figure 3.6. Infrared spectra of surface modified PMMA. Comparison of surface IR spectrum obtained from untreated PMMA and modified PMMA by oxygen plasma, d-CaP coating, p-CaP coating and 3-APTES.

Consistent with the FTIR analysis, EDX spectroscopy demonstrated a reduction of C atoms and increased O atoms in the plasma group (**Figure 3.7**) compared to untreated PMMA (**Figure 3.7**), which suggested the creation of surface O-H groups by oxygen plasma treatment. EDX further corroborated our FTIR analysis, which showed relatively more phosphate groups produced by d-CaP than p-CaP. This resulted in lower Ca/P ratio in d-CaP group (1.21 ± 0.03) (**Figure 3.7**) and p-CaP (1.29 ± 0.06) (**Figure 3.7**). However, the difference in Ca/P ratio was not significantly different ($p=0.069$). EDX revealed the presence of $10.2 \pm 1.0\%$ Si atoms in the 3-APTES group (**Figure 3.7**). The atomic composition of untreated and modified PMMA surfaces is summarized in **Table 3.3**.

Table 3.3. Surface composition of untreated PMMA and modified PMMA detected by EDX.

	C	O	Ca	P	Si
Untreated PMMA	$71.2 \pm 0.3\%$	$28.8 \pm 0.3\%$	-	-	-
Plasma	$69.7 \pm 0.8\%$	$30.3 \pm 0.8\%$	-	-	-
d-CaP	-	-	$54.8 \pm 0.6\%$	$45.2 \pm 0.6\%$	-
p-CaP	-	-	$56.4 \pm 1.2\%$	$43.6 \pm 1.2\%$	-
3-APTES	$60.9 \pm 2.0\%$	$28.9 \pm 1.3\%$	-	-	$10.2 \pm 1.0\%$

3.3.5 Surface morphology and roughness after surface modifications

Macroscopically, plasma (**Figure 3.7**) and 3-APTES (**Figure 3.7**) treated PMMA appeared transparent and similar to untreated PMMA (**Figure 3.7**). In contrast, d-CaP (**Figure 3.7**) and p-CaP (**Figure 3.7**) appeared opaque and were completely covered by the bone-like apatite. Under SEM, nano-sized particles, indicative of bone-like apatite growth [26], were seen covering the entire PMMA surface of d-CaP (**Figure 3.7**) and p-CaP (**Figure 3.7**). Agglomerates of the particles were present in some regions, attributing to the surface roughness of both CaP-coated surfaces. This observation was confirmed by cross-sectional SEM image of d-CaP (**Figure 3.8A**) and p-CaP (**Figure 3.8B**), which revealed that multi-layered particles in some regions were thicker than another due to agglomeration of the apatite-like

particles. AFM showed an average roughness of 343 ± 88 nm for d-CaP group (**Figure 3.9C**) and 301 ± 34 nm for p-CaP group (**Figure 3.9D**). No significant difference was found between d-CaP and p-CaP groups ($p=0.235$).

Figure 3.7. Morphology and elemental composition of surface modified PMMA assessed by SEM and EDX. (Left panels) Macroscopic appearance of untreated and surface modified PMMA sheets. (Middle panels) SEM images of untreated and surface modified PMMA sheets. (Right panels) EDX spectrographs showing surface elemental composition of untreated and surface modified PMMA.

Figure 3.8. Cross-sectional SEM images of CaP-coated PMMA surfaces. Both d-CaP (A) and p-CaP (B) produced multi-layered nano-sized particles with some regions thicker than another due to agglomeration of particles. The thickness of the CaP coating appeared to be ranging from 20-40 μm .

Table 3.4. Surface roughness of untreated PMMA and modified PMMA.

	Mean RMS (nm)	<i>p</i> value*
Untreated PMMA	1.5 ± 0.3	
Plasma	18.7 ± 1.0	5.83×10^{-13}
d-CaP	342.8 ± 88.1	2.74×10^{-6}
p-CaP	300.7 ± 34.1	4.66×10^{-9}
3-APTES	8.1 ± 1.1	2.84×10^{-8}

*Significant difference relative to untreated PMMA

$p < 0.05$ was considered to be statistically significant

The untreated PMMA surface appeared relatively smooth under both SEM (**Figure 3.7**) and AFM (**Figure 3.9A**), interspersed with some nano-sized grooves. Nanoscale surface undulations, created by plasma etching, were observed in the plasma group (**Figure 3.7**). Under AFM, there was an increase in surface roughness after 5-minute plasma treatment (18.7 ± 1.0 nm; **Figure 3.9B**). As shown in SEM images, coating with 3-APTES smoothed the initially plasma etched surface, evidenced by the barely visible nanoscale undulations seen in the plasma group earlier (**Figure 3.7**). This was confirmed by AFM, which showed a significantly smoother surface in 3-APTES group (8.1 ± 1.1 nm; **Figure 3.9E**) compared to the plasma group ($p < 0.001$). The mean RMS value for untreated and all modified PMMA surfaces is summarized in **Table 3.4**.

Figure 3.9. Surface roughness of untreated and modified PMMA analyzed with AFM. (A) Untreated PMMA. (B) Plasma group. (C) d-CaP group. (D) p-CaP group. (E) 3-APTES group.

3.3.6 Adhesion of collagen hydrogel on modified PMMA surfaces

Figure 3.10 summarizes the resulting interfacial bonding strength, measured 1 day after the hydrogels were casted on untreated and modified PMMA surfaces. The raw data indicated that there was a mild stick-slip phenomenon (relatively small fluctuations along the force-displacement curves), except on untreated PMMA surface. It appeared that all adhesions failed at the peak load. The force-displacement curves suggested that normal force vector was applied, instead of peel force vector, which typically produced a relatively plateaued curve after the peak load had been reached. There was significant improvement in the interfacial bonding strength after any surface modification ($p < 0.001$ compared to untreated PMMA). CaP coating of PMMA, either pre-treated with plasma or dopamine and 11-MUA, produced the best bonding strength to collagen hydrogel (177 ± 27 mN/cm² for p-CaP group and 168 ± 31

Figure 3.10. Shear adhesion strength of collagen hydrogel on surface modified PMMA. (Left column) Raw data obtained from the shear adhesion tests. (Right column) Bar graph depicting the interfacial adhesion strength after various PMMA surface modifications. The d-CaP and p-CaP groups, which yielded the best adhesion strengths, were subjected for longer term study, whereby both groups were incubated in PBS at 37°C for a further 14 days and 28 days. At the end of each time point, shear adhesion tests were then performed on the constructs. Height of error bars indicates standard deviation of means. * $p < 0.001$ in comparison to untreated PMMA group. ** $p < 0.001$.

mN/cm² for d-CaP group). No significant difference was found between p-CaP and d-CaP groups ($p=0.647$). However, d-CaP ($p=0.010$) and p-CaP ($p<0.001$) produced significantly stronger interfacial adhesion than the plasma group. Coating with 3-APTES yielded the next best adhesion strength (145 ± 12 mN/cm²), followed by plasma treatment (119 ± 10 mN/cm²). This result ruled out the use of plasma treatment due to its inferior bond strength with collagen when compared to the shear stress that can be created by the eyelid wiper.

A much in depth look into the failure analysis of the hydrogel-PMMA interface following the shear adhesion revealed bare PMMA surface with no remnants of hydrogel in both plasma group (**Figure 3.11A**) and 3-APTES group (**Figure 3.11B**).

Figure 3.11. Failure analysis of PMMA-hydrogel interface following shear adhesion test viewed under SEM. (A) Plasma group. (B) 3-APTES group. (C) d-CaP group. (D) High magnification SEM image of area enclosed in the red box in pane (C). (E) p-CaP group. (F) High magnification SEM image of area enclosed in the green box in pane (E).

These observations showed that the failure occurred at the interface between the hydrogel and modified PMMA surface. In d-CaP group (**Figure 3.11C** and **3.11D**) and p-CaP group (**Figure 3.11E** and **3.11F**), it was obvious the removal of hydrogel had delaminated most CaP coating on the PMMA surface. Some residual CaP could be seen on the bare PMMA surface. There was a clear demarcation line between region with intact coating and region where the coating had been delaminated. This SEM observation suggested that the weakness of d-CaP was primarily at the interface between the coating and PMMA. The remnants CaP on the affected region suggested that, although in a much smaller extent, the cohesion of the CaP coating could be the secondary factor of the bonding failure.

The KPro is designed to survive for a long period of time in patients. Therefore, it is paramount to assess the stability of the coating and the interfacial adhesion between the modified PMMA and hydrogel. I selected the two coating methods, that yielded the best adhesion strength from the tests performed 1 day after hydrogel casting, for a longer term study. Interestingly, I found that d-CaP produced a more stable adhesion with collagen over time (**Figure 3.10**). At day 14, the adhesion was $164 \pm 25 \text{ Nm/cm}^2$ ($p=0.906$ relative to adhesion at day 1) and was slightly reduced to $131 \pm 20 \text{ Nm/cm}^2$ at day 28 ($p=0.053$). In contrast, the hydrogel adhesion strength on p-CaP was significantly reduced at day 14 ($86 \pm 26 \text{ Nm/cm}^2$; $p<0.001$ compared to day 1 adhesion) and was further reduced at day 28 to $66 \pm 22 \text{ Nm/cm}^2$. The stick-slip phenomenon became less apparent for both d-CaP and p-CaP on day 28, an indication of weakening of the interfacial bond. For both time points, the d-CaP produced significantly better adhesion than the p-CaP ($p=0.001$). As expected, pristine PMMA could not provide long term stability on the interfacial adhesion. Most of the hydrogels casted on untreated PMMA floated after a few days incubation in the PBS.

I conducted further study to seek for an explanation of the reduction of interfacial adhesion in the post-incubation p-CaP group. Macroscopically, the CaP coating on the d-CaP group remained intact at day 28, except the region where the hydrogel lied on (**Figure 3.12A**). It appeared that the CaP coating was attached to and removed along with the hydrogel. A contrasting observation made on the p-CaP group revealed that most of the CaP coating was lifted off during the incubation process

Figure 3.12. Assessment of bare CaP-coated PMMA surfaces after shear adhesion tests. (A) Left: Macroscopically, the CaP coating on the d-CaP group remained intact on day 28, except the region where the hydrogel used to lie on. Right: A contrasting observation made on the p-CaP group revealed that most of the CaP coating was lifted off during the incubation process. (B) When water contact angle tests were performed on the bare PMMA surface, where the hydrogel used to lie on, d-CaP (day 14) and d-CaP (day 28) had similar wettability to the dopamine+11-MUA activated PMMA (d-PMMA). However, the surface of the bare p-CaP (day 14) and p-CaP (day 28) had become significantly more hydrophobic than the surface after plasma treatment. $*p < 0.001$. (C) ATR-FTIR performed on the bare areas of d-CaP (day 14) and d-CaP (day 28). (D) EDX analysis was performed on the bare surface site to assess the difference in surface atomic composition of the post-incubation p-CaP.

(**Figure 3.12A**). When I conducted water contact angle tests on the region where the hydrogel used to lie on, I found that d-CaP (day 14) and d-CaP (day 28) had similar wettability to the dopamine and 11-MUA activated PMMA (d-PMMA) (**Figure 3.12B**). However, the surface of the bare p-CaP (day 14) and p-CaP (day 28) had become more hydrophobic than the surface after plasma treatment (both $p < 0.001$) (**Figure 3.12B**). FTIR performed on the bare d-CaP (day 14) and p-CaP (day 28) site showed characteristic peaks, albeit relatively weak bands, of phenyl group due to the presence of dopamine, such as the overtones from $1850\text{--}1750\text{ cm}^{-1}$ and the aromatic C-C stretch from $1600\text{--}1500\text{ cm}^{-1}$ (**Figure 3.12C**, right panel). The IR spectrum of d-CaP (day 14) and d-CaP (day 28) appeared similar to d-PMMA, where there was a lack of C-H stretching peaks (2951 and 2995 cm^{-1}) of the pristine PMMA (**Figure 3.12C**, left panel). Due to difficulty in assigning distinct spectral peaks in the p-CaP (day 14) and p-CaP (day 28) groups with FTIR, I performed EDX on the bare surface site to assess the difference in surface atomic composition of the post-incubation p-CaP. There was a decrease in the percentage of oxygen composition on post-incubation p-CaP compared to the plasma treated PMMA (**Figure 3.12D**). By taking the increased water contact angle and decreased number of O atoms on the post-incubation p-CaP into consideration, I can postulate that there was a recovery of surface hydrophobicity after a period of time on the plasma activated surface even with CaP deposited on it. This could be due to the degradation of the plasma induced O-H group over time in PBS, a phenomenon well-documented in the literature [27,28]. Because of the hydrophobic surface and lack of active functional groups to anchor on, the CaP coating could be easily peeled off and ultimately reduced the adhesion of hydrogel on the p-CaP (**Figure 3.13A**). On the other hand, although the CaP was lifted off along with the hydrogel, the dopamine and 11-MUA still remained grafted on the surface, providing better anchorage to the CaP coating (**Figure 3.13B**). Hence, the d-CaP resulted in better hydrogel adhesion over time compared to the p-CaP.

In my study, aminosilane produced the next best adhesion strength after d-CaP and p-CaP. The terminal --NH_2 functional groups of 3-APTES could form amide bonds with --COOH functional groups, which are present in collagen type I, via carbodiimide chemistry and as a result, enhanced the bonding strength between collagen hydrogel and PMMA. The amide bonds, which are generally stronger than

Figure 3.13. Illustrations showing mechanism of interactions between collagen and bone-like apatite deposited on two differentially activated PMMA surfaces after 28 days of incubation in PBS. (A) p-CaP group. (B) d-CaP group.

hydrogen and electrostatic bonds, should have resulted in stronger adhesion strength than the CaP-coated PMMA. The reason of our contradictory finding may be two-fold. collagen hydrogel and PMMA. The amide bonds, which are generally stronger than hydrogen and electrostatic bonds, should have resulted in stronger adhesion strength than the CaP-coated PMMA. The reason of our contradictory finding may be two-fold. First, the coating thickness of 3-APTES after overnight deposition is usually in the range of 10-15 nm [29]. The extremely thin coating was most likely not as effective in bearing the weight of the hydrogel vertically as the much thicker CaP coating, which can be in the range of 20-40 μm -thick after 14 days of deposition in SBF (**Figure 3.8**). Second, most of the free carboxyl groups of the collagen were already crosslinked to the amine groups of the collagen when the solution was dispensed on the 3-APTES-modified PMMA surface [30], leaving only a little carboxyl groups to react with terminal amine groups of the 3-APTES.

3.3.7 Cell viability and proliferation on modified PMMA surfaces

As part of the optical core of a KPro, the modified PMMA surface will be transplanted in corneal stroma. Thus, it is necessary to study if the surface coating affects stromal properties, such as cytotoxicity and stromal cell proliferation. In **Figure 3.14A**, I showed the in vitro effect of the PMMA surface modifications on the proliferation of human corneal stromal fibroblasts at 48h and the cell viability at 24h and 72h post-seeding. The cells grown on glass coverslips, acting as the control group, showed a proliferative capacity of $62 \pm 14\%$ and negligible cell death at both 24h and 72h time points. On untreated PMMA, relatively few cells were detected and the proliferation rate was reduced to $43 \pm 10\%$ ($p=0.033$ relative to control). In addition, live/dead cell ratio at 24h and 72h post-cell seeding was about 50%, and they were significantly less than the control group at the respective time point ($p<0.001$). Cell proliferation and viability of plasma, d-CaP, and p-CaP groups were not significantly different than the control group. On 3-APTES coated surface, more live cells were found at 24h post-seeding; however, fewer cells were seen at 48h. The cell proliferation rate was significantly reduced ($31 \pm 15\%$) ($p<0.001$). After 72h of culture, no live cells were detected, suggesting that 3-APTES coating might have a latent cytotoxic effect. This could be related to the partial hydrolysis and the presence of leachable toxic products of 3-APTES during cell culture [31]. The summary of cell proliferation rate and live/dead cell ratio for all treatment groups was depicted in **Figure 3.14B** and **3.14C**, respectively.

From the live/dead cell assay, I also measured the initial cell attachment efficiency on the modified PMMA surfaces. **Figure 3.14D** showed an efficiency of $17.52 \pm 5.21\%$ for the control group. Untreated PMMA with hydrophobic surface had the significantly lowest cell attachment efficiency ($7.62 \pm 1.83\%$; $p<0.001$ compared to any other group). The efficiency increased after PMMA surface modifications. However, only p-CaP ($29.59 \pm 5.58\%$) and 3-APTES ($57.14 \pm 5.06\%$) treatments significantly improved the efficiency compared to the control (both $p<0.001$). The presence of $-NH_2$ terminal group after 3-APTES coating most likely enhanced the initial cell attachment, a phenomenon reported previously, of which human umbilical vein endothelial cells showed the best attachment efficiency on $-NH_2$ functionalized

surface compared to $-\text{COOH}$, $-\text{OH}$ and $-\text{CH}_3$ grafted surfaces [32]. However, the adverse effects of 3-APTES coating were seen after 3 days of culture.

Figure 3.14. Viability and proliferation rate of human corneal fibroblasts on surface modified PMMA. (A) Left panel: Proliferative corneal fibroblasts on 48 hours after cell seeding on differentially modified PMMA surfaces. Middle and right panel: Live/dead cell assay performed on primary human corneal fibroblasts on 24 and 72 hours after cell seeding. Scale bars=100 μm . (B) Percentage of cell proliferation rate on untreated and modified PMMA surfaces. Height of error bars indicate standard deviation of means. * $p<0.05$ in comparison to control. ** $p<0.001$ in comparison to control. (C) Quantification of live/dead cell ratio on 24 (red bars) and 72 (green bars) hours after cell seeding. * $p<0.001$ compared to control. ** $p<0.001$ compared to live/dead cell ratio after 24-hour-culture on 3-APTES-treated PMMA. (D) Percentage of cell attachment efficiency on untreated and modified PMMA surfaces on 24 hours after cell seeding. * $p<0.001$ in comparison to control. ** $p<0.001$ in comparison to 3-APTES group.

3.3.8 Cell morphology and adhesion on modified PMMA surfaces

The cell morphology and expression of cell adhesion markers of human corneal stromal fibroblasts at day 3 after seeding on glass coverslip (control), untreated PMMA and modified PMMA surfaces are shown in **Figure 3.15**. Cell morphology observed in plasma group appeared similar to the control, which showed typical slender morphology of fibroblast with prominent actin cytoskeleton spanning the length of cells [33]. In addition, prominent anchorage of cells to the modified

Figure 3.15. Cell morphology and attachment on modified PMMA surfaces on cell culture day 3. Cell morphology was assessed by staining of f-actin marker, phalloidin (red fluorescence). The ability of cells to attach on the modified PMMA surfaces was studied by expression of cell adhesion markers, paxillin and vinculin (green fluorescence). Nuclei (blue fluorescence) was stained with DAPI. Scale bars = 20 μm .

surfaces was evident by the expression of focal adhesion proteins, paxillin and vinculin, at the cell periphery. In contrast, fewer cells stayed on untreated PMMA and 3-APTES coated PMMA, and they appeared round with reduced adherence to the surface. Cells on d-CaP and p-CaP, showed more prominent actin cytoskeleton. Most of them however, appeared thinner and more spindle-shaped compared to cells on glass surface. Although CaP agglomerates generate huge autofluorescent background, I still could detect the expression of paxillin and vinculin in the adherent cells.

This cell adherence phenomenon on different coated PMMA surfaces was confirmed by SEM. The fibroblasts seeded on glass coverslip showed extensive filopodia protrusions at the cell periphery, indicating prominent cell anchorage (**Figure 3.16A**). Similar observation was made for cells grown on plasma treated PMMA (**Figure 3.16C**). Cells seeded on either d-CaP (**Figure 3.16D**) or p-CaP (**Figure 3.16E**) appeared more spindle-shaped, but still featured prominent filopodia protrusions. In contrast, fibroblasts cultured on untreated PMMA (**Figure 3.16B**) and 3-APTES treated PMMA (**Figure 3.16F**) appeared round with rare filopodia protrusions.

It remains to be seen whether the biointegration of soft tissue, such as the cornea, to hard materials, such as PMMA can be enhanced by direct cell adhesion and/or by material interaction with cell-secreted extracellular matrix and proteins. However, studies on cells contributing to the adhesion and biointegration of hard tissue, such as bone, on hard materials appear to suggest this possibility [34,35]. The fact that corneal stromal cells survived and formed good adhesion on the CaP-coated PMMA surface suggests a potential benefit of the CaP coating in long-term conservation of the material biointegration and thus, improving the clinical outcomes of the KPro device. A limitation associated with the current study is that the hydrogels were acellular; however, the initial adhesion occurred with the acellular collagen. Similar to other implants, long-term survival of a KPro in the body is subject to many events including (but not limited to) cellular infiltration. In vivo, corneal stromal cells are normally in quiescent state and their migration rate is slow [36].

Figure 3.16. SEM of human corneal stromal fibroblasts on modified PMMA surfaces on day 3 after cell seeding. Prominent filopodia protrusions could be seen at the edge of the cells seeded on glass coverslip (A), plasma treated PMMA (C), d-CaP treated PMMA (D) and p-CaP treated PMMA (E), suggesting good cell adhesion. Cells seeded on untreated PMMA (B) and 3-APTES (F) appeared round and not adherent on the surface.

It should also be mentioned that the acellular hydrogel-modified PMMA constructs that I showed here were intended to model a synthetic core-skirt KPro, which would eliminate the use of human donor cornea as skirt material. Not only will the use of hydrogel reduce our reliance on donor corneas, but it can also reduce long-term complications normally associated with donor corneal transplantation due to immunologic reaction elicited by donor cells [37]. The collagen hydrogels are similar to those that have been implanted as an acellular artificial cornea in an ongoing human clinical trial, and have been shown to promote host cellular regeneration within months post-implantation [38]. Nevertheless, it is still important to maintain the stability of the interfacial adhesion before the stromal cells populate the scaffold, biointegrate and subsequently provide further and more permanent stability to the KPro.

3.4 Conclusion

I have shown that d-CaP not only enhanced the interfacial adhesion with collagen type I hydrogel, but also provided better bonding over a longer period of time compared to p-CaP. In addition, d-CaP provided a surface that was biocompatible, which allowed good biointegration with corneal stromal fibroblasts. Aminosilane (3-APTES) produced the next best adhesion; however, the coating was not biocompatible to human corneal stromal fibroblasts. Ultimately, the modified core-skirt KPro with PMMA core and collagen hydrogel skirt is aimed at reducing the heavy dependence on transplant-grade human donor corneas in the near future and reducing long-term complications associated with conventional KPro.

This study was published in: Riau AK, Mondal D, Yam GH, Setiawan M, Liedberg B, Venkatraman SS, Mehta JS. Surface modification of PMMA to improve adhesion to corneal substitutes in a synthetic core-skirt keratoprosthesis. *ACS Appl Mater Interfaces*. 2015;7:21690-21702.

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Chapter 4

Immobilization of hydroxyapatite nanoparticles on PMMA surface via dip coating method

Currently, the most common technique of depositing hydroxyapatite (HAp) on polymeric substrates is by incubation in simulated body fluid (SBF). The drawbacks of the method, however, are the long processing time, that it requires replenishment of constant pH SBF, and the resulting calcium-deficient CaP coating. The other HAp coating techniques, such as plasma spraying and magnetron sputtering, involve extremely high processing and/or annealing temperature which render them unsuitable for polymeric materials with lower melting temperature, such as PMMA. In this chapter, a new non-thermal coating method, which is simple and inexpensive to execute for immobilization HAp nanoparticles on PMMA, was described. A combination of 20% (w/v) HAp nanoparticles in 5% (w/v) PMMA/chloroform, followed by cleaning with plasma irradiation appeared to offer the best formulation to produce a uniform and homogenous coating, that was hydrophilic (water contact angle of $14.3 \pm 3.4^\circ$), resistant to delamination and still preserved the crystallinity of the HAp Ca/P ratio of 1.64 ± 0.04). These advantages resulted in better and more stable adhesion (over 28 days in artificial tear fluid) of collagen hydrogel on HAp-coated PMMA than on untreated PMMA. Corneal fibroblasts were viable and able to form good adhesion and proliferate on the HAp-coated PMMA. The new dip coating technique may have a potential to be applied to a KPro optical cylinder to provide a good bonding surface for collagen-based skirt material and a modified surface that is still biocompatible and can promote biointegration over time.

4.1 Background of study

Although incubation in SBF leads to formation of bone-like apatite on PMMA surface, the resulting CaP did not attach well on dopamine activated PMMA surface and did not exhibit intrinsic properties of pure HAp. These could be the contributing factors of the adhesion strength reduction of overlying collagen hydrogel from 164 ± 25 mN/cm² at day 14 to 131 ± 20 mN/cm² at day 28. Although the reduction was not statistically significant, it was on the borderline of significance ($p=0.053$). From **Figure 3.11C** and **Figure 3.12A**, it was obvious the CaP coating was lifted together with the collagen hydrogel following the shear adhesion test, suggesting a better bonding between collagen and CaP than CaP and dopamine and 11-MUA activated PMMA. A tape adhesion test using Scotch filament tape 892 (with 65.4 N/100 mm adhesion strength rating) showed complete delamination of the coating, further confirming the observation in the earlier study (**Figure S2.1A**). Another factor that could cause the poor coating retention probably is the poor crystallinity and impurities of SBF-assembled CaP, where the calcium deficient bone-like apatite exhibited a lower Ca/P ratio than pure HAp (**Figure S2.1C**). Poor CaP crystallinity and impurities are associated with increased resorption rate of the apatite, reducing the longevity of the SBF-mediated CaP coating in biological microenvironment [1]. In addition, the underlying dopamine and 11-MUA (laid before CaP deposition) did not form a homogenous coating layer that attached well on the PMMA surface. Aggregates of varying sizes could be seen interspersed on the PMMA surface (**Figure S2.1B**). The combination of non-homogenous poly-dopamine layer and desorption of the CaP layers might have led to reduced adhesion of collagen on d-CaP surface (**Figure S2.1D**). Although the coating improved the adhesion and biointegration of corneal tissue or collagen hydrogel on PMMA surface [2,3], the coating technique could be improved further in order to create a HAp coating that is more resistant to biodegradation and has better affinity to PMMA.

Currently, the most common technique of depositing CaP on polymeric substrates is by incubation in SBF owing to its low processing temperature. The drawbacks of the method, however, are the long processing time (2 weeks in my earlier study), that it requires replenishment of constant pH SBF, and the resulting

calcium-deficient CaP coating. There are other CaP coating techniques that have been described in the literature, but they usually involved high processing and/or annealing temperature ($>400^{\circ}\text{C}$) which render them unsuitable for polymeric materials with relatively low melting temperature, such as the PMMA (110°C melting temperature). Some examples of these CaP coating method are plasma spraying [4,5], electrophoretic deposition [6,7], magnetron sputtering [8,9], and sol-gel dipcoating [10,11].

In the current study, a new dipcoating technique of HAp nanoparticles on a polymeric substrate was described and characterized. The rationale behind the selection of the method is that it is a non-thermal coating technique, does not require any annealing process, is simple to execute, does not require elaborate and expensive equipment to perform, and only requires short processing time. Following the dipcoating process, biocompatibility and biointegration of corneal stromal fibroblasts were also investigated on the HAp nanoparticles-immobilized PMMA surfaces.

4.2 Materials and methods

4.2.1 Immobilization of nanoparticles on PMMA surface

PMMA sheets with 0.5 mm thickness were purchased from Goodfellow (Huntingdon, England). The sheet was cut into 0.5 x 2 cm pieces, washed in 70% ethanol for 15 minutes and rinsed extensively with distilled water before being dried in 37°C vacuum incubator for 24 hours. Several concentrations of needle-shaped, 60-nm HAp nanoparticles (MKnano, Mississauga, Canada): 0%, 10% and 20% (w/v) were mixed into chloroform without PMMA or with 5% (w/v) PMMA (MW 120,000; Sigma-Aldrich, St. Louis, MO). The mixture was then probe sonicated for 5 minutes at 50% amplification level with 5-second pause every 30 seconds to allow for gentle shaking. Immobilization of the HAp nanoparticles on PMMA sheet was performed by dip coating technique using a KSV NIMA dip coater (Biolin Scientific, Stockholm, Sweden). The PMMA sheet was secured with a customized clamp (**Figure 4.1**) and dipped for 5, 15, 30 or 60 seconds. The PMMA sheet was both lowered and withdrew with a speed of 240 mm/min. Following this, the coated PMMA sheet was left on the

dip coater for 30 minutes to allow for evaporation of the chloroform and nanoparticles settlement at the surface. The substrate was then washed with 70% ethanol, rinsed with copious amounts of distilled water, and dried in 37°C vacuum incubator overnight.

Figure 4.1. Dip coating setup to immobilize HAp nanoparticles on PMMA surface.

The following day, the surface of the substrates was subjected to oxygen plasma treatment to remove remnant contaminants and thin layer of PMMA that had possibly coated the HAp surface during the dip coating process. Oxygen plasma treatment was performed in a Covance multi-purpose plasma system (Femto Science, South Korea). The samples were treated with radio frequency oxygen plasma with 200W power for 30 seconds. The PMMA sheets were placed between two parallel plate electrodes enclosed in the plasma reactor chamber. Air was removed with vacuum application for at least 30 minutes before the power was turned on. The pressure at the moment of plasma discharge was 0.2 Torr. The flow rate of oxygen was set at 20 sccm. After the discharge stopped, the plasma treated PMMA sheets were removed from the plasma chamber, washed with 70% ethanol and copious amounts of distilled water, and dried in 37°C vacuum incubator overnight before being used for further experiments. The most optimized treatment was referred to as *HAp-coated* group in the later part of the text.

4.2.2 Analysis of surface morphology and elemental composition

Surface morphology and roughness of functionalized PMMA sheets was observed with a Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA). Topographic images were captured in tapping mode employing monolithic silicon NCH-50 Point Probe (NanoWorld AG, Neuchatel, Switzerland). The scan size was set at 5 x 5 μm . The surface was scanned at a rate of 1 Hz with a resolution of 512 x 512 pixels. Three-dimensional topographic images, amplitude and roughness (RMS value) data were obtained using Nanoscope image processing software version 5.12r5 (Digital Instruments).

In addition to AFM, surface morphology of HAp nanoparticles-immobilized PMMA sheets was also analyzed by SEM. In brief, the PMMA sheets were mounted on a stub secured by carbon adhesive tape. The sheets were sputter-coated with 10-nm-thick layer of gold and examined with a JSM-7600F microscope (JEOL, Tokyo, Japan) at accelerating voltage of 5 kV. Surface elemental composition was assessed by EDX attached to the microscope.

4.2.3 Water contact angle analysis

The water contact angle was measured by a FTA200 contact angle goniometer (First Ten Angstroms, Inc., Portsmouth, VA) using a static sessile drop technique. At room temperature, distilled water was pumped out of a syringe at a rate of 5 $\mu\text{l/s}$ and allowed to settle for 15 seconds on the samples before the image was taken. The contact angle was generated from the image with FTA32 software version 2.0 (build 276.2) (Portsmouth, VA).

4.2.4 Analysis of surface functional groups by ATR-FTIR

IR spectra of the modified PMMA surfaces were collected in the range from 4000 to 650 cm^{-1} using a PerkinElmer Frontier FTIR spectrometer (Waltham, MA). The spectrometer was equipped with an ATR sampling universal accessory supplied with a top plate for ZnSe crystal. The plate was cleaned with 70% ethanol before

acquisition of each spectrum. The PMMA sheet was placed on the plate and tightened down to the 40% gauge mark shown on the software for the instrument. Spectra were obtained with 16 scans and 4 cm^{-1} resolution.

4.2.5 Assessment of biomechanical properties

PMMA samples of $3 \times 1 \times 0.5\text{ cm}$ were subjected to 3-point bending tests using a Chatillon mechanical tester (Largo, FL). The supporting beam distance was maintained at 2 cm for all tests. The crosshead speed was set at 1 mm/min and 10N load cell was used. Maximum flexural stress, flexural strain at break, and flexural modulus of samples were obtained from the tests.

4.2.6 Casting of collagen hydrogel on PMMA surface

Casting of collagen hydrogel on the modified PMMA surfaces was performed after the substrates had been dried for 24 hours in 37°C vacuum incubator. The collagen hydrogel was constructed as described previously [12]. Bovine atelocollagen type I was purchased from Koken (Tokyo, Japan). Briefly, 0.5 ml aliquot of 10% (w/v) atelocollagen in acidic solution (pH 3.0) was loaded into a syringe mixing system. The collagen solution was adjusted to pH 5.0 with 1N NaOH, followed by thorough mixing by pumping the syringes. Calculated amounts of 10% (w/v) EDC (Sigma-Aldrich) and 10% (w/v) NHS (Sigma-Aldrich) were added to produce a 2:1 molar ratio of EDC to NHS and mixed with the collagen solution. The mixed solution (0.1 ml) was dispensed onto the center of the PMMA sheet and flattened with a glass slide, resulting in a cylindrically shaped hydrogel. Thickness of the hydrogels was consistently maintained by placing 0.5-mm-thick spacers under the glass side at the edges of the modified PMMA sheet. The hydrogel was cured in a humidified chamber at room temperature for 24 hours before being subjected to the shear bond strength test.

4.2.6 Shear adhesion strength test

Samples (hydrogel adhered on PMMA sheets; $n=6$ of each group) were secured at the base of a Chatillon mechanical tester. The position of the sample was adjusted

until the chisel-shaped fixture (attached to 10 N load cell) aligned at the center of the hydrogel at the bonding interface. The crosshead speed was set at 5 mm/min. The test was stopped and maximum force was recorded when the hydrogel was completely detached from the surface of PMMA sheets. To study the stability of the bonding in a longer term, the hydrogel-PMMA constructs were incubated in pH 7.4 artificial tear fluid (ATF) at 37°C for 14 and 28 days, with the ATF refreshed daily. ATF was prepared as previously described by mixing 68 g of NaCl, 22 g of NaHCO₃, 0.08 g CaCl₂·2H₂O and 14 g of KCl in 1000 ml of distilled water [13]. Shear adhesion strength test was then performed at the pre-determined time points.

The performance of the HAp nanoparticles dip coated PMMA was compared to the CaP coating deposited via SBF incubation that was described in the previous chapter and untreated PMMA. Briefly, cleaned PMMA sheets (2 x 3 cm in size) was treated with 2 mg/ml dopamine (Sigma-Aldrich) in 10 mM Tris buffer, pH 8.5 (Sigma-Aldrich) overnight at room temperature, followed by immersion in 50 µM of 11-MUA (Sigma-Aldrich) for 4 hours at room temperature, before being placed in 1.5x SBF solution at 37°C for 14 days. The SBF solution was refreshed daily. After 14 days, the sheets were washed extensively in distilled water and dried in a vacuum incubator at 37°C. This treatment was referred to as *d-CaP* group.

4.2.7 Quartz crystal microbalance analysis

HAp nanoparticles-coated and PMMA film-coated gold sensors were purchased from Biolin Scientific and cleaned before each measurement. Briefly, the crystal surfaces were treated for 10 min to an ultraviolet oxidation (UVO) treatment, followed by 30 min soak in 70% ethanol. They were then rinsed with distilled water followed by blow drying with nitrogen gas and finally subjected to another 10 min UVO treatment. Quartz crystal microbalance (QCM) experiments were carried out in a Q-Sense E4 system (Biolin Scientific) using flow modules in parallel. Rat tail collagen type I (Thermo Scientific, Waltham, MA) solution was flowed into the modules at 50 µl/min using a 4-channel Ismatec IPC-N4 peristaltic pump (Wertheim, Germany). Once 200 µl of collagen solution was flowed through, the pump was stopped and the

system was allowed to record the interactions between collagen and the crystals for 40 additional minutes.

In QCM, changes in resonance frequency (Δf) of the quartz crystal were recorded to measure the amount of material that bound onto the sensor [14,15]. The crystal was excited at its fundamental frequency, approximately 5 MHz, and changes can be observed at the fundamental frequency ($n=1$) and overtone frequencies ($n=3, 5, 7, 9, 11$). The observation from the fundamental frequency is usually not used as it tends to be subjected to artifacts from the sensor clamp [15]. A decrease in Δf was associated with an increase in hydrodynamic mass (molecules and water) bound or adsorbed onto the crystal surface according to the Sauerbrey equation for non-viscous films [16], which can be simplified as follows:

$$\Delta m = - \frac{C \cdot \Delta f}{n}$$

where Δm = change in mass of material bound to the sensor, n = overtone number ($n = 1, 3, 5, 7, \dots$), Δf = change in resonance frequency, and C = constant that describes the sensitivity of the sensor to the changes in mass.

4.2.8 Culture and seeding of corneal stromal fibroblasts

Research grade cadaveric corneal tissues were purchased from Lions Eye Institute for Transplant and Research (Tampa, FL). They were preserved in Optisol-GS (Bausch&Lomb Surgical, Irvine, CA) and transported at 4°C to the laboratory. Central button (8 mm in diameter) was trephined and treated with dispase II (20 mg/ml, Roche, Basel, Switzerland) followed by gentle scraping to completely remove corneal epithelium and endothelium. The stromal tissue was trimmed into small pieces and digested with collagenase I (1 mg/ml, Worthington, Lakewood, NJ) in DMEM/F12 (Life Technologies, Carlsbad, CA) for 9-12 hours at 37°C. Cells were cultured in DMEM/F12 containing 10% fetal bovine serum (FBS; Life Technologies) and 1% antibiotic/antimycotic (penicillin, streptomycin sulfate and amphotericin B; Life Technologies). At passage 3 to 5, the stromal fibroblasts with 1000 cells/cm² starting density was seeded on sterile modified PMMA sheets and cultured for 24 and

72 hours before further tests. Cells seeded on glass coverslips were considered as control.

4.2.9 Cell viability assay

The cytotoxicity was analyzed using Live/Dead® Viability/Cytotoxicity assay kit (Life Technologies) according to the manufacturer's protocol. In brief, the cells were incubated with calcein AM and ethidium homodimer-1 (EthD-1) for 45 minutes, washed and mounted in Fluoroshield containing DAPI. Samples were viewed using a Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss) at 100x magnification. Live cells were stained green by calcein AM and dead cells in red by EthD-1. Both live and dead cells were quantified from 2 random fields on each sample (n=3 of each group), and the live/dead cell ratio was calculated. In addition, initial cell attachment efficiency was calculated by using the following formula:

$$\text{Cell attachment efficiency (\%)} = \frac{\text{Actual number of cells in a viewing field}}{\text{Theoretical number of cells expected to attach in a viewing field}} \times 100\%$$

where the theoretical number of cells expected to attach in a 100x viewing field (4.5 x 3.5 mm dimension) was equivalent to 157 after accounting for the 1000 cells/cm² seeding density.

4.2.10 Thermogravimetric analysis to detect presence of chloroform

Thermogravimetric analysis (TGA) using TGA Q500 (TA Instruments, New Castle, DE) was performed to detect the presence of residual chloroform. TGA was carried out for pristine PMMA and HAp nanoparticles-immobilized PMMA sheets with 10°C/min temperature increment from 25°C to 800°C in N₂ atmosphere.

4.2.11 Cell proliferation assay

Proliferation of corneal fibroblasts on modified PMMA surfaces was assessed using a 5-ethynyl-2'-deoxyuridine (EdU) assay kit (Life Technologies) according to

the manufacturer's protocol. In brief, the cells were incubated in EdU (10 μ M) containing medium for 24h. They were then washed with PBS, fixed with 4% paraformaldehyde (Sigma), followed by blocking and permeabilization in 0.1% Triton X-100 (Sigma) in 3% bovine serum albumin (Sigma) at room temperature. Incorporated EdU was detected by Alexa Fluor 488 fluorescent-azide coupling Click-iT reaction. Finally, the samples were mounted in Fluoroshield containing DAPI (4',6-diamidino-2-phenylindole; Santa Cruz Biotechnology, Santa Cruz, CA) and viewed under Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at 100x magnification. Total number of cells and EdU-positive cells were quantified from 3 random fields of each sample (n=3 of each group).

4.2.12 Immunocytochemistry of cell attachment marker

Cells on glass coverslips and surface modified PMMA sheets were fixed with freshly-prepared neutral buffered 2% paraformaldehyde (Sigma-Aldrich). After quenching of free aldehyde with 50 mM ice-cold ammonium chloride and PBS washes, cells were permeabilized and blocked with PBS containing 0.425% saponin (Sigma-Aldrich), 1% bovine serum albumin (BSA; Sigma-Aldrich) and 2% normal goat serum (Life Technologies), followed by incubation of mouse monoclonal anti-vinculin (Sigma-Aldrich). After PBS washes, cells were incubated with donkey anti-mouse IgG AlexaFluor 488-conjugated secondary antibody (Jackson ImmunoRes Lab, West Grove, PA) and AlexaFluor 568-conjugated phalloidin (Life Technologies). The samples were mounted in Fluoroshield containing DAPI and viewed under Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss).

4.2.13 Assessment of cell morphology

Cell morphology on functionalized PMMA sheets was observed by SEM. Cells were fixed in 2% glutaraldehyde (Sigma-Aldrich) in 0.1M sodium cacodylate, pH 7.4 (Millipore) overnight at 4°C. The samples were then washed twice in distilled water for 10 minutes each before being immersed in 1% osmium tetroxide (FMB, Singapore) for 2 hours at room temperature. Following washing in distilled water (twice for 10 minutes each), the samples were dehydrated in increasing concentrations of ethanol for

10 minutes each (from 25%, 50%, 75%, 95% to 100%, with 95% and 100% concentrations being performed twice). Critical point drying was then performed using Bal-Tec dryer (Balzers, Liechtenstein). Finally, the samples were mounted on stubs secured by carbon adhesive tape, sputter coated with 10-nm thick gold and examined with a JSM-5600 microscope (JEOL).

4.2.14 Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). Statistical significance between groups was calculated by one-way ANOVA and post hoc Tukey comparison test. A value of $p < 0.05$ was considered to be statistically significant. All statistical analysis was performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL).

4.3 Results and discussion

4.3.1 General considerations for selection of nanoparticles immobilization technique

Other coating techniques, such as knife casting and spray coating, to immobilize HAp nanoparticles on PMMA were considered and attempted. There were two issues that ruled knife casting method out. First, most of the nanoparticles appeared to sink and settled relatively deep from the surface. When scanned with FTIR, characteristic peaks of HAp were barely observed. Second, knife casting method could not be applied to coat PMMA cylinders. Spray coating could be performed to coat PMMA cylinders; however, due to the propensity of nanoparticles to agglomerate, the nozzle of the spray coater was frequently blocked during the coating process.

The selection process was narrowed down to dip coating technique because it could be used to immobilize HAp nanoparticles on flat PMMA sheets, as well as on PMMA cylinders. In addition, the technique was simple and did not require expensive equipment to be performed with, which may add its appeal to be applied in other

biomedical fields. Besides the dip coating apparatus, the method only required dip coating solution containing 3 components: HAp nanoparticles, PMMA and chloroform. The use of chloroform to dissolve PMMA was a concern due to its cytotoxicity. Other organic solvents, such as acetone and toluene, were considered; however, the dissolving rate of these solvents was extremely slow. Uniform immobilization of the nanoparticles, which agglomerated in a faster rate, could not be achieved with these solvents.

4.3.2 Optimization of nanoparticles immobilization via dip coating

Optimization of the dip coating technique was first performed before shear adhesion and biocompatibility tests were performed on the modified PMMA surface. Dip coating of PMMA sheets in chloroform (with and without addition of 5% PMMA) without HAp nanoparticles revealed the formation of craters (**Figure 4.2** and **4.3**). The diameter of the craters increased as the dip coating time was increased, from $0.51 \pm 0.12 \mu\text{m}$ after 5s, $0.60 \pm 0.11 \mu\text{m}$ after 15s, and $0.84 \pm 0.04 \mu\text{m}$ after 30s to $1.19 \pm 0.10 \mu\text{m}$ after 60s. The depth of the craters also increased with dip coating time as indicated by the increasing RMS value and R_{max} (**Table 4.1**). Because of the relatively short dip coating time, the organic solvent did not cause significant damage to the bulk of the PMMA sheets. The idea would be to populate the craters with the

Table 4.1. Surface roughness of PMMA after dip coating in 5% PMMA in chloroform without HAp nanoparticles.

Dip coating time	RMS value (nm)	<i>p</i> value*	R_{max} (nm)	<i>p</i> value*
5s	43.3 ± 10.9	3.15×10^{-7}	210.0 ± 21.8	4.05×10^{-11}
15s	98.9 ± 6.6	2.52×10^{-7}	616.1 ± 45.6	4.05×10^{-11}
30s	111.7 ± 9.0	6.32×10^{-5}	746.2 ± 25.1	4.58×10^{-4}
60s	145.6 ± 4.2		816.5 ± 10.7	

*Significant difference relative to 60s treatment time

$p < 0.05$ was considered to be statistically significant

Figure 4.2. Surface morphology of PMMA after dip coating in 5% PMMA in chloroform without HAp nanoparticles. Craters with increasing diameter could be seen as the dip coating time was increased.

Figure 4.3. Illustration showing increasing depth and diameter of craters created by chloroform on PMMA surface as dip coating time is increased.

HAp nanoparticles during the dip process, followed by ‘locking’ the nanoparticles at the sub-surface level after evaporation of the solvent to create HAp-coated PMMA surface.

The HAp coating was not uniform and homogenous when the solvent was not mixed with 5% PMMA (**Figure 4.4A**). Clumps of HAp nanoparticles were observed on the PMMA surface. Increasing the dip coating time also did not improve the homogeneity of the coating. Some craters could be seen on areas that were not covered by the HAp. I was not able to perform AFM on these surfaces due to their surface roughness that exceeded the z-scan limit of the AFM that I used in the study. The SEM results suggested that the nanoparticles agglomerated in a relatively rapid rate in the chloroform, forming clumps on the surface of the PMMA during the dip coating process (**Figure 4.4B**). **Figure 4.5A** showed the nanoparticles had started to settle to the bottom of the glass container after 30 min. In contrast, the nanoparticles were still dispersed evenly in the chloroform that had been added with 5% PMMA. Nanozetasizer revealed a polydispersity index (PdI) of 0.828 ± 0.191 when PMMA was

Figure 4.4. Coating outcomes on PMMA surfaces when HAp nanoparticles were added in chloroform without addition of 5% PMMA in dip coating solution. (A) SEM showed that clumps of nanoparticles were formed on the surface during the dip coating process regardless of the length of the dip coating time. (B) Illustration depicting a rapid agglomeration rate of HAp nanoparticles in the chloroform without addition of 5% PMMA, leading to the formation of clumps of HAp upon settling on the PMMA surface.

not added into the solvent, which was significantly higher than the PDI of the mixture when 5% PMMA was added (0.313 ± 0.090 ; $p=0.002$) (**Figure 4.5B**). This suggests that the agglomeration rate was reduced when 5% PMMA was added in the solvent. Adding 5% PMMA would increase the viscosity of the mixture and reduce the agglomeration propensity of the nanoparticles, providing ample time for the HAp to be laid on the surface in a more uniform and homogenous fashion.

Figure 4.5. Dispersion of nanoparticles in chloroform with and without 5% (w/v) PMMA. (A) After 30 minutes, most HAp nanoparticles had agglomerated and settled at the bottom of the glass vial containing chloroform without 5% PMMA. In contrast, the nanoparticles were still well dispersed in the solution with 5% PMMA. (B) Nanozetasier revealed a significantly higher polydispersity index (PDI) of the nanoparticles in the solution without PMMA than that containing PMMA. $*p<0.05$.

In the next set of experiments, I added 10% (w/v) HAp nanoparticles in the mixture of 5% PMMA and chloroform and dipped the PMMA sheets in the solution. Craters with irregular sizes were formed on the surface and the nanoparticles were not laid in a uniform and homogenous fashion (**Figure 4.6**). Both SEM and AFM showed that increasing the dip coating time did not improve the uniformity of the HAp coating. In fact, the craters became more irregular in depths and sizes when the dip coating time was prolonged, which were also indicated by the increasing RMS and $R_{\max}$ values, as well as increasing SD of both the RMS and $R_{\max}$ values (**Table 4.2**). A high magnification SEM image in **Figure 4.7** showed that most nanoparticles laid at the bottom of the craters and less settled on the surface of the PMMA. It is possible that 10% concentration of the nanoparticles was not sufficient to bring the HAp out on

Table 4.2. Surface roughness of PMMA after dip coating in 5% PMMA in chloroform with 10% (w/v) HAp nanoparticles.

Treatment time	RMS value (nm)	<i>p</i> value*	$R_{\max}$ (nm)	<i>p</i> value*
5s	60.5 ± 18.3	0.013	568.5 ± 159.7	0.011
15s	78.9 ± 18.1	0.043	733.6 ± 132.6	0.066
30s	92.0 ± 27.5	0.114	665.1 ± 143.2	0.031
60s	132.3 ± 48.5		1020.6 ± 294.4	

*Significant difference relative to 60s treatment time

$p < 0.05$ was considered to be statistically significant

Figure 4.6. Surface morphology of PMMA after dip coating in 5% PMMA in chloroform with 10% (w/v) HAp nanoparticles. Craters with more irregular sizes could be seen as the dip coating time was increased.

the surface (**Figure 4.7**). Higher concentration of nanoparticles in the mixture may be necessary to fill the craters up and subsequently lay layers of HAp on the PMMA surface.

Figure 4.7. Illustration showing the possible mechanism of non-uniform appearance of HAp coating when 10% nanoparticles were added. The amount of the nanoparticles added was insufficient to populate the craters and elevate most HAp on the PMMA surface. Green arrows in the high magnification SEM image and illustration indicate the nanoparticles that were laying at the bottom of the craters. Red arrows indicate some nanoparticles that were elevated from the craters and started to appear on the surface.

Adding 20% (w/v) nanoparticles in the mixture appeared to result in significantly better HAp coating (**Figure 4.8**). Craters were no longer seen on the PMMA surface after 5s dip coating. There were, however, a few regions on the surface that appeared relatively smooth and not completely covered by the nanoparticles (**Figure 4.8**, top panel). Increasing dip coating time to 15s and 30s did not improve the coverage of the coating, whereby some craters could be observed on the surface. Dip coating time of 60s appeared to produce the most optimized HAp coating on PMMA surface (**Figure 4.8**, bottom panel). Both SEM and AFM showed complete coverage of HAp nanoparticles on the surface. Individual rod shaped HAp nanoparticles could

Figure 4.8. Surface morphology of PMMA after dip coating in 5% PMMA in chloroform with 20% (w/v) HAp nanoparticles.

Table 4.3. Surface roughness of PMMA after dip coating in 5% PMMA in chloroform with 20% (w/v) HAp nanoparticles.

Treatment time	RMS value (nm)	p value*	R _{max} (nm)	p value*
5s	83.5 ± 24.1	0.141	815.5 ± 360.2	0.913
15s	81.1 ± 26.4	0.126	675.7 ± 272.2	0.376
30s	93.7 ± 23.7	0.472	763.2 ± 154.6	0.719
60s	102.2 ± 14.4		797.3 ± 164.8	
60s + plasma	92.5 ± 10.5	0.213	787.5 ± 87.9	0.900

*Significant difference relative to 60s treatment time
 $p < 0.05$ was considered to be statistically significant

be seen interspersed on PMMA surface in the high magnification SEM image in **Figure 4.9**. In terms of surface roughness, it was increased the most after 60s dip coating, but it was not significantly different than that after shorter dip coating time

Figure 4.9. Illustration showing the possible mechanism of immobilization of HAp nanoparticles on PMMA surface when 20% nanoparticles were added in 5% PMMA/chloroform. High magnification SEM image showed individual rod shaped nanoparticles interspersed on the PMMA surface.

(Table 4.3). Figure 4.9 explained the possible mechanism of nanoparticles immobilization on the PMMA surface, whereby prolonging the dip coating time allowed more nanoparticles to accumulate and fill the craters, and eventually they form layers of HAp coating on the surface. Based on these SEM and AFM observations, I determined that dip coating of 20% (w/v) nanoparticles in 5% (w/v) PMMA/chloroform was the most optimized combination to immobilize HAp nanoparticles on PMMA surface.

4.3.3 Characterization of HAp-coated PMMA surface

Following the dip coating, plasma treatment was performed on the HAp-immobilized PMMA surface to remove contaminants and to etch regions that were covered by PMMA. Plasma treatment was able to reveal more HAp on the PMMA surface as shown by both SEM (Figure 4.10A) and AFM (Figure 4.10B). Plasma treated surface was also marked by relatively smoother surface (Table 4.3) and weaker characteristic IR spectra of PMMA, such as peaks near $1100\text{--}1300\text{ cm}^{-1}$ and 1726 cm^{-1} , which were attributed to the presence of C-O and C=O stretching modes of the ester group, respectively, as well as spectral peaks at 2951 and 3009 cm^{-1} which indicated the CH_2 group of PMMA (Figure 4.10C). After plasma treatment, the HAp nanoparticles-immobilized PMMA surface featured spectral peaks that closely resembled HAp, such as intense peaks at 960 , 1029 and 1090 cm^{-1} which were attributed to the phosphate group and a small peak at 3576 cm^{-1} which was indicative of the hydroxyl group of HAp (Figure 4.10C). For simplicity, the plasma irradiated HAp-immobilized substrate is referred to as *HAp-coated PMMA* from this point forward. Surface hydrophilicity was also altered from $79.2 \pm 5.6^\circ$ before plasma treatment to $14.3 \pm 3.4^\circ$ after treatment ($p < 0.001$) (Figure 4.10D). The HAp-coated PMMA surface was also significantly more hydrophilic than pristine PMMA ($66.1 \pm 0.5^\circ$; $p = 0.006$) (Figure 4.10D). In addition, the dip coating process and subsequent plasma treatment did not significantly alter the Ca/P ratio of the HAp (Figure 4.11).

Figure 4.10. Surface characterization HAp nanoparticles-immobilized PMMA following plasma treatment. (A) SEM showed that plasma treatment removed some PMMA from the surface and revealed more HAp nanoparticles on the surface. Macroscopic view of the HAp-coated PMMA showed uniform and homogenous surface coverage of the HAp. (B) AFM confirmed the observation with SEM and in addition, showed a relatively smoother surface after plasma treatment. (C) ATR-FTIR demonstrated negligible IR features of PMMA after plasma treatment. (D) Surface of the HAp-immobilized PMMA became more hydrophilic after plasma treatment.

Figure 4.11. Elemental composition of HAp nano-powder and plasma treated HAp-coated PMMA obtained with SEM-EDX.

There was a concern whether the dip coating process would damage the bulk material and whether the HAp nanoparticle could penetrate deep into the bulk of the PMMA. If the nanoparticles would indeed penetrate deep into the core of the PMMA, the HAp-coated PMMA would become not suitable to be used as a visual optics of a KPro because the nanoparticles could easily impede the visual axis of the patient. Low magnification cross-sectional SEM image showed that the HAp did not penetrate deep into the PMMA (**Figure 4.12**). HAp, which appeared as electron-intense layers both anteriorly and posteriorly, was only observed at 30-50 μm from the surface. High magnification images of both anterior and posterior surfaces of the HAp-coated PMMA revealed presence of rod shaped nanoparticles, consistent with the shape of nanoparticles that were used in this study. EDX further confirmed that Ca and P elements were only detectable at the surfaces of the PMMA, whereas only C and O elements were found in the center of the PMMA (**Figure 4.12**). High magnification images of both anterior and posterior surfaces of the HAp-coated PMMA revealed presence of rod shaped nanoparticles, consistent with the shape of the nanoparticles used in this study (**Figure 4.12**).

Figure 4.12. Cross-sectional SEM images of plasma treated HAp-coated PMMA. Electron-intense layers, suggesting the presence of HAp, along the anterior and posterior surfaces of the PMMA. Individual rod shaped nanoparticles could be seen in high magnification (30,000x) images. SEM of the bulk of the PMMA did not reveal the presence of HAp nanoparticles. EDX analysis further confirmed that HAp (Ca and P elements) could only be detected at the surfaces of the PMMA, but not in the bulk of the PMMA.

Dip coating and plasma treatment process did not significantly alter the flexural stress and flexural stress at break of the PMMA sheets (**Figure 4.13** and **Table 4.4**). Stress-strain graph revealed a different bending pattern between untreated and HAp-coated PMMA. Untreated PMMA resulted in immediate failure at the peak flexural stress. In contrast, HAp-coated PMMA had 2 stages of bending failure. The first stage of failure did not completely split the sheet possibly due to the presence of HAp coating on the reverse side of the HAp-coated PMMA sheet. This layer of

coating provided extra support before the complete failure occurred. In addition, the HAp coating also added significantly more rigidity to the PMMA sheets ($p=0.005$).

Figure 4.13. Stress-strain graph of plasma treated HAp-coated PMMA. Biomechanical properties of untreated and HAp-coated PMMA sheets were obtained by 3-point bending tests.

Table 4.4. Key biomechanical properties of untreated and plasma treated HAp-coated PMMA.

	Untreated	HAp-coated	<i>p</i> value
Ultimate flexural stress (MPa)	23.1 ± 4.0	21.0 ± 5.5	0.481
Flexural strain at break (%)	2.5 ± 0.5	2.2 ± 0.6	0.279
Flexural modulus (MPa)	1024.3 ± 47.3	1220.6 ± 109.6	0.005

$p < 0.05$ was considered to be statistically significant

Unlike the CaP coating described in the earlier chapter, the HAp nanoparticles immobilized on the PMMA via dip coating technique were anchored strongly to the surface. Tape adhesion test, similar to that carried out by Ryu et al. [17] and was earlier performed for d-CaP coating (**Figure S2.1B**), did not delaminate the coating on the HAp-coated PMMA surface (**Figure 4.14**). The improved surface hydrophilicity, maintenance of Ca/P ratio and better HAp coating cohesion and adhesion on PMMA of the HAp-coated PMMA sheets should then result in improved bonding strength between the substrate and collagen hydrogel. Indeed, using a QCM to demonstrate the

strength of interactions between 2 materials on nanoscale level, I found that there were approximately 4 times more collagen being bound on HAp nanoparticles-coated crystal than on PMMA film-coated crystal in the first few minutes after the collagen was introduced in the system (**Figure 4.15A**). The interactions remained stable in the next 40 minutes of the experiment in the collagen on HAp group, indicated by the Δf that remained constant at -100 Hz. In contrast, the Δf changed from -30 Hz to -25 Hz at the 12th minute mark and appeared to increase gradually over time in the collagen on PMMA group, suggesting shearing of collagen from the underlying PMMA-coated crystal. The QCM study provided an initial indication that collagen was able to bind stronger and more stably on HAp than on PMMA.

Figure 4.14. Macroscopic and microscopic views of HAp-coated PMMA surface after tape adhesion test. The test showed that the HAp nanoparticles were strongly anchored on the surface.

4.3.4 Shear bonding strength between HAp-coated PMMA and collagen hydrogel

The shear adhesion strength tests were performed using the same method described in the previous chapter. In this chapter, the substrate-hydrogel constructs were incubated in ATF, instead of in PBS, to mimic the physiological condition closer to where the artificial cornea is intended to be used in. There was a marked improvement in the shearing resistance on HAp-coated PMMA compared to the d-CaP

group and especially to the untreated PMMA group at all time points ($p<0.001$) (**Figure 4.15B**). The shearing resistance remained stable over time on the HAp-coated surface (474 ± 43 mN/cm² at day 1, 495 ± 59 mN/cm² at day 14 and 485 ± 51 mN/cm²). In contrast and consistent with the findings in the previous chapter, the shear adhesion strength of the hydrogel on d-CaP surface reduced over time from 190 ± 18 mN/cm² at day 1 and 180 ± 33 mN/cm² at day 14 to 138 ± 39 mN/cm² at day 28.

Figure 4.15. Interactions and shear bonding strength between collagen and HAp-coated substrate. (A) QCM showing strength of interactions between collagen and HAp, and between collagen and PMMA on nanoscale level. (B) Shear adhesion strength test results of collagen hydrogel on HAp-coated PMMA, untreated PMMA and CaP-coated PMMA via SBF incubation (d-CaP). Height of error bars indicates standard deviation of means. $*p<0.001$. (C) Illustration showing the HAp nanoparticles acting as an intermediate layer that interlocks PMMA and collagen hydrogel. This interlocking mechanism improves the shear adhesion strength of collagen on PMMA.

The improvement in shear adhesion strength of the hydrogel on HAp-coated PMMA relative on the d-CaP surface could possibly be attributed to: (i) better anchorage of the apatite layer on the PMMA; and (ii) crystallinity resembling pure

HAp, which potentially reduced the resorption rate of the apatite layer over time (**Figure 4.15C**). Contrary to the poor cohesion of d-CaP coating shown in **Figure 3.11C**, the immobilized HAp nanoparticles remained intact and not delaminated following the shear adhesion test (**Figure 4.16**), which suggested that the adhesion failure occurred at the interface between the hydrogel and HAp. In addition, the HAp nanoparticles acted as an intermediate layer that interlocked the PMMA and collagen hydrogel (**Figure 4.15C**). The dip coating technique allowed the HAp to be physically immobilized in the sub-surface of the PMMA. This physical immobilization obviated the need to perform surface chemical modifications, which were necessary for the d-CaP coating described in the previous chapter. Consistent with the QCM results, the more macroscopic shear adhesion test using a mechanical tester, showed improvement and stability of adhesion on HAp-coated substrate than on untreated PMMA surfaces.

Figure 4.16. Failure analysis of HAp-coated PMMA-hydrogel interface following shear adhesion test viewed under SEM.

4.3.5 Cell viability and proliferation on HAp-coated PMMA

There was a concern whether the chloroform could still be present on the HAp-coated PMMA even after rigorous washing steps. In order to ascertain chloroform had been completely evaporated after washing steps, TGA analysis of HAp-coated PMMA and pristine PMMA was performed. Pristine PMMA showed two main stages of decomposition, with a maximum degradation temperature of approximately 300°C and 400°C at each stage (**Figure 4.17**, red outline). Similar observation could be made

with HAp-coated PMMA decomposition pattern (**Figure 4.17**, purple outline). There was no additional derivative thermogravimetric (DTG) peak present other than the DTG peaks of the PMMA. DTG peak of HAp, which is normally beyond 900°C [18,19], did not appear in the DTG curve of the HAp-coated PMMA group. About 5% residual mass remained from the HAp-coated PMMA suggesting the undecomposed HAp (**Figure 4.17**, green outline). Chloroform under 100 ppm in quantity might not be able to be detected with TGA due to the limit of detection of the equipment [20]. According to the National Advisory Committee for Acute Exposure Guideline Levels for Hazardous Substances, the lethal dosage for exposure to chloroform in human is 1600 ppm for 8 hours continuously [21]. This value, which is substantially higher than the limit of detection of TGA, would be easily detected if residual chloroform of lethal dosage was still present in the HAp-coated PMMA. It is safe to conclude that no lethal amount of chloroform remained in the HAp-coated PMMA following rigorous washing and overnight drying in vacuum incubator.

Figure 4.17. TGA analysis of HAp-coated PMMA and pristine PMMA. TGA analysis was performed to detect chloroform in HAp-coated PMMA that might be present even after rigorous washing.

Cell viability on HAp-coated PMMA surface at day 1 was not great ($76\pm3\%$) compared to that seeded on glass coverslip ($99\pm1\%$; $p=0.002$) (**Figure 4.18A** and **4.18B**). However, it was significantly better than the cell viability on untreated PMMA

Figure 4.18. Proliferation and viability of corneal stromal fibroblasts on HAp-coated PMMA surface. (A) Cell viability detected with live/dead cell assay. Green and red fluorescence indicate live cells and dead cells, respectively. Scale bars = 100 μm . (B) Graph comparing % cell viability on glass coverslip (control), untreated PMMA and HAp-coated PMMA. * $p < 0.05$ relative to HAp-coated PMMA group. ** $p < 0.001$ relative to HAp-coated PMMA group. (C) Bar graph showing % cell attached on each material surface after 24 hours. * $p < 0.05$ relative to HAp-coated PMMA group. (D) Cell proliferation detected with ClickIT-EdU assay. Green fluorescence marks proliferative cells. Scale bars = 100 μm . (E) Bar graph showing % proliferative cells on each substrate after 48 hours of cell culture.

($45\pm1\%$; $p<0.001$) (**Figure 4.18A** and **4.18B**). This could possibly be attributed by the relatively poorer cell attachment efficiency on HAp-coated PMMA surface than the glass coverslip (**Figure 4.18C**). However, the cell viability improved significantly in the subsequent time points, whereby there were $93\pm6\%$ live cells on day 3 ($p=0.286$ compared to control group) and $92\pm6\%$ on day 7 ($p=0.195$ compared to control group) (**Figure 4.18A** and **4.18B**). It was possible that the adherent cells had started to lay more recognizable foundation for growth on the substrate and undergone proliferation [22]. In contrast, cell viability remained significantly low in the untreated PMMA group on day 3 and 7 (**Figure 4.18A** and **4.18B**), most likely due to the surface that was unfavourable for cell proliferation (**Figure 4.18D** and **4.18E**). Stromal fibroblasts had a proliferative capacity of $22\pm3\%$ on untreated PMMA surface and it was significantly lower than the control group ($35\pm4\%$; $p=0.009$) (**Figure 4.18E**). On the other hand, the cell proliferative capacity on HAp-coated PMMA ($36\pm8\%$) was similar to the control group ($p=0.913$) (**Figure 4.18D** and **4.18E**).

4.3.6 Cell morphology and adhesion on HAp-coated PMMA

Stromal fibroblast on HAp-coated PMMA appeared thinner (**Figure 4.19C**) than on the glass coverslip (**Figure 4.19A**). However, they both showed similarly expansive lamellipodia and abundant presence of vinculin, suggesting good cell adhesion (**Figure 4.19A** and **4.19C**). This observation could be confirmed by SEM on both surfaces, which also demonstrated presence of multiple filopodia that extended from the edge of the cells (**Figure 4.19B** and **4.19D**). In contrast, the fibroblast appeared roundish and non-adherent on pristine PMMA surface (**Figure 4.19E** and **4.19F**). Cell adhesion components, such as vinculin and filopodia, were not that distinctly present.

Figure 4.19. Cell attachment components assessed with immunohistochemical staining of vinculin and SEM. (A,C,E) Immunohistochemical staining of vinculin (red fluorescence) complemented with phalloidin staining (green). Scale bars = 20 μm . (B,D,F) SEM images showing cell morphology and cell adhesion components. (A,B) Control group. (C,D) HAp-coated PMMA group. (E,F) Untreated PMMA group.

4.4 Conclusion

I have established a new non-thermal coating method, which is simple and inexpensive to execute for immobilization HAp nanoparticles on a polymeric substrate. A combination of 20% (w/v) HAp nanoparticles in 5% (w/v) PMMA/chloroform, followed by cleaning with plasma irradiation appeared to offer the best formulation to produce a uniform and homogenous coating, that was hydrophilic, resistant to delamination and still preserved the crystallinity of the HAp. These advantages resulted in better and more stable adhesion between the HAp-coated PMMA and collagen hydrogel than untreated PMMA. Although corneal stromal fibroblasts did not have excellent attachment after 1 day on the HAp-coated PMMA surface, cells that remained were able to form good adhesion and proliferate in the subsequent days. The dip coating technique described here may have a potential to be applied to a KPro optical cylinder to provide a good bonding surface for collagen-based skirt material and a modified surface that is still biocompatible and can promote biointegration over time.

This study was published in: Riau AK, Mondal D, Setiawan M, Palaniappan A, Yam GH, Liedberg B, Venkatraman SS, Mehta JS. Functionalization of the polymeric surface with bioceramic nanoparticles via a novel, nonthermal dip coating method. *ACS Appl Mater Interfaces*. 2016;8:35565-35577.

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Chapter 5

Collagen-based skirt material with anti-infective capability for prevention of perioperative infections

*Infections following keratoprosthesis (KPro) implantation is a serious complication. Conventional antibiotic prophylaxis, including topical vancomycin application, is limited by low bioavailability, high dosing requirement and poor patient compliance. The ideal option to overcome these issues is an antibiotic-eluting skirt material that sustains the local release of drug. In this study, vancomycin was loaded in collagen hydrogels (VH) to create KPro skirt material with anti-infective capability. The incorporation of vancomycin did not significantly alter the Young's modulus, transparency and refractive index of the VH, which were 0.79 ± 0.04 MPa ($p=0.233$ relative to blank hydrogel), $94.3 \pm 2.3\%$ ($p=0.115$) and 1.346 ± 0.005 ($p=0.264$), respectively. The drug elution could be sustained for up to 7 days in vitro. The VH was implanted intrastromally in rabbit corneas. A corneal infectious keratitis model was then created by injection of *Staphylococcus aureus* inoculate on day 2 post-implantation. On day 3 post-infection, the VH-implanted corneas were clear and non-edematous and showed a reduction of log 2.5 in bacteria compared to the blank hydrogel-implanted corneas, which appeared hazy, edematous, and had excessive inflammation. Immunohistochemistry of CD18 demonstrated a significant reduction in inflammatory cells in VH-implanted corneas (49 ± 9 cells/unit area) compared to blank hydrogel-implanted corneas (523 ± 15 cells/unit area) ($p<0.001$). In conclusion, the antibiotic-eluting collagen hydrogel was efficient in preventing perioperative bacterial infections.*

5.1 Background of study

As with any implantable medical device that extends from the surface of the body (i.e. the skin or ocular surface) into a sterile environment (i.e. the anterior chamber or blood), implantation of a KPro carries a high risk of infections [1]. Contamination of the medical device by inoculation of microorganisms from the patient's skin or mucous membrane surface during implantation and to a lesser extent, from the surgical tools or the hands of the surgeon often contribute to the risk of perioperative infection [2]. Ocular bacterial infection remains one of the most devastating complications after implantation of any type of artificial cornea [3,4], which can rapidly affect the surrounding ocular tissues and cause a visual loss if interventions are not undertaken quickly [5]. Implantable medical device-associated infections often have serious repercussions on the patient's quality of life, are difficult to manage, and require lengthy hospital admission [6,7].

In many cases of KPro-related infections, Gram-positive bacteria have been identified as the most prominent isolates [8,9]. The use of topical vancomycin has been shown to be effective against almost all clinical strains of Gram-positive bacteria and has reduced the incidence of KPro-related infectious endophthalmitis dramatically [10]. However, the bioavailability of topical vancomycin is poor especially in the presence of an intact corneal epithelium [11]. Although in patients with advanced corneal disease, the corneal epithelial barrier is not always intact or is usually more 'leaky', the efficacy of topical antibiotic application is still limited by rapid tear fluid turnover and patient compliance [12,13], which result in variations in drug administered concentration. Topical administration of vancomycin is required for at least 4 times daily after surgery [14,15], a regimen that must continue indefinitely and can lead to poor patient compliance. To overcome these limitations, the ideal solution would be an antibiotic-eluting corneal prosthetic device capable of sustained and localized drug release.

In the current study, I loaded vancomycin in a collagen hydrogel, which has been used as the major component of an artificial cornea used in a clinical trial [16,17] and proposed as the substitute skirt material of a core-skirt KPro. The drug could be

released for up to 7 days in vitro with concentrations above the minimum inhibitory concentration (MIC) of vancomycin against *S. aureus* (2 µg/ml) [18]. To further validate the antimicrobial capability of the vancomycin-eluting hydrogel (VH) in reducing perioperative infection risk in vivo, I implanted the hydrogel in a rabbit model of infectious keratitis, where *S. aureus* was injected intrastromally on day 2 post-implantation. On the third day after bacterial injection, I found that the drug-loaded hydrogel was able to inhibit bacterial growth and maintain corneal clarity.

5.2 Materials and methods

5.2.1 Drug loading in collagen hydrogel

The collagen hydrogel was constructed as described previously with minor modifications [17]. Bovine atelocollagen type I from Koken (Tokyo, Japan) was used in the study. Briefly, aliquot of 10% or 15% (w/v) atelocollagen (0.4 ml) was loaded into a syringe mixing system. Vancomycin (Sigma-Aldrich, St. Louis, MO) was then dissolved in 0.625M 2-(N-morpholino)ethanesulfonic acid (0.1 ml) buffer (Sigma-Aldrich) and added into the collagen solution (vancomycin:collagen weight ratio = 1:1). Next, 10% (w/v) NHS (Sigma-Aldrich) and 10% (w/v) EDC (Sigma-Aldrich) were added (EDC:NHS molar ratio = 2:1) and mixed with the collagen solution. The mixture was dispensed onto a glass slide and flattened with another glass slide. For in vitro tests, 0.5-mm-thick hydrogels were made. For in vivo study, 0.2-mm-thick hydrogels were made to be inserted in rabbit corneas. The hydrogels were trephined to a diameter of 6 mm. Excess NHS/EDC in the hydrogel was removed by washing with 0.01M PBS containing 1% chloroform for 3 times 5 minutes and with water for 3 times 5 minutes. Drug loading efficiency was calculated using the following formula:

% drug loading efficiency

$$= \frac{\text{Actual amount of loaded in the hydrogel}}{\text{Amount of drug loaded in the syringe mixing system}} \times 100\%$$

where the actual amount of drug loaded in the hydrogel was obtained by spectrophotometry of vancomycin after complete digestion of collagen hydrogel by

collagenase enzyme. Blank hydrogels with no vancomycin (BH) were constructed as negative control. Vancomycin-loaded 10% and 15% hydrogels were referred to as VH-10% and VH-15%, respectively, whereas blank 10% and 15% hydrogels were referred to as BH-10% and BH-15%, respectively.

5.2.2 Biomechanical and optical properties of hydrogel

Mechanical properties of the hydrogels were determined using an Instron 3345 uniaxial tensile tester (Norwood, MA) with the use of a 10N load cell under a crosshead speed of 5 mm/min. Strips of VH and BH with 20 x 3 x 0.5 mm dimension were prepared for the tensile tests. Gauge length was maintained at 5 mm (refer to Appendix A for a more detailed analysis method of the mechanical properties of the hydrogels).

Hydrogels were hydrated in 0.01M PBS for 1 hour before measurements of optical transparency and refractive index (RI). To measure the optical transparency, hydrogels were cut to fit the bottom of a 96-well plate. Absorbance across visible wavelengths (390-700 nm) was then measured using a Tecan Infinite 200 microplate reader (Maennedorf, Switzerland). The acquired absorbance values were then converted to % transmittance. To measure the material RI, an Abbemat 200 refractometer (Anton Paar, Graz, Austria) was used.

5.2.3 In vitro drug release profile

Hydrogels were incubated in 1 ml of 0.01M PBS with constant shaking at 37°C. At various time intervals, 1 ml of the above solution was collected and refreshed with PBS. The amount of vancomycin in the withdrawn solution was determined by spectrophotometry (Tecan Infinite 200 microplate reader) at 280 nm wavelength. The percentage of drug release at each time interval was calculated by the following formula:

$$\% \text{ drug release} = \frac{\text{Amount of drug released in the supernatant}}{\text{Total amount of drug loaded in the hydrogel}} \times 100\%$$

where the total amount of drug loaded in the hydrogel was obtained by spectrophotometry of vancomycin after complete digestion of trephined collagen hydrogel, which was prepared from the same batch, by collagenase enzyme.

5.2.4 Circular dichroism analysis of released drug

Far UV–circular dichroism (CD) spectra of the vancomycin released from the hydrogels were recorded using a Chirascan CD plus spectropolarimeter (Applied Photophysics, UK) using a quartz cuvette with a path length of 0.1 cm (Hellma, Müllheim, Germany). The spectra were recorded from 260 nm to 190 nm at a scan rate of 50 nm/min. The CD data were expressed as mean residual weight ellipticity (deg.cm²/dmol). The mean residual weight (MRW) ellipticity ($[\theta]_{\text{MRW}}$) was estimated using the following equation:

$$[\theta]_{\text{MRW}} = \frac{[\theta]_{\lambda} \times \text{MRW}}{10 \times l \times c}$$

where $[\theta]_{\lambda}$ is the observed ellipticity, l is the path length of the cuvette and c is concentration of released vancomycin in mg/ml. The data obtained was plotted using Origin 8.0 software.

5.2.5 Minimum inhibitory concentration of released drug

MIC values of vancomycin released in the supernatant at day 1, 3, 5 and 7 time interval were determined by the microdilution assay. Overnight cultivated *S. aureus* (ATCC 29213) was suspended in yeast extract-dextrose broth at a starting concentration of $\sim 10^8$ CFU/ml in a microtiter plate. A serial double dilution of the released vancomycin in the same broth was mixed with the inoculum to give a final concentration ranging from 0.125–128 µg/ml and incubated in 37°C for 24 hours. The antimicrobial activity was assessed by measuring the optical density at 600 nm using a Tecan Infinite 200 microplate reader (Maennedorf).

5.2.6 Animals

Eight 12- to 15-week-old New Zealand White rabbits (3–4 kg body weight) were purchased from InVivos, Singapore. Six rabbits underwent bilateral hydrogel implantation, where one eye was implanted with VH and the fellow eye with BH. The implantation of the hydrogel, since being transparent, did not cause blinding of the rabbit; hence was approved for bilateral surgery by the Institutional Animal Care and Use Committee (IACUC) of SingHealth, Singapore. Three rabbits were monitored for 4 weeks to assess the biocompatibility of the VH in the corneas. The other 3 rabbits were injected with *S. aureus* intrastromally on day 2 after hydrogel implantation and the tissue responses to the infection were studied for 3 days further before being euthanized. Two rabbit with eyes not operated were used as control. The rabbits were monitored closely daily to ascertain that the bacterial infection did not cause binocular visual deprivation at any point of time during the experiment. Before any surgical procedure or post-operative examination, the rabbits were anesthetized with xylazine (5 mg/kg intramuscularly) and ketamine (50 mg/kg intramuscularly). The rabbits were euthanized under anesthesia by overdose intracardiac injection of sodium pentobarbitone.

5.2.7 Implantation of hydrogel in rabbit corneas

A thin stromal tissue was first removed by small incision lenticule extraction (SMILE) technique using a VisuMax femtosecond laser system (Carl Zeiss Meditec, Jena, Germany) as described in a previous study [19]. All rabbits (n=3) underwent removal of ~91 µm-thick tissue (equivalent to -7 diopters correction). Following completion of the laser procedure, a lamellar dissector (Asico, Westmont, IL) was inserted into the lamellar plane of dissection to release adhesions of the anterior portion of the lenticule from the overlying stroma and then the posterior surface of the lenticule from the underlying stroma (**Figure 5.1A**). Once the lenticule was detached from both surfaces, a Tan DSAEK forceps (Asico) was used to grasp and extract the lenticule from the corneal stromal bed (**Figure 5.1B**).

Hydrogel (5 mm in diameter and 0.2 mm in thickness), which had been washed with copious amounts of 0.01M PBS containing 1% chloroform and sterilized with UV for 15 minutes, was grasped across with a forceps and inserted intrastromally through the small incision (**Figures 5.1C and 5.1D**). An interrupted suture was finally placed at the small incision to prevent the hydrogel from being displaced. A one-time sub-conjunctival injection of dexamethasone (Hospira, Lake Forest, IL) was given after the implantation procedure. Topical prednisolone acetate (1%; Allergan, Irvine, CA) was administered 4 times a day for the next 3 days.

Figure 5.1. Snapshots captured during the femtosecond laser-assisted hydrogel implantation procedure. (A) A lamellar dissector was inserted into the lamellar plane of dissection to release adhesions of the anterior portion of the lenticule and then the posterior surface of the lenticule from the adjacent corneal stroma. (B) Once the lenticule was detached from both surfaces, a forceps was used to grasp and extract the lenticule (indicated by an asterisk) via a small incision that was created by the laser. (C) A hydrogel was then grasped across with a forceps and inserted intrastromally through the small incision. (D) Finally, the hydrogel was spread out and positioned centrally.

5.2.8 In vivo biocompatibility of drug-loaded hydrogel

IVCM was performed to assess the biocompatibility of the VH implanted in the rabbit corneas for up to 4 weeks using an in vivo confocal microscope with cornea module (Heidelberg retina tomography HRT3; Heidelberg Engineering GmbH, Heidelberg, Germany). A carbomer gel (Vidisic; Mann Pharma, Berlin, Germany) was applied on the tip of the microscope objective as immersion fluid to prevent the corneal epithelium to be excessively disrupted during the examination. The position of the objective lens was adjusted to the center of the cornea. The z-axis scan from the epithelium to endothelium was then performed and controlled with a manual micrometer knob. The resulting micrographs were analysed with Heidelberg Eye Explorer version 1.5.1 (Heidelberg Engineering GmbH). En-face images of the corneas at the level of epithelium, anterior stroma (60-70 μm -deep), mid stroma (140-150 μm -deep), posterior stroma (240-250 μm -deep) and endothelium were selected and presented.

5.2.9 Post-hydrogel implantation bacterial infection

On day 2 following hydrogel implantation, 3 rabbits were subjected to bilateral *S. aureus* (ATCC 29213) intrastromal injection. A 50 μl bacterial solution ($\sim 10^8$ CFU/ml concentration) was inoculated into the intrastromal pocket above the hydrogel. These rabbits were euthanized 3 days later. The corneas were then dissected from the eye globe and cut into two; one half was used for immunofluorescence staining analysis and the other half for in vivo bacterial quantification.

5.2.10 Post-hydrogel implantation and bacterial infection examinations

Slit lamp photography and AS-OCT were performed before surgery, before bacterial injection (post-hydrogel implantation day 2), and on day 3 after bacterial injection. Slit lamp photographs were taken using a zoom slit lamp NS-2D (Righton, Tokyo, Japan). Post-infection slit lamp images were clinically graded using a method described by Johnson and colleagues [20]. The infection was graded based on

evaluation of symptoms, such as conjunctival infection, chemosis, iritis, fibrin formation, hypopyon formation, percentage of stromal infiltrated area and percentage of stromal edematous area, with a total score ranging from 0, indicating no infection symptoms, to 28, indicating severe bacterial infection.

5.2.11 Quantification of bacteria from infected corneas

The corneas (n=3 in each group) were homogenized in 0.01M PBS using pestles, followed by fine homogenization with bead beating using 2 mm glass beads. A serial dilution of the homogenates was plated on tryptic soy agar (TSA) plates and incubated at 37°C for 24 hours. The number of colonies on each plate was counted and reported as log CFU.

5.2.12 Tissue sectioning, histology and immunohistochemistry

Corneas were first embedded in optimum cutting temperature cryocompound (Leica Microsystems, Nussloch, Germany). Sagittal corneal sections with 8 µm thickness were cut using a Microm HM525 cryostat (Walldorf, Germany). For histological analysis, hematoxylin and eosin (H&E) staining was performed on the cryosections and viewed with a Carl Zeiss Axioplan 2 light microscope (Oberkochen, Germany). For immunofluorescence staining analysis, the cryosections were first fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 minutes, washed with 0.01M PBS, and then blocked with 4% bovine serum albumin (Sigma-Aldrich) in 0.01M PBS, 0.15% Triton X-100 (Sigma-Aldrich) for 1 hour. The sections were then incubated with mouse monoclonal antibody against CD18 (Novus Biologicals, Littleton, CO) diluted 1:100 at 4°C overnight, followed by incubation with Alexa Fluor 488 conjugated secondary antibody (Life Technologies, Carlsbad, CA) at room temperature for 1 hour. Finally, the sections were mounted with UltraCruz Mounting Medium containing DAPI (Santa Cruz Biotechnology, Santa Cruz, CA). The tissue sections were imaged with a Carl Zeiss AxioImager Z1 microscope.

5.2.13 Statistical analysis

Data were expressed as mean $\pm$ SD. Statistical significance or p value was determined using two-tailed independent samples t-test with SPSS software version 17.0 (Chicago, IL). A value of $p<0.05$ was considered to be statistically significant.

5.3 Results and discussion

5.3.1 Vancomycin in vitro release profile

Being a protein, collagen contains many charged amino acid side chains, which could have formed ionic interactions with the vancomycin, slowing the drug release rate (**Figure 5.2A**). A small degree of crosslinking of vancomycin could also occur in the collagen network through NHS/EDC crosslinking reaction since both vancomycin and collagen contain $-NH_2$ and $-COOH$ in their structure (**Figure 5.2A**). Delayed drug release due to crosslinking and ionic reaction between pilocarpine and collagen has previously been documented [21].

The drug loading efficiency in the collagen hydrogels was $90.4\pm1.7\%$. The approximately 10% loss was probably due to the drug that was still entrapped in the remaining collagen, which could not be completely dispensed from the syringe mixing system. After further washing with PBS containing 1% chloroform and distilled water, the final drug loading efficiency was $78.7\pm3.8\%$. Overall, there was approximately 22% drug loss from hydrogel casting process and subsequence washing steps.

There was no difference in the percentage of vancomycin release at any time interval over 10 days between VH-10% and VH-15% ($p>0.05$ at all tested time intervals) (**Figure 5.2B**). Although the percentage drug release on day 7 appeared to be small ($0.6\pm0.1\%$ from VH-10% and $0.9\pm0.4\%$ from VH-15%), the concentration of vancomycin released from both hydrogels was still above the theoretical MIC against *S. aureus* (5.6 ± 1.2 $\mu\text{g/ml}$ from VH-10% and 9.8 ± 4.3 $\mu\text{g/ml}$ from VH-15%) (**Figure 5.2C**). The presence of vancomycin could be detected on day 10, but the concentration

Figure 5.2. Vancomycin release profile from collagen hydrogel. (A) Percentage drug release over 10 days from vancomycin-loaded 10% hydrogel (VH-10%) and vancomycin-loaded 15% hydrogel (VH-15%). No difference of percentage drug release was found between the hydrogels at any time interval ($p > 0.05$). (B) Amount of vancomycin released over 10 days from VH-10% and VH-15% in $\mu\text{g/ml}$. (C) Percentage drug release over 10 days from VH-15% with same diameter but different thickness (0.2 mm vs 0.5 mm). No difference of percentage drug release was seen between the hydrogels at any time interval ($p > 0.05$). (D) Amount of drug released over 10 days from VH-15% with 0.2 mm thickness and 0.5 mm thickness.

was too low to be bactericidal effective. In **Figure 5.2D**, I showed that thinner VH-15% (0.2 mm-thick), which would be used in the in vivo study later, did not affect the percentage drug release at any time points ($p > 0.05$). The concentrations of vancomycin released over 10 days from 0.2 mm-thick hydrogel were depicted in **Figure 5.2E**. My study has shown a considerable improvement in sustaining drug

release from a collagen-based system. Drug delivery from collagen-based vehicles, including vancomycin-soaked collagen shield, has been attempted previously, but the drug release could not be sustained for more than a day [21-23].

5.3.2 Biomechanical and optical properties of vancomycin-loaded hydrogel

Similar to porcine collagen hydrogels [24], my 15% collagen hydrogel produced a biomechanically stronger artificial corneal scaffold than 10% hydrogel. **Figure 5.3A** showed the representative tensile strength vs strain curves of the BH and VH. BH-10% and BH-15% hydrogels yielded an ultimate tensile strength of 0.26 ± 0.01 MPa and 0.32 ± 0.02 MPa, respectively ($p=0.012$). The Young's modulus of BH-15% (0.82 ± 0.03 MPa) was superior to that of BH-10% (0.78 ± 0.08 MPa), but the difference was not significant ($p=0.385$). VH-10% and VH-15% produced an ultimate tensile strength of 0.23 ± 0.02 MPa ($p=0.532$ compared to BH-10%) and 0.29 ± 0.01 MPa ($p=0.144$ compared to BH-15%), respectively. With respect to Young's modulus, VH-10% and VH-15% yielded 0.69 ± 0.10 MPa ($p=0.297$ compared to BH-10%) and 0.79 ± 0.04 MPa ($p=0.233$ compared to BH-15%), respectively. There was a concern that during the crosslinking process of the hydrogel, the vancomycin (with two amine ends and one carboxyl end) would interfere with the curing of the hydrogel and consequently affect its mechanical properties. The addition of vancomycin in the hydrogels slightly reduced both the ultimate tensile strength and Young's modulus of the materials, although the differences did not reach statistical significance.

High optical transparency of any artificial corneal scaffold is paramount. The visible light transparency of both 10% and 15% hydrogels was slightly reduced after the addition of vancomycin (**Figure 5.3B**). The reduction, however, was not significant across any wavelength (all tested wavelengths showed $p>0.05$ when comparing the % transmittance between VH and BH). The average % transmittance of BH-10% and VH-10% was 95.8 ± 1.4 and 94.9 ± 1.4 , respectively ($p=0.337$), while that of BH-15% and VH-15% was 96.6 ± 1.1 and 94.3 ± 2.3 , respectively ($p=0.115$). There was also no significant difference in material's RI between VH and BH. The RI of BH-10% and VH-10% was 1.352 ± 0.002 and 1.346 ± 0.004 , respectively ($p=0.090$),

whereas the RI of BH-15% and VH-15% was 1.351 ± 0.002 and 1.346 ± 0.005 ($p=0.264$). The obtained RI values were comparable to the RI of healthy human corneal stroma, which range from 1.360-1.370 [25].

Figure 5.3. Biomechanical and optical properties of vancomycin-loaded hydrogel and blank hydrogel. (A) Stress vs strain curves of VH and BH. (B) Percentage visible light transmittance of vancomycin-loaded hydrogels and blank hydrogels. BH-10% (blue line): 10% (w/v) collagen hydrogel without vancomycin. VH-10% (orange): vancomycin-loaded 10% collagen hydrogel. BH-15% (red): 15% collagen hydrogel without vancomycin. VH-15% (green): vancomycin-loaded 15% collagen hydrogel.

The material characterization leading up to this point showed that VH-15% shared similar drug release pattern and optical properties as the VH-10%, but had superior biomechanical properties. I decided to use VH-15% for further in vitro characterization and the following in vivo implantation. A biomechanically superior hydrogel was preferred to ensure the maintenance of the integrity of the scaffold during implantation procedure, which inevitably required some physical manipulations to be performed, such as folding and grasping of the hydrogel with forceps.

Table 5.1. Key biomechanical and optical properties of blank and vancomycin-loaded collagen hydrogels.

	BH-10%	VH-10%	BH-15%	VH-15%
<u>Mechanical properties</u>				
Ultimate tensile strength (MPa)	0.26±0.01	0.23±0.02	0.32±0.02	0.29±0.01
Elongation at break (%)	56.1±5.9	57.2±1.9	67.2±1.9	65.5±3.5
Young's modulus (MPa)	0.78±0.08	0.69±0.10	0.82±0.03	0.79±0.04
<u>Optical properties</u>				
Visible light transmittance (%)	95.8±1.4	94.9±1.4	96.6±1.1	94.3±2.3
Refractive index	1.352±0.002	1.346±0.004	1.351±0.002	1.346±0.005

5.3.3 Structural integrity and antimicrobial activity of vancomycin released from collagen hydrogel

Far-UV CD spectra (**Figure 5.4A**) of the antibiotic released from collagen hydrogels shows the characteristic spectra of vancomycin [26,27]. Similar far-UV CD spectra between control vancomycin and vancomycin released from the collagen hydrogel on day 1, 3 and 5 were observed. A clear maxima around 230 nm ($n-\pi^*$) and a minima of equal intensity around 205 nm ($\pi-\pi^*$), originated from the peptidal backbone of vancomycin, validated the structural integrity of the drug eluted on day 1, 3 and 5. However, there was a change in spectra with elevated minima intensity between 190-210 nm for the drug released on day 7, which indicated perturbations in the $\pi-\pi$ interactions possibly due to shielding of ionizable groups or alteration in shape of the vancomycin molecule. It would be interesting to see the detailed structural changes to the vancomycin molecule; however, this could not be resolved with CD spectrophotometry. A more complex and specialized imaging technique, such as X-ray crystallography or 3-D NMR would be required for that purpose.

Figure 5.4. Far-UV CD spectra and MIC of vancomycin released from collagen hydrogel. (A) Similar far-UV CD spectra between control vancomycin and vancomycin released from the collagen hydrogel on day 1, 3 and 5 was observed. An increased $[\theta]_{MRW}$ intensity of the spectral minima at between 190-210 nm of vancomycin released on day 7 possibly due to structural perturbations. (B) The structural perturbations of vancomycin released on day 7 corresponded to 4-fold reduction of MIC against *Staphylococcus aureus*. The MIC of control vancomycin and vancomycin released on day 1, 3 and 5 was 1 µg/ml.

Digital images of microplate following the antimicrobial microdilution assay showed that control vancomycin had a MIC of 1 µg/ml against *S. aureus*, which was similar to the MIC of the vancomycin released from the hydrogel, collected on day 1, 3 and 5 (**Figure 5.4B**). Interestingly, the structural perturbations of the vancomycin collected on day 7 correlated with the change in the bactericidal efficiency, as seen by the reduction in MIC against *S. aureus* from 1 µg/ml to 4 µg/ml. This could possibly be due to the reduction in the stability of vancomycin after incubation in PBS for 7 days in 37°C. A previous study has also noticed the progressive reduction in the bactericidal killing ability of vancomycin released in the PBS from poly(D,L)-lactide-co-glycolide nanofibers over time [28].

5.3.4 In vivo biocompatibility of vancomycin-loaded hydrogel

Equipped with the knowledge of drug eluting capability, antimicrobial activity of the eluted drug, biomechanical properties and optical properties of the VH in vitro, I proceeded to implant the hydrogel in a rabbit experimental model. I was able to monitor and study the biocompatibility of the VH in the rabbit eyes by utilizing an IVCN for 4 weeks. The state of the control cornea and VH-implanted cornea was similar from epithelium down to the endothelium, where the epithelial cells in both groups appeared in high density and cobblestone-like, the stroma was interspersed with quiescent keratocytes and the endothelial cells appeared hexagonal with defined contact inhibition (**Figure 5.5A**). Presence of hydrogel could be seen in the mid stroma of VH-implanted cornea, which was devoid of keratocytes. The density of epithelial cells, keratocytes in anterior and posterior stroma, as well as endothelial cells was similar in control cornea and VH-implanted cornea (**Figure 5.5B**). There was 564 ± 34 and 540 ± 45 epithelial cells/mm² found in control and VH-implanted cornea, respectively ($p=0.489$). In terms of keratocyte density, there was 296 ± 16 and 313 ± 29 cells/mm² ($p=0.427$) in the anterior stroma and 137 ± 17 and 143 ± 17 cells/mm² ($p=0.667$) in the posterior stroma of control and VH-implanted cornea, respectively. The density of corneal endothelial cells was 1583 ± 54 cells/mm² in the control group and 1579 ± 37 in the VH group ($p=0.917$).

The initial burst release of the vancomycin (2-mm diameter VH-15%) reached a relatively high concentration of 326.5 ± 52.0 µg/ml on day 1 and 78.2 ± 5.6 µg/ml on day 3. There was a concern that the high drug concentration would be cytotoxic to the corneal cells. The post-implantation follow-up examinations up to 1 month showed no sign of substantial toxicity to cells in the corneal epithelium, stroma and endothelium. This comes as no surprise due to the relatively wide therapeutic index of vancomycin in the cornea, which has a peak concentration of 12.5-100 mg/ml and trough concentration of 0.25-2 µg/ml [29].

Figure 5.5. In vivo confocal micrographs showing the biocompatibility of vancomycin-loaded hydrogel in the rabbit cornea. (A) The temporal images of corneal epithelium, anterior stroma, mid stroma, posterior stroma and endothelium were captured on week 4 after hydrogel implantation and were compared to observations made in control cornea, which did not undergo any surgery. (B) Bar graphs showing comparable epithelial, keratocyte and endothelial cell density between control and VH-implanted corneas.

5.3.5 In vivo bactericidal effects of vancomycin-loaded hydrogel

Once the biocompatibility of the VH was assessed, a new batch of rabbits was used to create an infectious keratitis model [30]. From the slit lamp photography, the

severity of infection in the corneas implanted with BH was clearly evident. The corneas appeared hazy and edematous with presence of hypopyon in the anterior chamber (**Figure 5.6A**). In contrast, the corneas implanted with VH appeared transparent and no hypopyon was observed (**Figure 5.6A**). By using a clinical grading method, the corneas implanted with BH scored 21.67 ± 1.53 and those with VH 1.67 ± 1.55 ($p < 0.001$). AS-OCT revealed the presence of hydrogels within the corneas on day 2 post-implantation (before bacterial infection) (**Figure 5.6B**). On day 3 post-infection, the corneas implanted with BH were cloudy and edematous with irregular corneal thickness spanning the length of the cornea, whereas the corneas implanted with VH appeared normal (**Figure 5.6B**). The pre-surgical corneal thickness was 359 ± 15 μm and 361 ± 17 μm in BH and VH groups, respectively ($p = 0.872$) (**Figure 5.6C**). The thickness was slightly increased after hydrogel implantation because the hydrogel (0.2 mm-thick) was thicker than the thickness of the tissue removed (~ 0.091 mm-thick). The BH-implanted corneas measured 480 ± 64 μm and VH-implanted corneas at 489 ± 48 μm ($p = 0.859$) (**Figure 5.6C**). The post-infected corneas were significantly thicker in BH group (713 ± 73 μm) than in VH group (537 ± 56 μm) ($p = 0.029$). The bacterial quantification in the infected corneas showed the presence of 7.02 ± 1.11 log CFU of *S. aureus* in the BH group, which was significantly higher than 4.45 ± 0.10 log CFU found in the VH group ($p = 0.016$) (**Figure 5.6D**).

To the best of my knowledge, this was the first study that had shown the bactericidal capability of a drug-loaded hydrogel in infected rabbit corneas. Previous studies have only shown the efficacy of topical antibiotics on non-operated rabbit corneas that had been injected with *S. aureus* intrastromally [31-33]. Alternative methods to the intrastromal injection model include soaking of contact lenses in *S. aureus* prior to placement on rabbit corneas [34,35] and inoculation of *S. aureus* underneath LASIK flaps [36], and these rabbits were also treated with antibiotics topically.

Figure 5.6. Clinical observations and bacterial quantification in corneas implanted with vancomycin-loaded and blank hydrogels infected with *S. aureus*. (A) Slit lamp photography showed the corneal state before surgery, before infection, and 3 days after bacterial infection. (B) AS-OCT showing cross-section of the corneas. (C) Bar graph showing changes in corneal thickness from pre-surgery, pre-infection to 3 days post-infection. (D) Bar graph showing bacterial quantification of infected corneas implanted with BH and VH. Dashed line indicates the amount of bacteria injected intrastromally. * $p<0.05$.

5.3.6 Histology and inflammatory reaction following bacterial infection

I could see abundant inflammatory cells both anterior and posterior to the implant site from the H&E stained corneal sections that were implanted with BH (**Figure 5.7A**). In addition, inflammatory cells were also present in the anterior chamber (**Figure 5.7A**). The cornea also did not have an intact epithelial layer (**Figure 5.7A**). In contrast, corneal sections implanted with VH showed minimal inflammation and intact epithelium (**Figure 5.7B**). Control cornea, which did not undergo any

Figure 5.7. Histology and immunofluorescence staining analysis of corneas implanted with vancomycin-loaded hydrogel and blank hydrogel infected with *Staphylococcus aureus*. Hematoxylin and eosin (H&E) staining of cornea implanted with BH (A) and VH (B), and of control cornea (C). Immunofluorescence staining of CD18, an inflammatory marker, in cornea implanted with BH (D) and VH (E), and in control cornea (F) confirmed the H&E observations. Asterisk (*) indicates presence of inflammatory cells in the anterior chamber of BH implanted eyes. Scale bars = 100 μ m.

surgical procedure, showed intact, stratified epithelium and normal stroma that was interspersed with quiescent keratocytes (**Figure 5.7C**). Immunofluorescence staining of CD18, an inflammatory cell marker, in the cornea implanted with BH further confirmed the H&E staining where abundant CD18-positive cells were found throughout the full thickness of stroma as well as the anterior chamber (**Figure 5.7D**). CD18 was expressed in a few cells at the anterior and posterior of the VH implant site (**Figure 5.7E**). The number of inflammatory cells in the central corneas in the VH group (49 ± 9 cells/unit area), however, was significantly lower than in the BH group (523 ± 15 cells/unit area) ($p < 0.001$). Control cornea did not express any CD18 (**Figure 5.7F**).

5.4 Conclusion

Although precautions have been taken, the rate of KPro-related infectious endophthalmitis has been reported to be 5.4% (ranging from 0%-17%) [37]. Higher infection rates have been reported in less developed countries due to a combination of a few factors, such as poor medical infrastructure, microbiologically unsafe water and poor patients' compliance [38]. These circumstances are confounded in the fact that the majority of the global corneal blind patients that are in need of a KPro are in less developed countries. An antibiotic-loaded artificial cornea could possibly alleviate the incidence of intra-operative and early post-operative bacterial infection by providing localized antibiotic prophylactic treatment and confer protection to the patient from microbial insults during the recovery of the corneal epithelial function (as the first line of defense of the cornea), which normally occurs during the first week after surgery [16,17,39]. The vancomycin-loaded hydrogel was able to fulfil the requirement by releasing the drug for 7 days within its therapeutic index in the cornea. Furthermore, I demonstrated the efficacy of localized vancomycin delivery from the collagen hydrogel in preventing implantable device-associated *S. aureus* infections in vivo. The devastation of the infected corneas implanted with blank hydrogel was visibly obvious compared to those implanted with drug-loaded hydrogel.

This study was published in: Riau AK, Mondal D, Aung TT, Murugan E, Chen L, Lwin NC, Zhou L, Beuerman RW, Liedberg B, Venkatraman SS, Mehta JS. Collagen-based artificial corneal scaffold with anti-infective capability for prevention of perioperative bacterial infections. *ACS Biomater Sci Eng.* 2015;1:1324-1334.

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Chapter 6

Summary and future direction of studies

This chapter contains summary of the studies that were conducted with an aim to improve the interfacial adhesion between two materials (PMMA and collagen hydrogel) that comprised a modified core-skirt KPro, without sacrificing the biocompatibility and the necessity of biointegration of the corneal stromal cells on the surface modified substrate. Finally, tested hypothesis, additional hypothesis generated from the studies, and the future direction of the studies are elaborated in the following section.

6.1 Summary

This thesis has highlighted the use of apatite as an intermediate layer to improve the interactions and bonding between PMMA and collagen hydrogel. Calcium phosphate coating deposited via simulated body fluid incubation on dopamine activated PMMA (d-CaP) was able to produce better adhesion with collagen hydrogel compared to untreated PMMA. However, the d-CaP coating could easily undergo delamination. This reduces the potential of d-CaP coating to be used to modify the surface of KPro optical cylinders, which undergo constant shearing force by eyelid blinking in patients. In addition, the d-CaP coating exhibited a calcium deficient apatite with a significantly lower Ca/P ratio compared to pure HAp, potentially reducing its retention in hostile biological microenvironment of advanced corneal diseases that these KPros are intended to be used in.

A new dip coating method, which is relatively simple and does not require expensive equipment to execute for immobilizing HAp nanoparticles on PMMA appeared to address the problems associated with the d-CaP coating. Dip coating of PMMA in the mixture of HAp nanoparticles in PMMA/chloroform for 60 seconds, followed by surface cleaning with plasma irradiation (HAp-coated PMMA) offered the best formulation to produce a uniform and homogenous coating, that was hydrophilic, resistant to delamination and still preserved the Ca/P ratio of pure HAp. These advantages resulted in about 2.5 times improvement in adhesion strength (over 28 days in artificial tear fluid) between collagen hydrogel and HAp-coated PMMA than between hydrogel and d-CaP. Corneal stromal fibroblasts were viable and able to form good adhesion and to proliferate on the HAp-coated PMMA, which suggests that good biointegration could be expected in long-term. In contrast, the cells had lower viability, appeared unhealthy and non-adherent on untreated PMMA.

The dip coating method can also be applied to immobilize other types of nanoparticles, for example TiO₂ nanoparticles, giving a possibility of applications beyond just the modification of KPro optical cylinder. However, different types of nanoparticles may require optimization of the formulation due to differences in dimension, weight and shape of the nanoparticles. **Figure 6.1** showed successful

immobilization of 50 nm-diameter, spherical TiO_2 nanoparticles (purchased from MKNano, Mississauga, Canada) on PMMA sheets using one time 60-second dip in 25% (w/v) TiO_2 nanoparticles in 5% PMMA/chloroform formulation. Lower concentrations of nanoparticles were initially tried but could not produce uniform and homogenous coating. The possibility to apply this dip coating method on other types of polymeric substrates should also not be ruled out. Similarly, different types of polymer used may require optimization of the formulation and protocol of the dip coating method.

Figure 6.1. Immobilization of TiO_2 nanoparticles on PMMA surface. The new dip coating technique could also be applied for other types of nanoparticles, such as TiO_2 shown in the SEM image. Individual spherical nanoparticles (50 nm in diameter) could be differentiated in the high magnification image on the right.

More importantly, the dip coating technique could be extended to immobilize HAp nanoparticles on PMMA cylinders (**Figure 6.2**), bringing the studies closer to the clinical application of the coating for KPro optical cylinders. However, minor modification in the dip coating method (12 times 5-second-dips instead of 1 time 60-second-dip) was necessary to immobilize the nanoparticles on PMMA cylinders of 3 mm in diameter due to the curved surface and smaller surface area compared to PMMA sheets. Detailed steps covering immobilization of HAp nanoparticles on PMMA cylinders and the coating mechanism can be found in Appendix C.

Figure 6.2. Immobilization of HAp nanoparticles on PMMA cylinder viewed on SEM.

Intraoperative bacterial infection has been a serious threat in many cases of KPro implantation. Early bacterial infection can negatively affect the initial tissue wound healing and biointegration process of the KPro with the host corneal tissue. Therefore substitution of donor cornea with collagen hydrogel as the skirt material of a KPro offers an advantage, whereby an antibiotic (vancomycin) could be loaded in the hydrogel (VH) and be released locally for 7 days. VH was shown to be bactericidal effective both in vitro and in vivo. When implanted in the rabbit corneas, which were infected with *S. aureus*, the VH-implanted corneas were clear and non-edematous and showed a reduction of log 2.5 in bacteria compared to the blank hydrogel-implanted corneas, which appeared hazy, swollen, and had excessive inflammation.

6.2 Tested hypothesis, additional hypothesis generated and future direction of research

The hypothesis that was tested in this thesis is as follows: Appropriate surface modification on PMMA can improve the resistance to interfacial shear adhesion between PMMA and donor corneal substitute material (collagen type I hydrogel) exceeding the maximum shear stress of 130 mN/cm^2 generated by the eyelid wiping process and also enhance the biointegration of corneal stromal cells on PMMA, and that incorporation of antibiotic in the collagen hydrogel can improve the early-postoperative protection against bacterial invasion of a core-skirt KPro construct.

The first part of the hypothesis was validated. Application of surface modification on PMMA surface was able to improve its adhesion with collagen type I (the primary component of corneal stroma). All surface modification techniques

studied here showed marked improvement of interfacial bonding between the two materials. Surface coating with 3-APTES resulted in good interfacial adhesion; however, the coating was not biocompatible with human corneal stromal cells. CaP coating via SBF incubation initially demonstrated great potential, but the stability of the coating over a period of time was not satisfactory. Novel non-thermal dip coating method was then developed to immobilize bioactive HAp nanoparticles on the PMMA and the HAp-coated PMMA showed stable adhesion with collagen that had shear resistance above the maximum shear stress that could be generated by the eyelid wiper. Although the HAp-coated PMMA was biocompatible, it remains to be seen whether a much longer term biointegration of soft tissue, such as the cornea, to the modified PMMA can occur *in vivo*. Another limitation associated with the studies is that the hydrogels were acellular; however, the initial adhesion occurred with the acellular collagen. Similar to other implants, long-term survival of a KPro in the body is subject to many events including (but not limited to) cellular infiltration. *In vivo*, corneal stromal cells are normally in quiescent state and their migration rate is slow [1]. Studies on the interactions between HAp-coated PMMA and collagen (with cells), as well as biointegration of the material in an animal model can only follow in the wake of initial adhesion of the interfaces, and will be planned for later.

The second part of the hypothesis was also validated. Incorporation of vancomycin in the collagen hydrogel was able to prevent the bacterial growth for 7 days *in vitro* and *in vivo*. This provides sufficient protection to the cornea undergoing epithelial barrier recovery, which normally occurs in the first week after implantation. Obviously a longer-term drug release from the hydrogel is desired in any clinical scenarios. A possible solution to prolonging the drug release from the collagen hydrogel can be by a 2-step drug encapsulation, a method recently described [2]. This involves the encapsulation of the drug in either polymeric or liposomal nanoparticles, that can release the drug in significantly longer term, followed by encapsulation of the drug-loaded nanoparticles in the collagen hydrogel. Encapsulation of antibiotics with a wider bactericidal spectrum, such as levofloxacin, should also be explored in the future.

In terms of moving the research towards clinical application, it is obvious the next study would involve the application of the dip coating method for the KPro

optical cylinder that is used clinically. Further optimization of the dip coating method could be required due differences in dimension and shape of the KPro optical cylinder compared to the PMMA cylinders that I described in the previous chapter of this thesis (**Figure 6.3**). Another challenge would be to prevent surfaces, which are the visual axis of the patients and also are not intended to be bonded with the skirt material (areas above and below the dashed lines in **Figure 6.3**), from being exposed to the nanoparticles and solvent mixture during the dip coating process. Exposure to the organic solvent could cause opacity to the PMMA and obstruct the visual axis of the KPro patients. Caps to cover the anterior and posterior of the optical stem may need to be fabricated to address this problem.

Figure 6.3. Picture of a PMMA optical cylinder that is normally used to construct a core-skirt KPro. Areas above and below the dashed lines indicate regions that should not be exposed to the nanoparticles and chloroform mixture during the dip coating process.

Should the nanoparticles be able to be immobilized on the KPro optical cylinder, an animal experiment could then be planned and performed. Rabbits may be the most suitable experimental animal to be used due to the size of the eyes which are relatively big and could fit the human KPro. In addition, rabbits are the most widely used large animal in eye research due to the ease of handling and robust corneal wound healing ability.

6.4References

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Appendix A

Biomechanical properties of bovine collagen hydrogel

Biomechanical properties of 10% bovine collagen type I hydrogels were determined using an Instron 3345 uniaxial tensile tester (Norwood, MA) with the use of a 10N load cell under a crosshead speed of 5 mm/min. Hydrogels with 20 x 3 x 0.5 mm dimension were prepared for the tensile tests. Gauge length was maintained at 5 mm. Comparison of key biomechanical properties (ultimate tensile strength, elongation at break, and Young's modulus) was made between bovine collagen hydrogel and porcine collagen I hydrogel and also recombinant human collagen III hydrogel. Young's modulus was obtained from the slope of the tangent to the initial, relatively linear, elastic region of the stress-strain curve [1,2]. All hydrogels were 10% (w/v) in concentration and crosslinked using similar method.

Figure S1.1. Biomechanical properties of 10% bovine collagen type I hydrogel. Representative stress-strain curves of 3 different hydrogels were presented.

Table S1.1. Comparison of key biomechanical properties of bovine and porcine collagen type I hydrogels, as well as recombinant human collagen type III hydrogel.

Materials	Bovine collagen I hydrogel	Porcine collagen I hydrogel*	Recombinant human collagen III hydrogel*
Ultimate tensile strength (MPa)	0.26 ± 0.01	0.13 ± 0.42	1.70 ± 0.21
Elongation at break (%)	56 ± 6	49 ± 15	14 ± 1
Young's modulus (MPa)	0.78 ± 0.08	0.60 ± 0.26	20.26 ± 2.04

*Biomechanical properties of porcine and recombinant human collagen hydrogels were obtained from Liu and colleagues' work [3].

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Appendix B

Experiments related to delamination of d-CaP coating

Delamination of d-CaP coating was performed by a tape adhesion test using Scotch filament tape 892 (with 65.4 N/100 mm adhesion strength rating). Briefly, the tape was applied on one half of the d-CaP coated PMMA sheet and pressed against each other with a paper binder clip (19 mm width, SureMark, Singapore) that applied an average force of 1.37 ± 0.25 N/cm² for 5 minutes. After the paper bind clip and tape were removed, the PMMA sheet was rinsed with copious amounts of distilled water. The PMMA sheet was then mounted on a stub secured by carbon adhesive tape and sputter-coated with 10-nm-thick layer of gold. Delamination of the coating was viewed with a JSM-7600F microscope (JEOL, Tokyo, Japan) at accelerating voltage of 5 kV.

Detection of calcium and phosphorus atom composition was performed with EDX module attached to JSM-6300F microscope (JEOL) at accelerating voltage of 20 kV. EDX was performed on HAp nanoparticles (MKnano, Mississauga, Canada) and d-CaP coated PMMA, both of which were placed on sample stub with carbon adhesive tape.

AFM was performed on dopamine and 11-MUA activated PMMA sheet to show the irregularity and non-uniformity of the coating layer. AFM was chosen over SEM because the imaging method does not require the sample to be sputter coated with electron conducting material, such as gold, which could mask the details of the surface morphology of the object of interest. A Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA) was utilized in this experiment. Topographic images were captured in tapping mode employing monolithic silicon NCH-50 Point Probe (NanoWorld AG, Neuchatel, Switzerland). The scan size was set at 15 x 15 μ m. The surface was scanned at a rate of 1 Hz with a resolution of 512 x 512 pixels. Three-dimensional topographic images and amplitude data were obtained using Nanoscope image processing software version 5.12r5 (Digital Instruments).

Figure S2.1. Delamination of CaP coating produced by incubation in SBF. (A) Macroscopic view and SEM showing delamination of CaP coating on the taped half of the PMMA sheet. (B) EDX analysis showing low Ca/P ratio of SBF-assembled CaP coating, which might partly contribute to the weak coating attachment. (C) Another factor that might contribute to the weak attachment is the non-homogenous and irregular dopamine coating on PMMA. AFM showed patchy and aggregates of the dopamine layer on PMMA. (D) Illustrations showing combination of non-homogenous poly-dopamine layer and desorption of the CaP layers led to reduced adhesion of collagen on d-CaP surface.

Appendix C

Experiments related to immobilization of nanoparticles on PMMA cylinders

PMMA rods with 3 mm diameter and 5 cm length were purchased from Goodfellow (Huntingdon, England). The rods were washed in 70% ethanol for 15 minutes and rinsed extensively with distilled water before being dried in 37°C vacuum incubator for 24 hours. The previously optimized formulation was used to immobilize HAp nanoparticles on the rods. Briefly, 20% (w/v) needle-shaped, 60-nm HAp nanoparticles (MKnano, Mississauga, Canada) was mixed into chloroform with 5% (w/v) PMMA (MW 120,000; Sigma-Aldrich, St. Louis, MO). The mixture was then probe sonicated for 5 minutes at 50% amplification level with 5-second pause every 30 seconds to allow for gentle shaking. Immobilization of the HAp nanoparticles on PMMA rods was performed by dip coating technique using a KSV NIMA dip coater (Biolin Scientific, Stockholm, Sweden). The rod was secured with a customized clamp that was shown in the previous chapter. The lowering speed and withdrawal speed were both set at 240 mm/min. Several dipping strategies were employed to find the most optimal method:

- (A) One time dip for 60 seconds.
- (B) Three times dip for 60 seconds each with no pause at the top of each withdrawal.
- (C) Surface roughening with 1000 grit sand paper, followed by one time dip for 60 seconds.
- (D) Three times dip for 5 seconds each with no pause at the top of each withdrawal.
- (E) Six times dip for 5 seconds each with no pause at the top of each withdrawal.
- (F) Twelve times dip for 5 seconds each with no pause at the top of each withdrawal.

Following this, the coated rod was left on the dip coater for 30 minutes to allow for evaporation of the chloroform and nanoparticles settlement at the surface. The substrate was then washed with 70% ethanol, rinsed with copious amounts of distilled water, and dried in 37°C vacuum incubator overnight.

The following day, the surface of the substrates was subjected to oxygen plasma treatment to remove remnant contaminants and thin layer of PMMA that had possibly coated the HAp surface during the dip coating process. Oxygen plasma treatment was performed in a Covance multi-purpose plasma system (Femto Science, South Korea). Air was first removed with vacuum application for at least 30 minutes before the power was turned on. The pressure at the moment of plasma discharge was 0.2 Torr. The flow rate of oxygen was set at 20 sccm. The samples were treated with radio frequency oxygen plasma with 200W power for 1 minute. After the discharge stopped, the plasma treated PMMA rod was removed from the plasma chamber, washed with 70% ethanol and copious amounts of distilled water, and dried in 37°C vacuum incubator overnight before being used for further experiments.

Surface morphology of HAp nanoparticles-immobilized PMMA sheets was analyzed by SEM. In brief, the PMMA rods were mounted on a stub secured by carbon adhesive tape. The sheets were sputter-coated with 10-nm-thick layer of gold and examined with a JSM-7600F microscope (JEOL, Tokyo, Japan) at accelerating voltage of 5 kV.

Results and discussion

Surprisingly, the coating method established in the previous chapter did not work for coating a PMMA rod. No HAp nanoparticle was immobilized on the PMMA rod by using method A (**Figure S3.1**, top panel). The surface appeared smooth and featureless on SEM. I then attempted to do multiple dipping method (3 times 60 seconds each), but the coating outcome did not improve significantly (**Figure 3.1**, mid panel). Next, the surface of the PMMA rod was roughened with sandpaper so that the nanoparticles could be trapped and adhered to the grooves created by the sanding process. Sanding surface of a polymer has been shown to improve the outcomes of various coating techniques [1-3]. More HAp nanoparticles could be seen immobilized on the surface, but most surface area still appeared smooth and uncoated (**Figure 3.1**, bottom panel).

Multiple dips of PMMA rods in the coating mixture, albeit with shorter time (5 seconds for each dip), appeared to improve the immobilization of HAp nano-

Figure S3.1. Resulting HAp coating on PMMA rods from various dip coating strategies. *Method A: One time dip for 60 seconds. Method B: Three times dip for 60 seconds each with no pause at the top of each withdrawal. Method C: Surface roughening with sand paper, followed by one time dip for 60 seconds.*

particles on the surface. Method D (3 times dip for 5 seconds each) showed formation of craters on the surface and clumps of nanoparticles forming in some craters (**Figure S3.2**, top panel). Increasing number of clumps of HAp nanoparticles occupying the craters was obvious upon increasing the dipping to 6 times for 5 seconds each (**Figure S3.2**, mid panel). Low magnification SEM images, however, revealed fine surface cracks after dipping with method D and E. In contrast, these cracks were not apparent when method F was employed (**Figure S3.2**, bottom panel). The surface of the PMMA

rod was rough and resembled that shown on the surface of PMMA sheets in the previous chapter. Individual rod shaped HAp nanoparticle could easily be distinguished in the higher magnification (10,000x and 30,000x) SEM images (**Figure S3.2**, bottom panel and **Figure S3.3**). Therefore, method F, which involved 12 times dipping for 5 second each, is the most optimized strategy to immobilize HAp nanoparticles on PMMA cylinders.

Figure S3.2. Resulting HAp coating on PMMA rods after multiple short, 5-second dips.

Method D: Three times dip for 5 seconds each with no pause at the top of each withdrawal.

Method E: Six times dip for 5 seconds each with no pause at the top of each withdrawal.

Method F: Twelve times dip for 5 seconds each with no pause at the top of each withdrawal.

The reason behind the inability of the nanoparticles to be immobilized on PMMA rods using the one dip for 60 seconds method is unclear. However, it could be due to the smaller surface area and curved surface of the rod which do not provide much space for the nanoparticles to “land” and settle on. Nanoscopically, these nanoparticles constantly undergo Brownian motion in liquid medium [4,5]. With

smaller landing space and curved surface, the nanoparticles could not settle long enough to be fixated in the sub-surface of the PMMA. The multiple 5-second dips allow the HAp to settle and be fixated in the craters during the withdrawal process of dip coating and to gradually fill the craters and form layers of HAp coating during each short dipping process (**Figure 5.4**). After 12 cycles of dipping/withdrawal, the entire surface of the PMMA cylinder was finally covered with rod shaped HAp nanoparticles.

Figure S3.3. Illustrations showing the possible mechanism of HAp nanoparticles immobilization on PMMA rods by employing multiple short dips technique.

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