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ÉCOLE DOCTORALE DE CHIMIE

Par Yi-Shiang HUANG

Intraocular lenses with surfaces functionalized by biomolecules in relation with lens epithelial cell adhesion

Sous la direction de Marie-Claire Gillet et de Marie-Christine Durrieu

Soutenue le 8 Décembre 2014

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Résumé

L'Opacification Capsulaire Postérieure (OCP) est la fibrose de la capsule développée sur la lentille intraoculaire implantée (LIO) suite à la dé-différenciation de cellules épithéliales cristalliniennes (LECs) subissant une transition épithélio-mésenchymateuse (EMT). La littérature a montré que l'incidence de l'OCP est multifactorielle, dont l'âge ou la maladie du patient, la technique de chirurgie, le design et le matériau de la LIO. La comparaison des LIOs en acryliques hydrophiles et hydrophobes montre que les premières ont une OCP plus sévère, médiée par la transition EMT. En outre, il est également démontré que l'adhérence des LECs est favorisée sur des matériaux hydrophobes par rapport à ceux hydrophiles. Une stratégie biomimétique destinée à promouvoir l'adhérence des LECs sans dé-différenciation en vue de réduire le risque de développement de l'OCP est proposée. Dans cette étude, les peptides RGD, ainsi que les méthodes de greffage et de quantification sur un polymère acrylique hydrophile ont été étudiés. La surface fonctionnalisée des LIOs favorisant l'adhérence des LECs via les récepteurs de type intégrine peut être utilisée pour reconstituer la structure capsule-LEC-LIO en sandwich, ce qui est considéré dans la littérature comme un moyen de limiter la formation de l'OCP. Les résultats montrent que le biomatériau innovant améliore l'adhérence des LEC, et présente également les propriétés optiques (transmission de la lumière, banc optique) similaires et mécaniques (force haptique de compression, force d'injection de la LIO) comparables à la matière de départ. En outre, par rapport au matériau hydrophobe IOL, ce biomatériau bioactif présente des capacités similaires vis à vis de l'adhérence des LECs, le maintien de la morphologie, et l'expression de biomarqueurs de l'EMT. Les essais in vitro suggèrent que ce biomatériau a le potentiel de réduire certains facteurs de risque de développement de l'OCP.

Mots clés: Opacification Capsulaire Postérieure (OCP); lentille intraoculaire (LIO), peptide RGD; épithéliales cristalliniennes (LECs); surface fonctionnalisée

Summary

Posterior Capsular Opacification (PCO) is the capsule fibrosis developed onto the implanted IntraOcular Lens (IOL) by the de-differentiation of Lens Epithelial Cells (LEC) undergoing Epithelial-Mesenchymal Transition (EMT). Literature has shown that the incidence of PCO is multifactorial including patient's age or disease, surgical technique, and IOL design and material. Reports comparing hydrophilic and hydrophobic acrylic IOLs show the former has more severe PCO after EMT transition. Additionally, the LEC adhesion is favored onto the hydrophobic materials compared to the hydrophilic ones. A biomimetic strategy to promote LEC adhesion without de-differentiation to reduce PCO development risk is proposed. RGD peptides, as well as their grafting and quantification methods on a hydrophilic acrylic polymer were investigated. The surface functionalized IOL promoting LEC adhesion via integrin receptors can be used to reconstitute the capsule-LEC-IOL sandwich structure, which is considered to prevent PCO formation in literature. The results show the innovative biomaterial improves LEC adhesion, and also exhibits similar optical (light transmittance, optical bench) and mechanical (haptic compression force, IOL injection force) properties comparing to the starting material. In addition, comparing to the hydrophobic IOL material, this bioactive biomaterial exhibits similar abilities in LEC adhesion, morphology maintenance, and EMT biomarker expression. The in vitro assays suggest this biomaterial has the potential to reduce some risk factors of PCO development.

Keywords: Posterior capsular opacification (PCO); intraocular lens (IOL); Arginineglycine-aspartic acid (RGD); lens epithelial cells (LEC); surface functionalization

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Abstract

Cataract is the opacity of the crystalline lens or capsule of the eye, causing impairment of vision or even blindness. The cataract surgery, with damaged native lens extraction and IntraOcular Lens (IOL) implantation, is still the only currently available treatment. The materials for IOL require excellent optical properties for light transmission, mechanical properties for folding injection during surgery, and biological properties for preventing body rejection. Nowadays, the conventional materials for IOLs include PMMA (Poly(Methyl MethAcrylate)), silicone, hydrophobic acrylic and hydrophilic acrylic polymers [1]. The hydrophilic acrylic polymer, mainly composed by pHEMA (Poly(2-HydroxyEthyl MethAcrylate)), has several superior characteristics: surgeons benefit from its foldability and controlled unfolding behavior. Patients suffer less from glistening and the glare phenomenon [1]. For the manufacturers, the rigidity in dry state is helpful for easy machining. However, IOLs made from this material are prone to induce secondary cataract [2].

Secondary cataract, or Posterior Capsular Opacification (PCO), is the most common postoperative complication of the cataract surgery. PCO is a clouding of the posterior capsule by the cells forming a thick layer onto IOL and causing loss of vision again. Current treatment is using Nd:YAG laser capsulotomy to clear the cells. This method also potentially creates other complications such as damage to the IOL, higher intraocular pressure, cystoid macular oedema, and retinal detachment[1, 3]. The problem of PCO has been a challenge to scientists and ophthalmologists for decades.

Strategies to prevent PCO formation have been suggested including improvement of surgical techniques, IOL materials, IOL designs, use of therapeutic agents, and combination therapy [3]. Among these proposals, only the square-edge IOL design (a sharp-edge design preventing the cell migration onto the optic part of IOL) enters to the clinical practice and achieved significant decrease in PCO formation [4-6]. However, more and more evidence is showing that the square-edge design can only delay, rather than prevent PCO formation [7-9]. Therefore, PCO remains a challenge to the researchers and surgeons.

On the other hand, the biological basis of PCO has been investigated [10]. In the normal crystalline lens, the Lens Epithelial Cells (LECs) attach to the anterior capsule and form a monolayer. The LECs are quiescent in a contact-inhibition status. During cataract surgery, the structure is broken and the remnant LECs become active in proliferation and migrate into the space between the posterior capsule and the IOL. The LECs further undergo Epithelial-Mesenchymal Transition (EMT) and transdifferentiate to fibroblasts.

These cells express α -smooth muscle actin and secrete collagen I, III, V and VI which are not normally present in the lens. The extracellular matrix network and the overproliferated cells scatter light and lead to PCO. Another concept of tissue response to biomaterials is also suggested to explain the PCO formation[11]. The surgical trauma provokes breakdown of the blood–aqueous barrier (BAB) and the infiltration of macrophages and giant cells, further inducing foreign body reaction. These cells secrete cytokines including transforming growth factor β (TGF- β) and fibroblast growth factors (FGFs) that promote EMT. At the final stage, the fibrous encapsulation of IOL marks the end of tissue self-healing and the formation of PCO [10, 12].

In order to explore the relationship between PCO and IOL materials, clinical studies have been performed in different research groups. Although some studies show controversial results [13, 14], it has been generally accepted that the acrylic hydrophilic materials have higher PCO rate than acrylic hydrophobic ones [1-3, 15]. The molecular basis of this phenomenon has been speculated by protein adsorption behavior[16, 17]. Linnola et al. have shown that fibronectin is adsorbed more to the hydrophobic IOL than to the hydrophilic ones [18-20]. Therefore, the hydrophobic IOL can be considered as bio-sticky (i.e. stick to the capsular bag via the adsorbed proteins or via the adsorbed adhesion protein-induced cell layer mechanism) [21]. This glue effect could possibly inhibit the migration of residual LECs tending to invade the posterior capsule from the haptic-optic junction [2, 9]. In the *in vitro* culture experiments, LECs differentiation is drastically accelerated if the cells are not well attached [22]. The substrate-cell adhesion is considered as an exogenous trigger for fiber differentiation of human LECs in vitro. In this context, Linnola also proposed a "Sandwich Theory" model to control PCO [23]. In this model, a sandwich like structure of fixed LECs between the lens capsular bag and IOL can form by selecting a sticky IOL material. The LECs regain the mitotically quiescent status and diminish eventually without provoking PCO.

Our strategy of PCO control is to improve the hydrophilic IOL capsular biocompatibility by creating a higher LEC affinity surface. We recently highlighted their particular properties in terms of adhesion forces, LECs adhesion, and tissue response as indicators of posterior capsular opacification development risk [17]. In this study, we functionalized the surface of acrylic hydrophilic disks of 25% water content (HA25) with cell-adhesion peptides (RGD peptides [24]) and we characterized these surfaces using Xray photoelectron spectroscopy and contact angle measurements. To evaluate the PCO performance, the *in vitro* LEC culture was performed and compared to acrylic hydrophobic material named GF (glistening free) used here as PCO-negative reference. Additionally, in order to ensure that the surface functionalization process does not compromise the material properties, optical and mechanical properties of functionalized polymers were conducted and compared with control material.



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Glossary

- 6F-Val 4,4,4,4',4',4'-Hexafluoro-DL-valine
- BAB Blood-aqueous barrier
- CDAP 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate
- DAPI 4',6-Diamidino-2-phenylindole Dihydrochloride
- DMAP 4-Dimethylaminopyridine
- DMEM Dulbecco's modified Eagle's medium
- ECM Extracellular matrix
- EDC 1-ethyl-3-(3-dimethylaminopropyl) Carbodiimide
- EDTA Ethylenediaminetetraacetic Acid
- EMT Epithelial-mesenchymal transition
- FBS Fetal bovine serum
- FITC Fluorescein isothiocyanate
- GF Hydrophobic acrylic polymer, glistening free®
- HA25 Hydrophilic acrylic polymer, water uptake 25%
- HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- HOBt Hydroxybenzotriazole
- IOL Intraocular lens
- ISO International organisation for standardisation
- LEC Lens epithelial cells
- LECp Porcine lens epithelial cells
- MES 2-(N-morpholino)ethanesulfonate
- MTF Modulation transfer function

MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

Nd:YAG – Neodymium-Doped Yttrium Aluminium Garnet

- NEAA Non-essential amino acids
- NHS N-hydroxysuccinimide
- PBS Phosphate buffered saline
- PCO Posterior capsular opacification
- PE Poly(ethylene)
- PET Poly(ethylene terephthalate)
- PFTE Poly(tetrafluoroethylene)
- pHEMA Poly(2-hydroxyethyl methacrylate)
- PMMA Poly(methyl methacrylate)
- PP poly(propylene)
- RFGD Radio frequency glow discharging
- RGD Arg-Gly-Asp
- RGE Arg-Gly-Glu
- SD Standard deviation
- Sulfo-SDTB Sulfosuccinimidyl-4-O-(4,4'-dimethoxytrityl)butyrate
- TCPS Tissue culture-grade polystyrene
- TGFβ Transforming growth factor Beta
- WQPPRARI Trp-Gln-Pro-Pro-Arg-Ala-Arg-Ile
- lpha SMA Alpha smooth muscle actin

Chapter 1. Literature Review and Objective

1.1 Cataract

Cataract is the opacity of the crystalline lens or capsule of the eye, causing impairment of vision or even blindness (Fig. 1-1) [1, 25]. In native crystalline lens, the well-ordered arrangement of crystallin proteins confers the homogenous light transparency. The crystallins are highly concentrated in differentiated lens fiber cells and make up almost the entire composition of lens. The disruption of the crystallin proteins arrangement, mainly by unfolding, degradation, aggregation and precipitation, is the molecular basis of the opacity [26].

The factors of cataractogenesis (i.e. generation of cataract) have been investigated. In addition to the genetic basis, the main environmental factors include radiation, trauma, and aging. Inherited cataracts are found in patients with galactosaemia, Nance–Horan, and Down syndromes whereas the crucial genes (Table 1-1) in cataractogenesis are identified [27]. The cause of cataract is also found related to sunlight or UV exposure in surveys [28]. Cataract is also known induced by ocular trauma (blunt trauma to the eye globe or penetrating) [29, 30]. However, the most dominant factor of cataract is aging. In 1998, it was estimated that worldwide 19.4 million people were bilaterally blind from senile cataract [31]. Along with aging, the prevalence increases in population (Table 1-2) [32]. The current suggestion to prevent cataract is avoid UVB exposure and stop smoking [25]. However, there is yet no medicine to treat cataract [31] except surgery.

The modern cataract surgery applies a minimal traumatic method called "Phacoemulsification" (Fig. 1-2) [1]. In this technique, the ultrasound probe is introduced into the capsular sac through a small incision of 3.0–3.5 mm. The damaged hardened crystalline lens is broken into small pieces by ultrasound fragmentation. An irrigating and aspirating system eliminates the crystalline lens fragments. To restore the normal vision of the patients, the polymer-based implant called intraocular lens is further introduced into the empty capsular bag (Fig. 1-3) [1]. The intraocular lens is intended to provide light transmission and focusing as the natural lens.



Fig.1- 1 Comparison of cataract and normal eye. The cataract makes the lens opaque (upper). The light though the cataract lens is scattered (middle) and the patients have a blurred vision (lower).[1]

Locus	Gene	Protein	Mutation	Phenotype
1q21–25	GJA8	Connexin 50	Missense	Pulverulent
2q33–35	CRYG	γ-Crystallin	Pseudogene activation	Pulverulent, Coppock-like
10q24–25	PITX3	Pitx3	Missense	Total
13q11–12	CX46	Connexin 46	Missense	Pulverulent
21q22.3	CRYAA	α -Crystallin	Missense	Variable
22q	CRYBB2	β-Crystallin	Chain termination	Cerulean (blue-dot)

Table1-1 Identified gene mutations implicated in human congenital cataract [27]

Age	Prevalence (%)	
43–54	1.6	
55–64	7.2	
65–74	19.6	
75–85	43.1	

Table1- 2 Prevalence of cataract in the population of U.S. residents [32]



Fig.1- 2 Phacoemulsification: probe removing the lens nucleus [1]



Fig.1-3 Intraocular lens with different designs [1]

1.2 Posterior capsular opacification

Secondary cataract, or Posterior Capsular Opacification (PCO), is the most common postoperative complication of the cataract surgery. Posterior Capsular Opacification (PCO) is the capsule fibrosis developed onto the implanted IntraOcular Lens (IOL) by the Lens Epithelial Cells (LEC) undergoing Epithelial-Mesenchymal Transition (EMT). PCO is a clouding of the posterior capsule by the cells forming a thick layer onto IOL and causing loss of vision again (Fig. 1-4). According to the literature, the incidence of PCO is 11.8% at 1 year, 20.7% at 3 years, and 28.4% at 5 years after surgery [33].

There are two types of PCO: fibrosis and pearl (Fig. 1-5) [34]. Fibrosis-type PCO is caused by the proliferation and migration of LECs, which undergo EMT, resulting in fibrous metaplasia and leading to significant visual loss by producing folds and wrinkles in the posterior capsule [3]. Anterior LECs, which are generally mitotically quiescent (CZ region, Fig. 1-6 [35]), are considered important in pathogenesis of fibrosis PCO, because the primary types of response of these cells is to undergo fibrous metaplasia [36].

Pearl-type PCO is caused by the LECs located at the equatorial lens region (lens bow, EZ region, Fig. 1-6 [35]) causing regeneration of crystallin-expressing lenticular fibers and forming Elschnig's pearls and Soemmering's ring, responsible for most cases of PCO-related visual loss [3]. Elschnig's pearls and Soemmering's ring are ultra-structurally similar to lens fiber cells but opaque and scattering light due to a lack of the strict organization necessary for transparency. Soemmering's ring locates at the periphery of the capsular bag. In contrast, Elschnig's pearls locate in a central part when cells escaped from the periphery of the capsular bag [34].

The biological basis of PCO has been investigated [3, 37]. In the native lens, the LECs attach to the anterior capsule and form a monolayer. The LEC are quiescent in a contact-inhibition status (Fig. 1-6) [35]. During cataract surgery, the structure is broken and the residual LEC become active in proliferation and migrate into the space between the posterior capsule and the IOL (Fig. 1-7) [38]. The LEC further undergo Epithelial-Mesenchymal Transition (EMT) (Fig. 1-8) [39] and transdifferentiate to fibroblasts. These cells express α -smooth muscle actin and secrete collagen I, III, V and VI which are not normally present in the lens. The extracellular matrix network and the over-proliferated cells scatter light and lead to PCO.

Another concept of tissue response to biomaterials is also suggested to explain the PCO formation (Fig. 1-9) [11]. The host-implant interaction can be separated into several stages according to the implantation time. It is composed by injury, plasma protein adsorption, neovascularization, early chronic inflammation, foreign body reaction, and

fibrous capsule formation (Fig. 1-10) [40]. In the case of IOL implantation, the surgical trauma provokes the breakdown of the blood–aqueous barrier (BAB) and the infiltration of macrophages and giant cells, further inducing foreign body reaction. These cells secrete cytokines including transforming growth factor β (TGF- β) and fibroblast growth factors (FGFs) which promote EMT and fibroblast trans-differentiation. At the final stage, the fibrous encapsulation of IOL marks the end of tissue self-healing and the formation of PCO [10, 12].

Current treatment is using Nd:YAG laser capsulotomy to clear the cells [1, 41]. The procedure of this surgery involves focusing a Nd:YAG laser pulse to clear the visual axis by creating a central opening in the opacified posterior capsule. The applied laser energy is few milli-joules and duration is a few nanoseconds [41]. Although this is the only available treatment of PCO, however, this also potentially creates other complications such as damage to the IOL, higher intraocular pressure, cystoid macular oedema, and retinal detachment [1, 3].





Fig.1-4 Scheme and photo of PCO

Original figure legend from Ref [37]: A schematic representation of (A) the post-surgical capsular bag and (B) the extensive growth and modification that gives rise to PCO. (C) A dark-field micrograph of a capsular bag removed from a donor eye that had undergone cataract surgery prior to death that exhibits light scattering regions beneath an IOL.



Fig.1- 5 Fibrosis (left) and pearl (Elschnig, right) type of PCO [34]



Fig.1- 6 Lens epithelial cells inside the native lens capsule [35]



Fig.1- 7 Development of posterior capsular opacification [38]. After cataract surgery, the original structure of the lens epithelial cells at anterior capsular bag is destroyed. The residual lens epithelial cells migrate to the space between the posterior capsular bag and the intraocular lens





Downregulation of E-cadherin, ZO1 Cell-cell junction dissolution

Loss of apical-basolateral cell polarity

Actin reorganization

Upregulation of metalloproteases Increased deposition of extracellular matrix proteins

Migration and invasion

Fig.1-8 Epithelial-mesenchymal transition [39]



Fig.1-9 Biological reaction against an implanted IOL [11]

Original figure legend from Ref [11]: Foreign body reaction is mediated by macrophages (A) and foreign body giant cells generated through a fusion of many macrophages (B). Wound healing reaction occurs in lens epithelium. The equatorial region of the capsular bag is occupied by regenerated lenticular fibers of Soemmering's ring (C). Lens epithelial cells on the posterior capsule exhibit an elongated, fibroblast-like, shape (D).



Fig.1-10 Timeline of Host-Implant reaction [40]

1.3 Strategies to prevent PCO

PCO is known to be multifactorial: the incidence can be influenced by the patient's age or disease, surgical technique, and IOL design and material [42]. Research scientists and ophthalmologists worldwide have been attempted to alleviate PCO development. Strategies to prevent PCO formation have been suggested including improvement of surgical techniques, IOL materials, IOL designs, use of therapeutic agents, and combination therapies [3].

The improvement of the surgery technique is mainly focused on the removal of LEC at the time of lens extraction, which includes aspiration of the anterior capsule using an extensive irrigation/aspiration system during cataract surgery, pharmacological dispersion and aspiration of the anterior capsule, and manual polishing of the anterior and/or posterior capsule [3]. The proposed techniques removing anterior or include posterior capsule have been reported to delay but not to eliminate PCO, for the reason that PCO is mainly caused by germinative LECs in the equatorial region rather than the displaced metaplastic LECs already on the posterior capsule [3]. Hydrodissection, injection of saline fluid stream in-between the capsular bag and lens which facilitates the removal of retained cortical material and LECs, shown to be important for PCO prevention [43]. However, it does not completely eliminate LECs.

The research to the therapeutic agent is mainly focused on the selectively destroying residual LECs without causing toxic effects to other intraocular tissues. The routes of administration can be direct injection into the anterior chamber, addition to the irrigating solution, impregnation of the IOL. In the in vitro tests, the applied hypoosmolar agents and antimetabolites include pirenoxine (Catalin™)[44], methotrexate (dihydrofolate reductase inhibitor), mitomycin (DNA crosslinker), daunomycin (Intercalating DNA), and fluorouracil (thymidylate synthase inhibitor) [45]. The other studies tested cytotoxic and therapeutic agents, including diclofenac sodium (nonsteroidal antiinflammatory drug), saporin (inhibiting ribosome activity), thapsigargin (inhibiting the fusion of autophagosomes with lysosomes and leading cell death), salmosin (venom that interacts with integrin $\alpha_v \beta_3$ and inhibits cell adhesion and proliferation), minoxidil (inhibitor of lysyl hydroxylase involved in collagen formation), a matrix metalloproteinase inhibitor (llomostat) (preventing cell migration), and cyclo-oxygenase 2 inhibitors (controlling inflammation) [3]. Another *ex-vivo* study suggests actinomycin D (inhibiting cell transcription) may prevent PCO formation [46]. Recently, the immunosuppressant compound rapamycin (Fig. 1-11) (also known as Sirolimus) is suggested to prevent PCO by in vitro [47, 48]. Unfortunately, a wide range of pharmacological agents, cytotoxic as well as therapeutic agents, have shown the

potential to prevent PCO *in vitro* but exhibit toxic effects to the nearby ocular tissues *in vivo* [3]. The lack of selectivity currently limits their clinical use.

Scientists are also devoted to reduce PCO by developing IOL materials and designs. PCO was regarded as an inevitable consequence of lens implant surgery until 1993, when the clinical trial of the hydrophobic acrylic IOL (Acrysof IOL MA series, Alcon Laboratories) was conducted [42]. In 2000, Nishi and his colleagues proposed a PCO development prevention method by square-edge IOL design (Fig. 1-12), which involves the sharp-edge of IOL inhibiting the cell migration to the optic part along lens capsule [49]. The square-edge IOL is later improved with 360° design (Fig. 1-13) to prevent cell migration via haptic-optic junction and achieved significant decrease in PCO formation [4-6]. Recently, adhesion–preventing ring designs have been proposed to inhibit PCO by separating the anterior and posterior capsular flaps which allows aqueous humor to circulate in and out of the capsular bag and leads LEC proliferation inhibited [50, 51]. However, the biological mechanism of these designs in PCO inhibition is yet to be investigated. In addition, adhesion of the IOL material with the lens capsule also plays a role in PCO prevention by creating a sharp capsular bend, which inhibits LEC migration onto the posterior capsule [3]. Therefore, a sticky material may help to reduce PCO.

Among these proposals, only the square-edge (or sharp-edge) IOL design enters to the clinical practice and achieved significant decrease in PCO formation [4-6]. The sharp-edge optic IOL and the formation of a capsular bend are highly effective in reducing PCO by preventing the cell migration onto the optic part of IOL (Fig. 1-12) [5]. However, more and more evidences show that the posterior square-edge design can only delay, rather than prevent PCO formation [7-9]. Nowadays, it is generally accepted that the square edge design and hydrophobic acrylic material are relative better choice to eliminate PCO. PCO is, however, still a challenge to scientists and ophthalmologists.



Fig.1-11 Chemical structure of rapamycin



Fig.1- 12 Principle of square-edge design to prevent LEC migration toward optic part of IOL [5] The sharp-edge optic IOL forms a capsular bend and further prevents LEC migration from the side (right side, space between the posterior and the anterior capsule) to the center of vision field (left side, space between the posterior and the optic).



Fig.1-13 Example of 360° design square-edged IOL (Concept 360)

Original figure legend from Ref [34]: (a) Schematic drawing showing the design of the lens. The overall design is that of a disc-shaped lens with the appearance of a propeller; the space between the haptic components will decrease as a function of the diameter of the capsular bag. (b) Gross photograph of the lens experimentally implanted in a cadaver eye.

1.4 Relationship between PCO and IOL materials

The materials for IOL require excellent optical properties for light transmission, mechanical properties for folding injection during surgery, and biological properties for preventing unfavored body reaction. Biocompatibility is generally accepted as the ability of the biomaterials or medical devices to perform specific functions with appropriate host response [12]. For IOLs, the biocompatibility can be assessed in terms of uveal and capsular compatibility, which are the inflammatory foreign-body reaction of the eye against the implant and the relationship of the intraocular lens with remaining LECs within the capsular bag, respectively [52]. In acrylic materials, the hydrophilic acrylic materials are superior to hydrophobic materials in uveal aspect, which is inversely related to inflammation [15]. However, in capsular compatibility, hydrophilic is considered less capsular biocompatible, which is related to the higher PCO incidence [53].

Nowadays, the commercial materials for IOLs include PMMA (Poly(Methyl Methacrylate)), silicone, hydrophobic acrylic and hydrophilic acrylic polymers (Fig. 1-14) [1]. The hydrophilic acrylic polymer, mainly composed by pHEMA (Poly(2-hydroxyethyl methacrylate)), has several superior characteristics (Fig. 1-15) [1]. Surgeons benefit from its foldability and controlled unfolding behavior. Patients suffer less from glistening and the glare phenomenon [1]. For the manufacturers, the rigidity in dry state is helpful for easy machining. However, IOLs made from this material are prone to induce PCO. Therefore, it will be beneficial to improve the capsular biocompatibility of hydrophilic acrylic materials.

In order to explore the relationship between PCO and IOL materials, clinical studies have been performed in different research groups. Although some studies show controversial results [13, 14], it has been generally accepted that the hydrophilic acrylic materials have higher PCO rate than acrylic hydrophobic materials [1-3, 15]. The molecular basis of this phenomenon has been speculated by protein adsorption behavior[16, 17]. Linnola et al. show that fibronectin is adsorbed more to the hydrophobic IOL than to the hydrophilic ones [18-20]. Therefore, the hydrophobic IOL can be considered as bio-sticky (i.e. stick to the capsular bag via the adsorbed proteins or via the adsorbed proteininduced cell layer mechanism) [21]. This glue effect could possibly inhibit the migration of residual LEC tending to invade the posterior capsule from the haptic–optic junction [2, 9]. In addition, in the *in vitro* culture experiments, LECs differentiation is drastically accelerated if the cells are not well attached [22]. The substrate-cell adhesion is considered as an exogenous trigger for lens fiber cell differentiation of human LEC *in*
vitro. In this context, Linnola also proposed a "Sandwich Theory" model to control PCO (Fig. 1-16) [23]. In this model, a sandwich-like structure of fixed LEC between the lens capsular bag and IOL can form by selecting a sticky IOL material. The LECs regain the mitotically quiescent status and regress eventually without provoking PCO.



Fig.1-14 Common materials for IOL fabrication [1]

IOL type	Advantages	Disadvantages
Poly(MMA)	Long-term experience Good biocompatibility Low cost	Rigid, which needs large incisions Pits after YAG laser treatment High incidence of PCO
Silicone	Foldable – small incision Fairly low incidence of PCO (partial incidence for the second generation) Second generation of silicone with higher refractive index, thus thinner IOLs	Low refractive index of the first generation of silicone, thus thicker IOLs Pits after YAG laser treatment Rapid unfolding in the eye Dislocation after YAG laser treatment More decentration More Anterior Capsule Opacification Slippery when wet Cannot be used with silicone oil
Hydrophobic acrylic	Foldable – small incision High refractive index Very low incidence of PCO LEC regression Biocompatible Fewer pits after YAG laser treatment Slow controlled unfolding	Short experience Tacky surface – sticks to forceps, which is worse at higher temperature Difficult folding because of higher Tg Glistening Glare
Hydrophilic acrylic, hydrogel	Foldable – small incision Good biocompatibility – low inflammatory cell reaction Fewer pits after YAG laser treatment Controlled unfolding Less endothelial cell damage with corneal touch	LECs adhesion on the anterior IOL surface High incidence of PCO Possible calcification

Fig.1-15 Comparison of IOL materials [1]



Fig.1-16 Sandwich model of PCO control [23]

Original figure legend from Ref [23]: The sandwich theory: Phase 1. Schematic picture of an IOL with optic made of bioactive material in the capsular bag. The anterior capsule over the bioactive surface of the IOL bonds directly or through the remaining lens epithelial cells to the IOL. The IOL and the capsular bag have formed a closed system. Inside the bag the remaining lens epithelial cells proliferate and migrate behind the IOL. Phase 2. The bioactive bond is formed when a single lens epithelial cell has the posterior capsule on one side and the bioactive IOL surface on the other side. The sandwich is formed and the cell-posterior capsule junction and the cell-bioactive IOL surface junction prevent more cells from migrating behind the IOL. The posterior capsule remains clear. Phase 3. After some time, a part of these monolayer cells die. The reason may be aging, lack of nutrition or the pressure of the IOL against the posterior capsule. A true contact with the IOL and the posterior capsule is formed in these places.

1.5 Surface modification of IOL

Surface functionalization is the act of modifying the surface of a material by bringing physical, chemical or biological characteristics different from the ones originally found on the surface of a material. Methods for polymer surface modification includes high energy irradiation, polymer composite / blend, polymer coating, incorporation of ECM proteins, chemical modifications, and photochemical modifications (Fig. 1-17) [54].

Surface modification of the IOL to prevent PCO is considered as a simple and safe method because it requires no manipulation within the eye and no application of harmful agents during IOL implantation [3]. Bio-passive components such as COO⁻/SO₃⁻ functional groups [55], titanium [56], heparin[57], PFTE/fluorocarbon [58], poly(ethylene-glycol) and its derivatives [59-61] have been suggested to be generated, grafted, or coated onto the IOL materials to reduce the cell adhesion or inflammatory reaction. Recently, bio-active substances including anti-TGFβ2 antibody (as an inhibitor of EMT)[62], sulfadiazine (as a mimic of matrix metalloproteinases inhibitors, Fig. 1-18) [63], and 5-fluorouracil (an antimetabolite drug, Fig. 1-19) [64] have been proposed to immobilize for blockage of the processes involved in PCO. Despite applied similar surface functionalization technologies, another strategy can be considered: functionalize the biomaterial surface by proteins or bioactive molecules to improve cell adhesion (Fig. 1-20 and Fig. 1-16D) [54]. More specifically, use bio-active molecule to create a bio-adhesive IOL material to aid tissue repair and regenerate the native monolayer structure of LECs (Fig. 1-21).



Fig.1-17 Methods for polymer surface modification [54]

Original figure description from Ref [54]: To achieve a surface chemistry that would remain stable in biological environments the covalent grafting of functional groups is preferred over physical coating (adsorption only). The surface stability achieved via chemical modification is much higher than that obtained by the physical adsorption of the same biomolecules.



Fig.1-18 Chemical structure of sulfadiazine



Fig.1-19 Chemical structure of 5-fluorouracil



Fig.1- 20 Biomaterial surface modification for improved Cell/ Protein/Growth Factor immobilization. [54]



Fig.1- 21 Strategy of bio-active molecule to regenerate LEC native monolayer: use of biomolecule to attract cell adhesion. The biomolecule coated hydrophilic acrylic intraocular lens exhibits a bio-adhesive surface attracting thin cell layer formation and could tightly bind to the capsular bag via the cells, which diminish the space for potential over-proliferation of cells leading secondary cataract. (Adapted from Intraocular Lenses: Evolution, Designs, Complications, and Pathology. Baltimore, Williams & Wilkins 1989)

1.6 Biofunctional RGD peptide

In order to create a bio-active IOL via surface modification, it is critical to select a biomolecule that promote lens epithelial adhesion. As introduced in Section 1.4, fibronectin is known adsorbed more to the hydrophobic IOL than to the hydrophilic ones [18-20] and the RGD peptide is the minimal domain to attract cell adhesion found on fibronectin [65]. Therefore, it is worthy to explore the possibility of functionalization of these biomolecules on hydrophilic acrylic polymers and evaluate this design in PCO control.

Among all the major components in the body, proteins plays important role for their versatile structures and functions. The building units of proteins are amino acids and the minimal functional blocks are peptides. Scientists have already investigated series peptide sequences as scaffolds for tissue engineering purpose (Table 1-3) [66]. On the other hand, the extracellular matrix (ECM) proteins are secreted by the cells and play crucial role in intracellular communications, which are desired for host-implant integration in tissue engineering field. The study of peptide sequences of ECM proteins provides chances for scientists to design implants with better biocompatibility (Table 1-4) [67].

Comparing to proteins, use of peptides to functionalize the polymers take more advantages. Firstly, the peptides can be synthesized chemically whereas proteins are usually obtained by living organism. Purification and characterization of the target proteins makes the process complicated and expensive. In addition, endotoxin is needed to remove completely in a bacterial expression system to avoid Systemic Inflammatory Response Syndrome (SIRS) and sepsis [68]. Secondly, the accessibility and the specificity of the target molecule are important. The protein may comprise more than one domain and scientists may only use one domain of the protein to perform specific biological function. Therefore, existence of other domains may cause unwanted or even adverse effect. In addition, the steric effect of the bulk volume from other domains may prevent the interaction between desired domain and its target. Thirdly, the peptides have less chance to induce host immune response than proteins. Finally, the stability of peptide is much higher than the proteins. Stability, including thermal, chemical, structural and proteolytic aspects, is the key factor of the successful functionalized biomaterials. A portion of proteins are thermal and chemical sensitive, and easily denatured and degraded inside the body. Therefore, a biomaterial functionalized with proteins is needed replenish or renew functional proteins frequently, which is extremely difficult for the implanted biomaterials. In addition, the hydrolyzed protein fragment may

further induce the immune system response [65]. Therefore, to functionalize the intraocular lens, it seems better to consider applying a peptide rather than a protein.

The RGD (Arg-Gly-Asp) sequence was firstly reported by Pierschbacher and Ruoslahti in 1984 [69] . Some of the major glycoproteins in bone matrix also contain the amino-acid sequence Arg-Gly-Asp (RGD) which conveys the ability of the ECM protein to bind to the integrin class of cell surface receptors (Fig. 1-22) [65]. The bone matrix contains as long list of RGD-containing glycoproteins (Collagen(s), thrombospondin, bone sialoprotein, fibronectin, vitronectin, fibrillins, and dentin matrix protein 1). This three-residue peptide sequence specifically is recognized by integrins onto the cell surface. Through RGD – integrin binding, the cells are guided to adhere onto the surface and start to form focal adhesion and actin stress fiber organization (Fig. 1-23) [65]. Cell adhesion shows a sigmoidal increase when presented against RGD surface density with a critical minimum required for cell response (Fig. 1-24) [70].

The RGD peptide has been suggested to specifically and safely promote cell adhesion. First, the RGD sequence is the functional motif of fibronectin [18-20], which is found to be adsorbed abundantly onto hydrophobic IOL. Surface functionalization by RGD peptide is a bio-mimic strategy to restore the LEC monolayer structure. In addition, the RGD modification method has been widely proposed in orthopedic, cancer diagnostic and therapy research fields [71-74]. Therefore, the fundamental safety profile of the peptide has been constructed from basic researches. Moreover, although the RGD based drug Cilengitide failed in efficacy evaluation in Phase III [75], the safety tests of this peptide have already been passed in the clinical trial. From the current information, it is speculated that the RGD peptide can promote LEC adhesion without causing toxic effects.

Functionality	Peptide	Source protein
Heparin- binding domains	FAKLAARLYRKA	Antithrombin III
Cell–matrix interaction domains	RGD	Multiple extracellular matrix proteins
	REDV	Fibronectin
	IKVAV	Laminin
	YIGSR	Laminin
Cell–cell adhesion domains	GRALARGEANF	Neural cell adhesion molecule
	DWVIPPISCPENEKGPFPKNLVQIKSNRDK	E-cadherin
Structural domains	GGRPSDSYGAPGGGN	Resilin
	VPGXG	Elastin
	GAGAGS	Silk fibroin
Crosslinking domains	SKGPG and VPGQG	Tissue transglutaminase binding domain
Signaling domains	CDEHYYGEGCSVFCRPR	hDelta 1 (Notch signaling pathway)
Inorganic binding domains	R5 peptide	Silaffin
	RHTDGLRRIAAR	Tra1 copper-binding protein
Degradation domains	Variable	Matrix Metalloproteinase cleavage site
	GTAR, TSHR, DRIR	Tissue plasminogen activator cleavage sites
	DNRR, FFSR, SILR	Urokinase plasminogen activator cleavage sites
Tal	ole1- 3 Representative Peptides Used	in Modular Protein-Engineered
	Biomaterials [66]	

Synthetic sequences	Origin	Function
RGD	Fibronectin, Vitronectin	Cell adhesion
KQAGDV		Smooth muscle cell adhesion
YIGSR	Laminin B1	Cell adhesion
REDV	Fibronectin	Endothelial cell adhesion
IKVAV	Laminin	Neurite extension

RNIAEIIKDI	Laminin B2	Neurite extension
KHIFSDDSSE	Neural cell adhesion molecules (NCAM)	Astrocyte adhesion
VPGIG	Elastin	Enhance elastic modulus of artificial ECM
FHRRIKA	Heparin binding domain	Improve osteoblastic mineralization
KRSR	Heparin binding domain	Osteoblast adhesion
NSPVNSKIPKACCVPTELSAI	BMP-2	Osteoinduction
APGL		Collagenase mediated degradation
VRN		Plasmin mediated degradation
ΑΑΑΑΑΑΑ		Elastase mediated degradation
WQPPRARI	Heparin binding domain	formation of cell focal adhesion

Table1- 4 Peptide sequences of ECM proteins used in tissue engineering applications adapted from [67]



Fig.1-22 RGD peptide structure [65]



spread cell --> survival, proliferation



Fig.1-23 RGD peptide target: cell surface receptor integrin [65]



Fig.1- 24 Immobilized RGD peptides promote cell adhesion in different surface density [70]

Original figure legend from Ref [70]: SEMs of adherent human foreskin fibroblast cells on substrates containing covalently grafted GRGDY indicating the classification into types I-IV. Cells adherent on substrates with varying surface concentration of peptide were scored according to the four morphological types represented here (A) type I, spheroid cells with no filopodial extensions; (B) type II, spheroid cells with one to two filopodial extensions; (C) type III, spheroid cells with greater than two filopodial extensions (D) type IV, flattened morphology representative of well spread cells.

1.7 Bioconjugation

As introduced in section 1.5, the design is to use bio-active molecule to create a bioadhesive IOL material to help the tissue repair and regenerate LEC native monolayer structure. On the other way, the advantage of RGD peptide has been introduced in section 1.6. Therefore, the RGD peptide is selected as candidate to be immobilized onto the IOL surface.

On the other hand, the type of RGD peptide linkage onto the HA25 polymer is important. Non-covalent adsorption is sometimes appropriate for drug delivery application [76]. However, in our case, covalent bonding formation is more desirable for several advantages including higher stability [54], better cell adhesion promoting ability [77], and lower potential of uncontrolled desorption of RGD peptide in physiologic environments [78]. Therefore, it is preferred to graft the RGD peptides on targeting polymer.

From the literature, common functional groups in bioconjugation chemistry design include aldehydes, carboxylic acids, hydroxyls, thiols, and primary amines. Scientists have proposed numerous crosslinking agents for bioconjugation (Fig. 1-25) [76]. The selection of chemistry is generally based on the species of biomolecule and polymer, temperature, pH value, as well as the intended application. For certain biological applications, the steric effect is critical to the bioactivity. In such cases, the introduction of with spacer with several angstroms could decrease the stereo hindrance and provide a better bioactivity. In addition, the conversion of functional groups for further conjugation is also considered (Fig. 1-26) [76]. The oxidation, reduction, as well as chemical deviation may also ensure proper coupling between targeted functional groups.

In the present case, all the peptides have amine and carboxyl group in N terminus and C terminus. On the other hand, the hydroxyl group is available on the polymer surface. Therefore, design the bioconjugation chemistry could start from these three functional groups. The functional groups can be either activated and coupled directly via crosslinking reagents, or conversion hydroxyl group into carboxyl group and then followed by amide bonding formation between amine and carboxyl group can be considered.

Cross-linking agent		Abbr.
(a) Reagents to cross-link carboxylic acids and	d primary amines ^a	
1-Ethyl-3-(3-dimethylaminopropyl) carbodiim	ide	EDC
1-Cyclohexyl-3-(2-morpholinoethyl) carbodiin Disudahamul aasha diimida	nide	CMC
Disopropyl carbodiimide		DIC
N-ethyl-3-phenylisoxazolium-3'-sulfonate		Woodward's
		reagent K
N, N'-carbonyldiimidazole 4-(n-Azidosalicylamido) butylamine		CDI ASBA
4-(p-Azidosancylannido) butylannine		ASDA
(b) Reagents to cross-link thiols and thiols ^a 1.4 Di [2/2/ pyridyldithio)propionemidalbuta	24	DDDDD
Bismaleimidohexane	lie	BMH
1,5-Difluoro-2,4-dinitrobenzene		DFDNB
4,4'-Difluoro-3,3'-dinitrophenylsulfone		DFDNPS
N, N-ethylene-bis(iodoacetamide)		n/a n/a
N,N'-undecamethylene-bis(iodoacetamide)		n/a
α,α'-Diiodo-p-xylene sulfonic acid		n/a
tri(2-Chloroethyl)amine		TCEA
(c) Reagents to cross-link primary amines and	l primary amines ^a	
Glutaraldehyde		GL
Formaldehyde Dithio <i>hie</i> (succinimidylpropionate)		n/a DSP
3.3'-Dithio <i>bis</i> (sulfosuccinimidylpropionate)		DTSSP
Disuccinimidyl suberate		DSS
bis(Sulfosuccinimidyl)suberate		BS3
Disuccinimidyl tartarate		DST ⁰ BEOCOFE ^b
Ethylene glycol <i>bis</i> (succinimidylsuccinate)	ne	EGS ^b
Disuccinimidyl glutarate		DSG
N,N'-Disuccinimidyl carbonate		DSC
Dimethyl adipimidate		DMA
Dimethyl pimelimidate		DMP
Dimethyl 3,3'-dithiobispropionimidate		DTBP
N-Hydroxysuccinimidyl-4-azidosalicylic acid		NHS-ASA ^{b,c}
Sulfosuccinimidyl-2-(p-azido-salicylamido)ethy	/l-1,3'-dithiopropionate	SASD
N-Hydroxysuccinimidyl-4-azidobenzoate	olhavanaata	HSAB ^o SANDAH ^b
N-5-azido-2-nitrobenzovloxysuccinimide	ojnexanoare	ANB-NOS
Sulfosuccinimidyl-2-(m-azido-o-nitrobenzamid	o)-ethyl-1,3'-dithiopropionate	SAND
N-Succinimidyl(4-azidophenyl)-1,3'-dithiopropionate		
Sulfosuccinimidyl-4-(p-azidophenyl)butyrate	Sulfo-SAPB	
Sulfosuccinimidyl-2-(7-azido-3-methyl coumaria-	Sulfo-SAMCA	
p-Nitrophenyl diazopyruvate		
(d) Reagents to cross-link thicks and primary	amines ^a	
N-Succinimidyl 3-(2-pyridyldithio)propionate	annes	SPDP ^{c,d}
4-Succinimidyloxycarbonyl-a-methyl-a-(2-pyri	dyldithio)toluene	SMPT
Succinimidyl 4-(N-maleimidomethyl)-cyclohex	ane-1-carboxylate	SMCC ^b
m-Maleimidobenzoyl-N-hydroxysuccinimide e N-Succinimidyl(4-iodoacetyl)-aminobenzoate	ster	MBS ^o SIAB ^b
Succinimidyl 4-(p-maleimidophenyl)butyrate		SMPB ^b
N-\gamma-Maleimidobutyryl-oxysuccinimide ester		GMBS ^b
Succinimidyl 6-[6-(((iodoacetyl)amino)-hexand	yl) amino]hexanoate	SIAXX
Succinimidyl 6-[(iodoacetyl)-amino]hexanoate		SIAX
Succinimidyl 6-((((4-(iodoacetyl)amino)methyl	-cyclohexane-1-carbonyl)amino)hexanoate	SIACX
Succinimidyl 4-((iodoacetyl)amino)methyl)-cyd	clohexane-1-carboxylate	SIAC
p-Nitrophenyl iodoacetate		NPIA
1-(p-Azidosalicylamido)-4-(iodoacetamido)but	ane	ASIB
/v-[4-(p-azidosalicylamido)butyi]-3 -(2 -pyridyl	ditnio)propionamide	APDP
Functional targets	Cross-linking agent	Abbr.
(e) Additional cross-linking reagents ^a		
Nucleophile and nucleophile	1,4-Butanediol diglycidyl ether	n/a
Carbonyl and carbonyl	Adipic acid dinydrazide Carbohydrazide	n/a n/a
Phenol and primary amine	Formaldehvde	n/a
Hydroxyl and primary amine	N,N'-Carbonyldiimidazole	CDI
	N,N'-Disuccinimidyl carbonate	DSC
Primary amine and reactive hydrogen	p-Nitrophenyl-2-diazo-3,3,3-triffuoropropionate	PNP-DTP
Thiol and reactive hydrogen	Benzophenone-4-iodoacetamide	n/a
	Benzophenone-4-maleimide	n/a
Thiol and carbonyl	4-(4-N-Maleimidophenyl)butyric acid hydrazide hydrochloride)	MPBH
	4-(N-Maleimidomethyl)cyclohexane-1-carboxyl-hydrazide	M_2C_2H
	nydrocniofide 3-(2-Pyridyldithio) propionyl hydrazide	РПРИ
	5-(2-1 yridyununo) propionyr nydrazide	r Dr ff

Fig.1-25 crosslinking reagents for bioconjugation [76]

Functional conversion	Reagent	Abbr.
Primary amine to carboxylic acid	Succinic anhydride	n/a
	Glutaric anhydride	n/a
	Maleic anhydride	n/a
Primary amine to thiol	N-succinimidyl S-acetylthioacetate	SATA
	Succinimidyl acetyl-thiopropionate	SATP
	Succinimidyloxycarbonyl-alpha-methyl-alpha-(2-pyridyldithio)toluene	SMPT
	S-acetylmercaptosuccinic anhydride	SAMSA
Primary amine to aldehyde	Glutaraldehyde	GL
Carboxylic acid to primary amine	Cystamine	n/a
	Ethylenediamine	n/a
Thiol to primary amine	Bromoethylamine	n/a
	Aminoethyl-8 TM	n/a
	Ethylenimine	n/a
Aldehyde to primary amine	Ammonia	n/a
	Ethylenediamine	n/a
Aldehyde to thiol	2-acetamido-4-mercaptobutyric acid hydrazide	AMBH
Phosphate to primary amine	Cystamine	n/a
	Ethylenediamine	n/a
Hydroxyl to carboxylic acid	Chloroacetic acid	n/a
Carbonyl to thiol	3-(2-pyridyldithio) propionyl hydrazide	PDPH
Thiol to carboxylic acid	Iodoacetate	n/a

Fig.1- 26 reagents modifying functional groups for bioconjugation [76]

Chapter 2. Materials and Methods

Part I: Material preparation and characterization (section 2.1-2.5)

2.1 Materials

Cross-linked hydrophilic acrylic polymer of water uptake 25% (HA25, Fig. 2-1) was obtained from Benz Research and Development (BRD, Sarasota, USA), and the cross-linked hydrophobic acrylic polymer of Glistening-Free® (GF) polymer was obtained from PhysIOL (Liège, Belgium). Disks were diamond-turned and milled down to thickness of 1 mm and 16 mm in diameter then were treated according to procedure typically applied for IOL manufacturing. The HA25-based IOL were obtained from PhysIOL. KRGDSPC peptide (denoted as RGD, Fig. 2-2), KRGESPC peptide (denoted as RGE, Fig. 2-3), and fluorescein-labeled KRGDSPC peptide (denoted as FITC-RGD, Fig. 2-4), WQPPRARI (denoted as WQPPRARI, Fig. 2-5) were purchased from Genecust (Luxembourg) (purity > 95%). Molecule 4,4,4,4',4',4'-Hexafluoro-DL-valine (denoted as 6F-Val, Fig. 2-6)was purchased from Sigma-Aldrich, Belgium (759317). The chemical parameters of the peptides are shown in Table 2-1.



Fig.2- 1 Schematic representation of virgin HA25 polymer. The HA25 polymer is a copolymer mainly composed of poly(2-hydroxylethyl methacrylate) (~75%) and poly(2-ethoxylethyl methacrylate) (~25%).



Fig.2- 2 Chemical structure of KRGDSPC peptide (denoted as RGD peptide)



Fig.2- 3 Chemical structure of KRGESPC peptide (denoted as RGE peptide)



Fig.2- 4 Chemical structure of FITC-KRGDSPC peptide (denoted as FITC-RGD peptide)



Fig.2-5 Chemical structure of QWPPRARI peptide

$$F_3C$$
 H_2 H_2 $CF_3 O O H$ H_2 $CF_3 O H$ H_2 $CF_3 O H$ H_2 $CF_3 O H$ H_2 $CF_3 O H$ H_2 H_2

Fig.2- 6 Chemical structure of 4,4,4,4',4',4'-Hexafluoro-DL-valine (denoted as 6F-Val)

Name	RGD	RGE	FITC-RGD	WQPPRARI	6F-Val
Sequence	KRGDSPC	KRGESPC	FITC-	WQPPRARI	V
			KRGDSPC		
Molecular	761.85	775.88	1264.4	1023.22	225.09
weight					
Molecular	C29H51N11O11S1	C30H53N11O12S1	C56H73N13O17S2	C47H74N16O10	C5H5N1O2F6
Formula					
С%	56	64	64	64	36
N%	21	15	15	22	7
0%	21	19	20	14	14
S%	2	2	2	0	0
F%	0	0	0	0	43
рІ	8.97	8.97	N/A	12.4	N/A

Table2- 1 Chemical parameters of all the synthetic peptides/molecules and their theoretical elemental percentages in XPS

2.2 Peptide immobilization onto HA 25

In order to create a robust and lasting immobilization of the peptide, a covalent bond formation between the peptide and the polymer is an ideal solution. In order the achieve this, strategically selection of conjugating reactions according to the available functional groups are needed. As shown in section 2.1, the hydrophilic acrylic polymer HA25 (Fig. 2-1) is rich in hydroxyl group whereas the peptides have amine and carboxylic acid in common. Therefore, five methods were selected to test the performance of peptide immobilization and cell adhesion promotion.

In addition, since the project is surface functionalization of IOL material to prevent PCO, the functionalized material must at least share the same or similar optical properties in light transmission, mechanical properties for IOL injection, tissue response and cytocompatibility with the native IOL material.

IOL samples made by HA25 were specifically used in optical (optical bench measurement) and mechanical (haptic compression force, IOL injection force) properties analysis. Disk samples made by HA25 were used as models for all the other tests.

2.2.1 Tresyl chloride method

This method is based on the work of Hubbell's team to graft RGD peptide onto the polymer surface (Fig. 2-7) [79]. The HA25 disks were rinsed with deionized water before an overnight air drying. Dried disks were then activated in the solution of 20 mL dry ether containing 40 μ L of 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride, 324787, Sigma-Aldrich, Belgium) and 2 mL of triethylamine for 15 minutes at room temperature. Activated disks were then rinsed with 0.2 M sodium bicarbonate pH 10 buffer and placed in the same buffer containing increased concentrations of RGD peptide overnight at room temperature to make covalent linkage. After peptide treatment, the disk cleaning process is Aqueous Washes (section 2.3). In order to verify the efficiency, a fluorescent molecule fluorescein amine (Fig. 2-8, 46930, Sigma-Aldrich, Belgium) is test for coupling in the same condition. The activation and the rinse steps of the tested disks is exactly the same, whereas in the conjugation step, the solution of peptide is replaced by fluorescein amine at the same concentration.



Fig.2-7 Tresyl chloride mediated peptide grafting reaction [79]



Fig.2-8 Chemical structure of fluorescein amine

2.2.2 Permanganate oxidation – EDC/NHS method

This method is based on the work of Durrieu's team to graft RGD peptide onto the polymer surface (Fig. 2-9) [80]. The HA25 disks were rinsed with deionized water to remove the salt from the biological buffer. Disks were then oxidized in the solution of 0.15 g KMnO₄ dissolved 3 mL 1.2N H₂SO₄ by shaking 1 hr at 60 °C. After the reaction, the disks were washed with in 6N HCl for 1hr at 60 °C and then deionized water for 18hr at 60 °C with continuous shaking. To make the carboxylic acid activated for peptide conjugation, the samples were immersed into the coupling solution containing 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) 150 mM, N-hydroxysuccinimide (NHS) 100 mM, and 2-(N-morpholino)ethanesulfonate (MES) 100 mM at 4°C overnight. After rinsing by MilliQ, the samples were conjugated with peptides by incubation with 1 mM peptide solution for 24 hours at room temperature. After peptide treatment, the disk cleaning process is Aqueous Washes (section 2.3).



Fig.2-9 Permanganate oxidation and EDC/NHS coupling reaction

2.2.3 CDAP method

This method is based on the work of Warsinke's team to graft enzyme onto the polymer surface (Fig. 2-10) [81]. A CDAP stock solution was made by dissolving 50 mg CDAP (1-Cyano-4-dimethylaminopyridinium tetrafluoroborate, 28390, Sigma-Aldrich) in 1 mL acetonitrile. The HA25 disks were rinsed with deionized water to remove the salt from the biological buffer. A 2200 μ L activating solution made from 0 to 400 μ L CDAP stock solution, 200 μ l 0.2M triethylamine, and 2000 to 1600 μ l MilliQ water was prepared and the HA25 disks were activated in the activating solution for 1hr or 24hr. To stop the

activation, the disks were rinsed in 0.1 M acetic acid. The peptide conjugation was accomplished by incubating with 1 mM peptide solution for 24 hours at room temperature. After peptide treatment, the disk cleaning process is Aqueous Washes or Ultrasonication Washes (section 2.3).



Fig.2-10 CDAP mediated peptide grafting reaction

2.2.4 HOBt method

This method is based on the work of Tennikova's team to graft protein onto the polymer surface (Fig. 2-11) [82]. The HA25 disks were rinsed with deionized water to remove the salt from the biological buffer. Firstly 1 mM peptides were activated in 1 mM HOBt (Hydroxybenzotriazole, 54802, Sigma-Aldrich) with 1mM EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, 39391, Sigma-Aldrich) dissolved in 10 mM MES (2-(N-morpholino)ethanesulfonic acid, 76039, Sigma-Aldrich) buffer (pH5.5) at 4°C for 30 minutes and then at room temperature for 10 minutes. The sample disks then incubated with the activated peptide solution at 4°C for 24 hr. After peptide treatment, the disk cleaning process is Aqueous Washes or Ultrasonication Washes (section 2.3).





2.2.5 Plasma-EDC/NHS method

The schematic illustration of model molecule 6F-Val and peptide immobilization procedure is shown in Fig. 2-12 and Fig. 2-13, respectively. The disk and IOL samples were rinsed with deionized water before an overnight air drying. Dried samples were then subjected into the chamber of radio frequency glow discharge (RFGD) instrument (customized, Europlasma). The surface activation by plasma treatment was driven at 200 W for 10 minutes. The flow rate of oxygen gas was set at 15 Sccm, and the system pressure was maintained at 50 mTorr. After plasma treatment, the samples were immersed into the coupling solution containing 1-Ethyl-3-(3-

dimethylaminopropyl)carbodiimide (EDC) 150 mM, N-hydroxysuccinimide (NHS) 100 mM, and 2-(N-morpholino)ethanesulfonate (MES) 100 mM at 4°C overnight. After rinsing by MilliQ water, the samples were conjugated with peptides by incubation with 1 mM peptide solution for 24 hours at room temperature. After peptide treatment, the disk cleaning process is Ultrasonication Washes or C (section 2.3).



Fig.2-12 Oxygen plasma and EDC/NHS reaction for 6F-Val coupling



Fig.2-13 Oxygen plasma and EDC/NHS reaction for peptide coupling

2.3 Disk cleaning method after peptide treatment

Three disk cleaning methods with different stringency were applied as the progress of this project. The disk cleaning methods were applied differently to the peptide immobilization methods, because along the progress of the research, the stringency of washing step was found more and more important. In order to specify each method, a code name system is applied. Firstly, the disks were simply rinsed several times by MilliQ water (Aqueous Washes). Secondly, one hour of sonication (35 kHz, 60 w, Elmasonic One, Germany) and two weeks shaking in MilliQ water were combined (Ultrasonication Washes). Finally, overnight shaking in MilliQ water, one hour of sonication, and ten cycles of consecutive extraction in 10 mL MilliQ water by 45 minutes of autoclave (120°C, 1 bar) were applied (Autoclave Washes). In Ultrasonication and Autoclave Washes methods, the eluates (MilliQ water after extraction) were collected and analyzed for the fluorescence intensity of the FITC-RGD peptide served as model molecule.

2.4 Surface density determination

In addition to confirm the immobilization status (grafted or adsorbed), it is also important to know the surface density of the peptide. The surface density can be used as an index to evaluate the efficiency of different conjugation methods. In this section, three methods were proposed to determine the surface density of either the peptides or the activated hydroxyl groups on HA25 polymer.

2.4.1 Ninhydrin method

The compound ninhydrin reacts with primary amines and produce "Ruhemann's purple", which can be quantified by colorimetric method (Fig. 2-14). This method is based on the work of Wang's team to quantify the RGD peptide onto the polymer surface [83]. The 16 mm diameter sample disks were subjected to acid hydrolysis in 2 mL 6N HCl at 100°C for 24 hr. The extracted solutions were neutralized by 6N NaOH. 500 μ l sample solutions were then reacted with 250 μ l 2% Ninhydrin solution (N 7285, Sigma-Aldrich) at 100°C for 1 hr. The solutions were cooled down and added 2.5 mL 96% ethanol for absorbance detection at wavelength of 570 nm. Standard curve was made by know concentration of RGD peptide solutions with the same hydrolysis reaction.



Fig.2-14 Ninhydrin reaction [84]

2.4.2 Sulfo-SDTB method

Sulfo-SDTB (Sulfosuccinimidyl-4-O-(4,4'-dimethoxytrityl)butyrate, UP35300A, Interchim, France) reacts with amino groups in the presence of perchloric acid to release the 4,4'dimethoxytrityl cation with a very high extinction coefficient (ϵ_{498} = 70,000) (Fig. 2-15) [85]. The sulfo-SDTB method has been reported to quantify the surface density of RGD peptide onto PTFE surface previously [86]. A stock buffer consisting of 50mM sodium bicarbonate, pH 8.5 was prepared. Sulfo-SDTB (3mg) was weighed and dissolved in 1 mL dimethyl formamide. After thorough mixing, the sulfo-SDTB solution was brought up to a total volume of 50 mL with the stock sodium bicarbonate buffer (working sulfo-SDTB solution). Stock buffer (1 mL) and 1 mL working sulfo-SDTB solution were added to each tube and reacted for 40 minutes at room temperature on an orbital shaker. Segments were then removed and washed twice in 5 mL of distilled water on an inversion mixer. Immediately following the wash, 2 mL of a perchloric acid solution was added to each segment. Segments were reacted for 15 minutes on the inversion mixer. The reaction solution (1 mL) was then removed and absorbance at 498 nm was measured.



4,4 Dimotroxytity

Fig.2-15 Sulfo-SDTB reaction [85]

2.4.3 Alkaline hydrolysis method

The alkaline hydrolysis method is only used to determine the activation level of CDAPtreated samples (Section 2.2.3) (Fig. 2-16) because the activated intermediate structure can be hydrolyzed to produce dimethylaminopyridine with a high extinction coefficient. The CDAP-activated intermediate was hydrolyzed in 10 mM NaOH (pH 9.5) at 90°C for 1 h. The absorbance of the supernatant was measured at 282 nm and the number of active groups was calculated using an extinction coefficient of 14600 M⁻¹ [81]. If the experimental absorbance value was higher than 1.0, a solution of 10X dilution is then prepared and re-measured to ensure the linearity between the absorbance and concentration. This method provides the number of available sites for peptide coupling.



Fig.2-16 Alkaline hydrolysis reaction

2.5 X-ray photoelectron spectroscopy (XPS) characterization

The experiments were performed by Dr. Christine Labrugère in PLACAMAT, Bordeaux, France. A ThermoFisher Scientific K-ALPHA spectrometer was used for surface analysis with a monochromatized AlK α source (hv=1486.6 eV) and a 200 micron spot size. A pressure of 10⁻⁷ Pa was maintained in the chamber during analysis. The full spectra (0-1150eV) were obtained at a constant pass energy of 200 eV and high resolution spectra at a constant pass energy of 40 eV. Charge neutralization was required for all insulating samples. High resolution spectra were fitted and quantified using the AVANTAGE software provided by ThermoFisher Scientific. Part II: Cell-based assays (section 2.6-2.9)

2.6 Cell culture

Table 2-2 lists the mammalian cell lines used in this project.

2.6.1 Porcine lens epithelial cell

Porcine lens epithelial cells (LECp) were isolated from the lens crystalline anterior capsular bag of pig eyes (Pietrain-Landrace pig; from Detry SA. Aubel, Belgium). The complete culture medium was composed of 85% Dulbecco's Modified Eagle's Medium (BE12-733, Lonza), 10% fetal bovine serum (10270-106, Gibco), 1% penicillin/streptomycin antibiotics (BE17-602, Lonza), 1% non-essential amino acids (NEAA) (BE13-114, Lonza), 1% sodium pyruvate (BE 13-115, Lonza), 1% Glutamax (35050 Gibco, Invitrogen, Oregon, United States), and 1% HEPES (17-737, Lonza, Vervier, Belgium). The cells were cultured under the condition of 5% CO₂ at 37°C. Trypsin-EDTA (Gibco, Invitrogen) was used for cell detachment after one rinse with PBS without calcium and magnesium (BE17-516, Lonza).

2.6.2 Human lens epithelial cell

Human lens epithelial cell line B3 (HLE-B3) was purchased from American Type Culture Collection (CRL-11421, ATCC, USA). The complete culture medium was composed of 80% ATCC formulated Eagle's Minimum Essential Medium (30-2003, ATCC), 20% fetal bovine serum (10270-106, Gibco), and 50 μ g/mL Kanamycin. The cells were cultured under the condition of 5% CO₂ at 37°C. Trypsin-EDTA (Gibco, Invitrogen) was used for cell detachment after one rinse with PBS without calcium and magnesium (BE17-516, Lonza).

2.6.3 Mouse L929 fibroblast cell

Mouse fibroblast cell line L929 was purchased from European Collection of Cell Cultures (85011425, ECACC, UK). The complete culture medium was composed of 87% Dulbecco's Modified Eagle's Medium (BE12-733, Lonza), 10% fetal bovine serum (10270-106, Gibco), 1% penicillin/streptomycin antibiotics (BE17-602, Lonza), 1% sodium pyruvate (BE13-115, Lonza), and 1% Glutamax (35050, Gibco). The cells were cultured under the condition of 5% CO₂ at 37°C. Trypsin-EDTA (Gibco, Invitrogen) was used for cell detachment after one rinse with PBS without calcium and magnesium (BE17-516, Lonza).

2.6.4 Human MDA-MB-231 cell

Human breast cancer cell line MDA-MB-231, used as E-Cadherin negative control for EMT assay, was a kindly gift from Dr. Christine Gilles (University of Liège, Belgium). The complete culture medium was composed of 89% Dulbecco's Modified Eagle's Medium (BE12-733, Lonza), 10% fetal bovine serum (10270-106, Gibco), and 1% penicillin/streptomycin antibiotics (BE17-602, Lonza). The cells were cultured under the condition of 5% CO₂ at 37°C. Trypsin-EDTA (Gibco, Invitrogen) was used for cell detachment after one rinse with PBS without calcium and magnesium (BE17-516, Lonza).

2.6.5 Human MCF-7 cell

Human breast cancer cell line MCF-7, used as E-Cadherin positive control for EMT assay, was a kindly gift from Dr. Christine Gilles (University of Liège, Belgium). The complete culture medium was composed of 89% Dulbecco's Modified Eagle's Medium (BE12-733, Lonza), 10% fetal bovine serum (10270-106, Gibco), and 1% penicillin/streptomycin antibiotics (BE17-602, Lonza). The cells were cultured under the condition of 5% CO₂ at 37°C. Trypsin-EDTA (Gibco, Invitrogen) was used for cell detachment after one rinse with PBS without calcium and magnesium (BE17-516, Lonza).

Name	type	Origin	Features
LECp	Lens epithelial cell, non-transformed	Pig	Epithelial cells from our lab previously, used for cell adhesion assays
HLE-B3	Lens epithelial cell, transformed	Human	Epithelial cells in human system, used for cell adhesion assays
L929	Fibroblast cell	Mouse	Standardized cytotoxicity test by MTS method
MDA-MB-231	Breast cancer cell	Human	Standard of EMT assay, expression of mesenchymal cell markers
MCF-7	Breast cancer cell	Human	Standard of EMT assay, expression of epithelial cell markers

Table2- 2 List of mammalian cell lines

2.7 Cell adhesion assay

The peptide-immobilized polymer disk samples were cut into 14 mm diameter disks, washed and sterilized in PBS (with Ca²⁺ and Mg²⁺) (BE17-513, Lonza) at 1 bar, 120 °C, for 21 min. The polytetrafluoroethylene (PFTE) cell culture (SIRRIS customized, Liège, Belgium) inserts with inner diameter of 12 mm were also sterilized by autoclave. Each disk was put into a well of a 12-well culture plate (from Greiner Bio-One, Frickenhausen, Germany) and fixed by an insert for seeding cells. The LEC cell concentration was adjusted into $1.59*10^5$ cells/mL. For each well, 750 µl of cell suspension were added $(1.4*10^5 \text{ cells/cm}^2)$. The cells were seeded on surfaces without serum for 6 hours to allow the RGD peptides to act on receptors without the hassle of serum proteins. The unattached cells and the serum-free culture medium were removed after 6 hours of serum-free medium incubation. The remaining attached cells were further cultured in fresh complete medium for 3 days to follow cell spreading and proliferation. In order to evaluate the LEC adhesion, the cell culture was stopped for further immunofluorescence staining. The culture medium was removed, and the samples were carefully washed with PBS (with Ca²⁺ and Mg²⁺) in order to eliminate the non-adhering and dead cells.

EMT can be indicated by cell shape and spatial distribution. The epithelial cells are cuboid shaped and clustered whereas the mesenchymal cells are elongated and scattered. However, if the cultured cells are close to confluence, the cells can adapt a distinct growth form and overlapping cytoplasmic expansions. Therefore, for the EMT induction or inhibition by porcine TGF- β or rapamycin, the cell preparation and DMEM complete medium were the same as above-mentioned. Porcine TGF- β 20 ng/ μ L and rapamycin 20 nM were tested by addition of each component into TCPS culture flask. In addition, in order to see the individual cell shape, the cells were cultured for 1 day rather than 3 days to avoid confluence. Porcine TGF- β 1 (101-B1-001) was purchased from R&D systems (Minneapolis, USA) and rapamycin was purchased from Apollo Scientific (UK).

2.8 Cell staining and visualization

2.8.1 Immunofluorescence

The cell fixation step was performed with 4% paraformaldehyde in PBS at room temperature for 20 min. The cell permeabilization step was performed with 0.5% Triton X-100 in PBS at 4°C for 20 min. The blocking step was performed with 1% BSA in PBS at 37°C for 1 hour. The primary antibody incubation step was performed in 0.05% Tween-20 in PBS solution with corresponding dilution (Table 2-3) at 37°C for 1 hour. The secondary antibody incubation step was performed in 0.05% Tween-20 in PBS solution with a dilution of 1:200 (Table 2-4) at 37°C for 1 hour. The stained sample surface was observed with an IX81 optical inverted microscope equipped with a UPIanFL objective at various (2X, 2.5X, 10X, 40X) magnification and with an XCite-iris IX fluorescence unit and a C-BUN-F-XC50 charge-coupled-device camera (Olympus Optical Co., Ltd). The full image area was related to cell coverage of 100% in cell adhesion quantification. At least three images per condition were acquired. For quantification of attached LECs onto surfaces, we used the image analysis "CellSens" software (Olympus). Threshold values were determined empirically by selecting a setting, which appears similar to the original photomicrograph but with minimal background. After threshold selection, the resulting image was then converted to binary image and the coverage % was reported by the software automatically. The colored merged images were generated by the channels combining function of the same software.

In order to evaluate the progress of EMT of the cultured cells, the mesenchymal protein marker α -SMA was detected. In addition, the epithelial protein marker E-cadherin was used in the section of procine lens epithelial cell characterization, and another epithelial protein marker cytokeratin was used in the section of EMT analysis of the LECs on disks. Moreover, the internal controls (i.e. structural proteins that do not express differently though EMT) are F-actin or tubulin.

The primary antibodies including mouse anti- α -SMA (ab7817), rat anti-tubulin (ab6160), and mouse anti-cytokeratin (ab668) were purchased from Abcam (Cambridge, UK). The fluorescence secondary antibodies Alexa Fluor 594 goat anti-mouse (A-11032) and Alexa Fluor 488 chicken anti-rat (A-21470) were purchased from Life Technology (Gent, Belgium). Table 2-3 and Table 2-4 lists the primary antibodies and secondary antibodies used in this study, respectively. In addition, Alexa Fluor 488 Phalloidin (A12379, Life Technologies) was used for visualization cellular actin by fluorescence.

Name	Target species	Origin	Cat. #	Dilution Factor	
Anti-Tubulin [YL1/2] antibody - Loading Control	Human, Pig, etc.	Rat	ab6160	1:1000	
Anti-beta Actin antibody - Loading Control	Human, Pig, etc.	Rabbit	ab8227	1:400	
Anti-Actin antibody	Human etc.	Rabbit	Sigma A2066	1:40	
Anti-E Cadherin antibody	Human, Pig	Rabbit	ab15148	1:25	
Mouse Anti-E- Cadherin	Human etc.	Mouse	BD 610181	1:100	
Anti-alpha smooth muscle Actin [1A4] antibody	Human, Pig, etc.	Mouse	ab7817	1:200	
Anti-Cytokeratin 18 antibody [C-04]	Human, Pig, etc.	Mouse	ab668	1:200	
Table2- 3 List of primary antibodies					

Name	Target specie	Origin	Cat. #	Dilution Factor
Goat anti-Rabbit IgG (H+L) DyLight 350 conjugate	Rabbit	Goat	62270	1:200
Alexa Fluor 350 Donkey Anti-Rabbit	Rabbit	Donkey	A-10039	1:200
Alexa Fluor 488 Chicken Anti-Rat	Rat	Chicken	A-21470	1:200
Alexa Fluor 594 Goat Anti-Mouse	Mouse	Goat	A-11032	1:200

Table2- 4 List of secondary antibodies

2.8.2 Optical microscopy

For Bright field (BF) and Phase contrast (Ph) photos, the hardware and software for image acquisition are the same with the fluorescence photos.

2.9 MTS cytotoxicity assay

Each conditioned medium was prepared by immersing a 14.5 mm polymer disk into 1.2 mL of complete culture medium (87% Dulbecco's Modified Eagle's Medium (BE12-733, Lonza), 10% fetal bovine serum (10270-106, Gibco), 1% penicillin/streptomycin antibiotics (BE17-602, Lonza), 1% sodium pyruvate (BE13-115, Lonza), and 1% Glutamax (35050, Gibco)) in a 12-well culture plate and incubated at 37°C, 5% CO₂ for 3 days. These conditioned media were added to wells containing adherent mouse L929 cells. Mouse L929 cells were precultured in a 96-well culture plate. The seeding amount for each well was 2000 cells in 100 µL of culture medium. After one day in culture, the medium was removed and the wells were replenished with 100 µL of disk-conditioned medium or unconditioned fresh medium as controls (100 % viability). The cells were cultured for another 3 days. The medium was then replaced by fresh DMEM/F-12 (21041-025, Gibco) and an additional 20 μL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution (G5421, Promega) was added. The MTS compound was bio-reduced by cells into a colored formazan product that is soluble in culture medium. The quantity of formazan product is related to viable cell population. The cells were incubated in a CO₂ supplemented incubator for 1 hour and absorbance was read with a microplate reader (PowerWave, BioTek). The 490 nm absorbance was obtained and the cytotoxicity was calculated and normalized from the absorbance of control samples taken as 100% (cells in identical culture environment but with unconditioned medium).

Part III: Optical, mechanical, and physical-chemical assays (section 2.10-2.12)

2.10 Optical properties tests

2.10.1 Light transmittance assay

Test of light transmittance was accomplished by keeping disks hydrated and placed onto a plastic 96-well plate for optical density scanning. Spectrum scan was set from 200 nm to 999 nm with 1 nm interval (PowerWave, BioTek). The absorbance was obtained and transformed to light transmittance after blank subtraction. The spectrum was reported in visible and infrared region (wavelength 370 nm to 999 nm).

2.10.2 Optical bench measurement

The optical bench measurement protocol was described by D. Bozukova et al previously [87]. Modulation transfer function (MTF) is a quantitative measurement of image quality and very sensitive to image degradation. MTF measure loss of contrast sensitivity and image sharpness when light passes through the optical system [88]. For the IOLs in this project, the MTF was measured by an eye model with an optical bench (NIMO TR0815, Lambda X) according to ISO 11979-2.18. The IOLs (1 per IOL model) were positioned in a quartz cuvette filled with buffered salt solution (0.9% sodium chloride, Baxter). The measurement was performed at a 3.0 mm aperture and spatial frequency of 100 cycles/mm. The relative contrast sensitivity recovery was calculated according to the formula:

Relative contrast sensitivity = $(MTF^*/MTF^0) \times 100\%$

where MTF⁰ and MTF^{*} are the MTF values measured before injection and at each time point after injection, respectively. The relative contrast sensitivity is presented as the percentage of contrast sensitivity recovered after injection at different time points.
2.11 Mechanical properties tests

2.11.1 Haptic compression force

The optical bench measurement protocol was described by D. Bozukova et al previously [87]. The force was determined with a compression force tester (MFC-1385-IOL, Applied Micro Circuits Corp.); the possible value variation was less than 0.2%. Before the measurement, the equipment was calibrated according to the standard manufacturer's procedure. Intraocular lenses in their original packaging state (1 per IOL model) were placed between the 2 jaws, and the compression force, in milligrams force, was measured for well diameters of 11.0 mm, 10.5 mm, 10.0 mm, and 9.5 mm, corresponding to various sizes of the capsular bag. In all cases, measurements were taken 30 seconds after haptic compression to give the haptic material time to relax.

2.11.2 IOL injection force

The optical bench measurement protocol was described by D. Bozukova et al previously [87]. The injection system uses a standard cartridge (Accuject 2.2 -1P, Medicel AG) that is connected to the injector device. Sodium hyaluronate 1.55% (Physiovisc Integral) was used as the ophthalmic viscosurgical device (OVD), and the measurements were performed at 21°C with a compression/traction mechanical bench (FL Plus Lloyd Instruments, Ametek) with a possible value variation of less than 0.05%, simulating surgical manipulation. The test equipment is supplied with a load cell of 100 N and operates with Nexygen FM software (Chatillon, Ametek, Inc.). The typical injection procedure includes the following traditional operational steps: (1) filling the empty cartridge with OVD, (2) loading the IOL, (3) closing the cartridge and placing it on the mechanical bench, (4) pushing the piston 20.0 mm at a speed of 200.0 mm/min, (5) pushing the piston 18.0 mm more at a speed of 150.0 mm/min, (6) pulling the piston back 5.0 mm with a speed of 200.0 mm/min, and (7) pushing the piston 10.5 mm at a speed of 150.0 mm/min. The IOL was typically expulsed during step 7, and the value of the force maximum was taken into consideration.

2.12 Contact angle measurement

Before the measurement, the sample disks were rinsed in MilliQ water and then dried in an oven at 35°C for 2 days. The aqueous contact angle was measured (4 measurements/ water droplet, 2 droplets/ disk, 3 disks/ sample) with a dual-gradient density contact angle meter (DGD Fast/60) coupled to WindropCC software (Digidrop, GBX). The static contact angles were measured by the water-droplet method after deposition of 15 μ L deionized water on the dry disk surfaces.

2.13 Statistical Analysis

For all experiments, at least 3 disks / IOLs replicates were prepared and analyzed independently. The quantified data were subjected to statistical analysis with Prism software (GraphPad, San Diego, USA). Unpaired t-test was applied to compare between test groups using 95% confidence interval and two-tailed P value. Not significant (P>0.05) is denoted as "ns" and for P values smaller than 0.01 and 0.001, are denoted 2 and 3 stars, respectively. One-way ANOVA was applied to compare among test groups using parameter of 95% confidence interval and Tukey post-test. The error bar of all the graphs presented stands for standard deviation.

2.14 Nomenclature List of Samples and Controls

In order to evaluate the effect of bioactive peptide surface functionalization to HA25 disk or IOL material and its potential to promote LEC adhesion and prohibit PCO, a series of control samples were prepared.

Fig. 2-17 shows the control samples used to evaluate the effect of immobilization types to LEC adhesion promotion. Peptide adsorption effect control (RGD ads, FITC-RGD ads, and RGE ads) was made by direct incubation of virgin dried HA25 material with the same peptide solution followed by the same wash steps above mentioned; plasma effect control (HA25 plasma) was prepared only by the plasma and immersed into MilliQ water immediately after the treatment.



Other key samples and their acronyms are listed in Table 2-5

Fig.2- 17 Illustration of plasma, peptide adsorbed, and peptide graft samples from neat HA25 material

Name	O ₂ Plasma treatment	Peptide Incubation	Description
TCPS	N/A	N	Tissue culture polystyrene, used for normal LEC morphology / proliferation control
TCPS/TGF-β	N/A	Ν	LEC cultured onto TCPS with additional TGF- β (20 ng/ μ L) in normal culture medium, used for EMT positive control
TCPS/Rapamycin	N/A	Ν	LEC cultured onto TCPS with additional rapamycin (20 nM) in normal culture medium, used for EMT negative control
GF	Ν	Ν	Hydrophobic acrylic polymer Glistening-Free [®] , used for PCO negative control
HA25	Ν	Ν	Hydrophilic acrylic polymer water-uptake 25%, used for PCO positive control
HA25 plasma	Y	N	HA25 treated with oxygen plasma, used for plasma effect evaluation
HA25-RGD ads	Ν	Y (RGD)	RGD adsorbed onto HA25, used for adsorption effect evaluation
HA25-RGD graft	Y	Y (RGD)	RGD grafted onto HA25, used for LEC adhesion promotion
HA25-RGE ads	Ν	Y (RGE)	RGE adsorbed onto HA25, used for adsorption effect evaluation
HA25-RGE graft	Y	Y (RGE)	RGE grafted onto HA25, used for a negative control of LEC adhesion promotion
HA25-FITC RGD ads	N	Y (FITC-RGD)	FITC-RGD adsorbed onto HA25, used for adsorption effect evaluation by fluorescence tracing
HA25-FITC RGD graft	Y	Y (FITC-RGD)	FITC-RGD grafted onto HA25, used for LEC adhesion promotion and fluorescence tracing

Table2- 5 Nomenclature of key samples used in this study

Chapter 3. Results

3.1 Peptide immobilization

As described in Section 2.2, the RGD peptide is selected as candidate to be immobilized onto the IOL surface. To investigate the relationship between the peptide modified surface and LEC, the disk made of HA25 material is used to avoid IOL shape design effect to the cells. As long as the best method is confirmed, the surface functionalized IOLs are prepared for optical, mechanical tests for adverse effect examination.

In this section, there are two parts of result presented here. Firstly, although XPS is one of the best spectrometric methods to detect the surface immobilized peptides, the FITC-tagged RGD peptide (Fig. 2-4) is also used for rapid RGD tracing. The resulting fluorescence microscopy images are presented. Secondly, in order to evaluate of the efficiency of each method, the cell adhesion assay is used to evaluate the overall bioactivity of the peptide functionalized surfaces.

Unless mentioned elsewhere, the concentration of the peptide solution in the immobilization step is 1 mM.

3.1.1 Tracing peptide by fluorescence images

3.1.1.1 Tresyl chloride method (see Section 2.2.1 for material and methods)

The test of tresyl chloride method (Fig. 2-7, Section 2.2.1) using fluorescein amine (Fig. 2-8) as a model molecule suggests that the reaction may function normally (Fig. 3-1). The fluorescence backgrounds of the control sample without fluorescent compound treatment (left) is significantly lower than the sample with fluorescent compound treatment (right), suggesting that fluorescence is an appropriate tool to trace the peptides. Subsequently, the test using fluorescein amine (Fig. 3-2B) and FITC-RGD peptide (Fig. 3-2C) shows a higher fluorescence signal comparing to the control (Fig. 3-2A), suggesting that the tresyl chloride method could possibly conjugate RGD peptide onto the polymer surface (Fig. 3-2D). Attempt of Raman and ATR-FTIR spectrum showed no information regarding to this modification (data not shown) (see Section 4.4 for explanation).



Fig.3- 1 Fluorescence microscope image of HA25 disks incubated with 10 μM fluorescein amine overnight without (left) or with (right) tresyl chloride activation. The left disk is barely visible for the low background fluorescence intensity. The scale bar represents 5 mm. (Magnification 2X objective) (Aqueous Washes, Section 2.3)



Fig.3- 2 Optical (upper) and fluorescence (lower) microscope images of HA25 disks incubated with (A) buffer (0.2 M sodium bicarbonate pH 10), (B) fluorescein amine 10 μM, (C)FITC-RGD 20 μM, and (D) RGD 20 μM after tresyl chloride activation overnight. (Aqueous Washes, Section 2.3) The scale bar represents 2 mm. The scratch of the disks at the edge was made during cutting. The irregular section of the disks creates the nonhomogeneous fluorescence signal. (Magnification 2X objective)

3.1.1.2 Permanganate oxidation – EDC/NHS method (see Section 2.2.2 for material and methods)

The test of permanganate oxidation followed by EDC/NHS method (Fig. 2-9, Section 2.2.2) using FITC-RGD suggests that the reaction functions normally (Fig. 3-3). The fluorescence intensity of the surface was distributed homogeneously. However, the material became whitened and opaque: the opacification of the polymer disk irreversibly occurred at the step of MilliQ wash after 1.2N HCl wash. The disk under optical microscope exhibited massive tiny dark spots (Fig. 3-4) in every focal plane, which seems to be gas trapped inside of the HA25 polymer (see Section 4.1.2.1 for explanation).



Fig.3- 3 Fluorescence microscope images of HA25 disks

Left: virgin HA25 disk; Right: HA25 disk after permanganate oxidation – EDC/NHS coupling reaction of the FITC-RGD peptide (Magnification 2.5X objective). (Aqueous Washes, Section 2.3)



Fig.3- 4 Optical microscope images of samples at the same position in Fig. 3-3

3.1.1.3 CDAP method (see Section 2.2.3 for material and methods)

The test of CDAP (Fig. 2-10, Section 2.2.3) suggests that the reaction functions normally (Fig. 3-5, upper). As mentioned earlier of this section, the FITC-tagged RGD peptide was used to detect the adsorbed or grafted peptides by fluorescence. Fig. 3-5 upper shows the fluorescent image of HA25 disks incubated with 1 mM FITC-RGD without (left) or with (right) CDAP activation. Since the fluorescence signal of the sample is low, a longer exposure and higher gain value was applied to enhance the image of the treated sample, which also lead the virgin polymer exhibits a basic fluorescence level. However, the images in Fig. 3-5 were taken with the same parameter so that can be compared. By comparing the integrated values of the fluorescence signal in each image, the CDAP method enhanced the peptide immobilization onto the surface.

3.1.1.4 HOBt method (see Section 2.2.4 for material and methods)

The test of HOBt (Fig. 2-11, Section 2.2.4) suggests that the reaction is able to enhance the peptide density grafted onto the HA25 disk (Fig. 3-5, lower).



Fig.3- 5 Fluorescence microscope images of disks and their intensity integration values without (left) and with (right) CDAP (upper) or HOBt (lower) coupling reaction of the FITC-RGD peptide (Magnification 2.5X objective). (Aqueous Washes, Section 2.3)

3.1.1.5 Plasma-EDC/NHS method (see Section 2.2.5 for material and methods)

The test of plasma-EDC/NHS (Fig. 2-13, Section 2.2.5) suggests that the reaction increase the fluorescence signal onto the HA25 polymer disk (Fig. 3-6 left green channel). The plasma-EDC/NHS/FITC-RGD treated surface exhibits evenly distributed, intense fluorescence signal. In addition, cross section images of the disks show the fluorescence mainly distributed onto the surface with maximum penetration depth of 25 μ m (Fig. 3-7).

From the fluorescence based tests on FITC-RGD peptide graft, it seems all the methods promote peptide immobilization with different extent (observed by different fluorescence intensity level). It is also necessary to investigate the activity of the immobilized peptides to ensure the bioactivity of our modified surfaces. Next, the cell adhesion assay results are presented to evaluate the overall bioactivity of the peptide functionalized surfaces.



Fig.3-6 Fluorescent intensities of FITC-RGD peptide-immobilized HA25 surfaces and immunofluorescence images of LECp adhesion on the corresponding surfaces. The condition of cell adhesion assay is described in Section 2.7. The samples are tissue culture polystyrene (TCPS), HA25 virgin disk, FITC-RGD immobilized by CDAP, HOBt, or plasma-EDC/NHS methods. (red: F-actin, green: FITC-RGD, blue: DAPI, 2.5X objective magnification) (Ultrasonication Washes, Section 2.3)



Fig.3- 7 Cross section of Plasma-EDC/NHS/FITC-RGD treated disks. Left: virgin HA25 disk; Middle: HA25 disk soaked into 1 mM FITC-RGD peptide solution; Right: HA25 disk activated by Plasma-EDC/NHS method and then soaked into 1 mM FITC-RGD peptide solution (BF: bright field) Scale bar represents 500 μm. (Magnification 2X objective) (Ultrasonication Washes, Section 2.3)

3.1.2 Evaluating methods by cell response

3.1.2.1 Tresyl chloride method

This method enhances the fluorescence signal of the fluorescein amine and FITC-RGD treated samples (Section 3.1.1.1). It could possibly enhance the cell adhesion. However, the method uses nonpolar solvent which may potentially damage the hydrophilic polymer (altering volume, changing mechanical properties, and creating creases and cracks). This method was never subjected into cell adhesion assay.

3.1.2.2 Permanganate oxidation – EDC/NHS method

This method also enhances the fluorescence signal of the FITC-RGD treated samples (Section 3.1.1.2). It could possibly enhance the cell adhesion as well. However, since the polymer became opaque after treatment, the sample is not capable for serving as an ophthalmological implant and never subjected into cell adhesion assay.

3.1.2.3 CDAP method

This method also enhances the fluorescence signal of the FITC-RGD treated samples (Section 3.1.1.3). However, reported by cell adhesion test, the immobilized peptide exhibits biological function to promote cell adhesion (Fig. 3-6 blue/red channel CDAP (+) sample). Comparing the CDAP samples (CDAP +/-), the trend of cell adhesion is also in line with the fluorescence signal. In addition, on the CDAP (-) surface, there are several tiny blue spots, which may be the degraded DNA from the dead cells. The CDAP method shows moderate peptide immobilization ability and biological function.

3.1.2.4 HOBt method

This method also enhances the fluorescence signal of the FITC-RGD treated samples (section 3.1.1.3). However, reported by cell adhesion test, the immobilized peptide exhibits very low biological function to promote cell adhesion (Fig. 3-6, HOBt samples). In addition, like the CDAP samples, on the HOBt treated surface, there are tiny blue spots, suggesting that the cells can barely survive on the surface. The HOBt method showed lowest cell adhesion promoting ability among the tested methods in Fig. 3-6. Combining with the fluorescence results obtained in Fig. 3-5, the CDAP method shows poor peptide immobilization ability and biological function.

3.1.2.5 Plasma EDC/NHS method

This method also enhances the fluorescence signal of the FITC-RGD-treated samples (Section 3.1.1.5). Reported by cell adhesion test, the immobilized peptide exhibits very high biological function to promote cell adhesion (Fig. 3-6 Plasma EDC/NHS sample). The plasma EDC/NHS method showed highest cell adhesion promoting ability among the tested methods in Fig. 3-6. Combining with the fluorescence channel in Fig. 3-6, the plasma EDC/NHS method shows good peptide immobilization ability and biological function.

In addition, in Fig. 3-6 green channel, the cell shapes can be seen in CDAP or HOBt treated samples but not in plasma-EDC/NHS treated samples. The aggregation of the cells would scatter the light and render this observation. The LECs on the plasma-EDC/NHS treated surface may exhibit a relative flattened layer which leads the homogeneous green fluorescence from FITC-RGD peptides.

From the fluorescence-based tests on FITC-RGD peptide-grafted surfaces, it seems all the method promotes peptide immobilization and cell adhesion with different extent. It is also important to remove the adsorbed peptide as many as possible because the free soluble bioactive peptide may enter the aqueous or blood system to arouse unexpected effect. On the other hand, the loss of biological activity of surface grafted peptide while removing adsorbed peptides is not acceptable. Therefore, the study of disk cleaning method is tested with different stringencies.

3.2 Disk cleaning method after peptide treatment (see Section 2.3 for material and methods)

Three disk cleaning methods with different stringencies were applied as the progress of this project and the results are shown here. The disks simply rinsed several times by MilliQ water (Aqueous Washes, Section 2.3) exhibit high fluorescence background (Fig. 3-5). The disks become lower fluorescence background after applying 1 hour of sonication and followed by 14 days of vigorous shaking (Ultrasonication Washes, Section 2.3) (Fig. 3-8). The extracts (eluates) of the disks were also collected for quantification by fluorescence intensity (Fig. 3-9). Comparing to Fig. 3-5, the fluorescence intensity contrast between CDAP (-) and (+) samples illustrate that Ultrasonication Washes is able to remove adsorbed FITC-RGD peptides and therefore reduce the background. Nevertheless, the washing reduces fluorescence intensity of the HOBt (+) sample to the level of HOBt (-) sample, suggesting extremely low or no covalent binding between peptides and HA25 polymer by HOBt method. In addition, both CDAP (-) and HOBt (-) show a higher fluorescence background than the virgin HA25 control sample (no FITC-RGD peptide treatment), indicating the washing step can be further improved. This finding is also corresponding to the fluorescence tracing result after autoclave (Fig. 3-9).

The cleaning procedure was further improved by adding 10 cycles of high temperature MilliQ extraction in the autoclave chamber (Autoclave Washes, Section 2.3) and the extracted solutions were collected for quantification as well (Fig. 3-11). The concentration is less than 10 μ M after 10 cycles of extraction no matter the sample types or starting concentration of the peptide in first autoclave. Taking Fig. 3-9 and 3-10 together, the "autoclave" sample can be considered as the "1" sample in Fig 3-10. This sample will be definitely higher than the later same autoclave extraction samples (sample 2 to 10, Fig 3-10). However, this sample exhibits higher concentration than the previous sample (D14, Fig 3-9), suggesting that elevating temperature by autoclave may greatly enhance the stringency, which leads more adsorbed peptides been extracted out.

From the fluorescence based tests on FITC-RGD peptide graft, it seems the method C is most stringent method among the tested 3 methods (Aqueous, Ultrasonication, and Autoclave Washes, Section 2.3). In addition, Fig. 3-11 shows Autoclave Washes (10X autoclave) protocol exhibits higher contrast in cell adhesion promoting ability than Ultrasonication Washes (1X autoclave). Moreover, Fig. 3-9 and Fig. 3-10 suggest that a large quantity of FITC-RGD peptides has been desorbed from the surface whereas the amount of the adhered cells remains high on the FITC-RGD-grafted surface in Fig. 3-11. Therefore, Autoclave Washes protocol could be a better choice to remove the surface adsorbed peptides on HA25 material.

CDAP (-) FITC-RGD	CDAP (+) FITC-RGD	HOBt (-) FITC-RGD	HOBt (+) FITC-RGD	Virgin HA25 Ctrl
253.298	6491.168	253.848	256.654	211.934

Fig.3- 8 Fluorescence microscope images of HA25 disks and their mean intensity values prepared by CDAP or HOBt coupling reaction of the FITC-RGD peptide after 1 hour of sonication and followed by 14 days of vigorous shaking (Ultrasonication Washes, Section 2.3) (2.5X objective magnification).



Fig.3- 9 Fluorescence tracing of the extract of HA25 disks immobilized (adsorbed and grafted) with FITC-RGD peptide. The grafted sample was prepared by plasma-EDC/NHS method and the adsorbed sample is prepared by direct incubating a dried virgin HA25 disk into FITC-RGD peptide solution. The eluates were collected during every step in Ultrasonication Washes (Section 2.3). Stage A stands for the 1 mM FITC-RGD standard solution, stage Incubation stands for peptide solution after incubation, and stage B stands for the time point of ultrasonication. D1 to D14 represent the two weeks shacking in MilliQ. At the final stage Autoclave, the disks were sterilized by autoclave and solutions were collected for fluorescence tracing.



Fig.3- 10 Peptide concentration of the extract of HA25 disks immobilized (adsorbed and grafted) with FITC-RGD peptide. The concentration was determined by comparing the fluorescence intensities with a series of standard solutions. The grafted sample was prepared by plasma-EDC/NHS method and the adsorbed sample is prepared by direct incubating a dried virgin HA25 disk into FITC-RGD peptide solution. In grafted and adsorbed groups, three disks were washed separately and the eluates were collected during every autoclave step in Autoclave Washes (Section 2.3).



Fig.3- 11 Fluorescence microscope images of cell adhesion assay. The samples are HA25 disks immobilized (adsorbed or grafted) with FITC-RGD by plasma EDC/NHS method with 1 cycle (Equivalent to Ultrasonication Washes) or with 10 cycles of autoclave (Equivalent to Autoclave Washes). The condition of cell adhesion assay is described in Section 2.7. The cells were stained with cytokeratin (red) and DAPI (blue) (Magnification 10X, Scale bar = 100 µm)

3.3 Surface RGD peptide density determination

In addition to enhance the peptide functionalized surfaces by improvement of grafting or washing techniques, it is also helpful to know the surface density (immobilized molecules/unit area) if possible. The development of surface density method is parallel to the development of surface grafting and washing technique. Not all the methods above mentioned were subjected into this part of study. The goal here is to find a method suitable for detecting and quantifying the peptides on HA25 surface.

3.3.1 Ninhydrin Method (see Section 2.4.1 for material and methods)

The ninhydrin can react with the α -amino acids of the peptide from the HA25 surface. This method was success to quantify the surface density with the simplest cleaning method (Aqueous Washes, Section 2.3) (Fig. 3-12). However, along with the cleaning procedure improvement (Ultrasonication or Autoclave Washes, Section 2.3), the method cannot react with sufficient α -amino acids and make color change (ie. no absorbance detected at 562 nm). Removal of the majority of the peptide by Ultrasonication Washes or Autoclave Washes renders the total amount of peptides below the detection limit, no matter the peptides are adsorbed or grafted.



Fig.3- 12 Ninhydrin reaction results of RGD immobilized HA25 disks prepared by CDAP method (Aqueous Washes, Section 2.3). The samples are CDAP treated disks with activation time of 1hr or 24hr. The control is the same solution without CDAP with incubation of 24hr, which can be considered as an adsorption-only control.

3.3.2 Sulfo-SDTB Method (see Section 2.4.1 for material and methods)

Theoretically the Sulfo-SDTB can react with the primary amine and form a product with a high extinction coefficient. Table 3-1 shows the raw data and the calculation of surfaces functionalized by CDAP method and analyzed by Sulfo-SDTB method. CDAP (+) and (-) indicate the surface with (200 μ L, 24hr) and without CDAP activation (buffer only

control), respectively. 1 hr and 24 hr indicate the time of surface incubated with the CDAP solution. "Hydrolyzed" specifically means the surface has treated with NaOH solution (Fig. 2-16). The quantification suggests that the blank sample "CDAP (-) 24hr hydrolyzed" exhibits even higher surface amine group than CDAP activated surface or peptide immobilized surface. This phenomenon is not reasonable and could be explained by the low absorbance value (Raw 2: Abs 498 nm) (Table 3-1) approaching the detection limit. Therefore, the calculated surface density from this raw data is unreliable.

	Formula	CDAP(-) 24hr /RGD	CDAP(+) 24hr /RGD	CDAP(+) 1hr hydrolyzed	CDAP(+) 24hr hydrolyzed	CDAP(-) 24hr hydrolyzed
Abs 498 nm	Abs	0.0028	0.0044	0.0072	0.0045	0.0082
Extinction Coefficient (M ⁻¹ cm ⁻¹)	٤	70000	70000	70000	70000	70000
Calculated concentration (µM)	C=Abs/ (ε*b) given b=1cm	0.0400	0.0629	0.1029	0.0643	0.1171
Volume (mL)	V	2	2	2	2	2
total amount (nmole)	n=C*V	0.0800	0.1257	0.2057	0.1286	0.2343
NH ₂ surface density (nmole/cm ²)	D _{NH2} =n/Area given Area= 2πr ²	0.0199	0.0313	0.0512	0.0320	0.0583

Table3-1 Raw data and the calculation of peptide surface density by Sulfo-SDTB Method (Ultrasonication Washes, Section 2.3). CDAP (-) 24hr /RGD is the control for peptide adsorption behavior. CDAP (+) 24hr /RGD is the test sample for peptide conjugation. The CDAP (+) 1hr and 24hr hydrolyzed samples contains primary amine (Section 2.4.3, Fig. 2-16) which can react with Sulfo-SDTB theoretically and reflect the activated hydroxyl group amount of the HA25 surface. The CDAP (-) 24hr hydrolyzed is the control for no amine group of the HA25 surface (ie. background).

3.3.3 Alkaline hydrolysis method (see Section 2.4.3 for material and methods)

The alkaline hydrolysis method was performed to determine the activation level of CDAP-treated samples. The method is only applicable to CDAP treated method because the intermediate activated structure can be hydrolyzed by alkaline and form a compound with high extinction coefficient (Fig. 2-16).

Table 3-2 shows the CDAP activation time effect. Notably, this result is comparable to the peptide surface density determined by ninhydrin method (Fig. 3-12). However, the

CDAP non-activated sample (CDAP (-), 24 hr) also shows a high background of this assay, suggesting that the real activated OH surface density is not as high as calculated. In addition, the result of CDAP amount effect is listed in Table 3-3. The low concentration of CDAP results in low absorbance value which is even lower than that of the blank, suggesting that the assay is approaching the detection limit.

From Table 3-2, a longer activation time makes a higher amount of activated OH group. In addition, from Table 3-3, a higher concentration of CDAP makes a higher amount of activated OH group. Overall, it seems combining 24 hr of activation time and 400 μ L of CDAP solution could maximize the OH group activation among tested conditions. The higher background also illustrates the activation of CDAP low efficiency or close to the detection limit.

	(Formula)	CDAP (+) 1hr	CDAP (+) 24hr	CDAP (-) 24hr	blank
Abs	Abs ₀	0.337	0.697	0.056	0.006
Abs w/o background	Abs=Abs ₀ - Abs _{blank}	0.330	0.690	0.049	0
Extinction Coefficient (M ⁻¹ cm ⁻¹)	3	14600	14600	14600	
Dilution factor	Ν	10	10	1	
Calculated activated OH concentration (µM)	C=N*Abs/ (ɛ*b) given b=1cm	226.357	472.885	3.387	
Volume (mL)	V	2	2	2	
total amount (nmole)	n=C*V	452.714	945.769	6.773	
calculated activated OH surface density (nmol/cm ²)	D _{OH} =n/Area given Area= 2πr ²	112.638	235.313	1.685	
calculated peptide surface density (nmol/cm ²)	D _{peptide} from Fig 3.12	15.069	16.555	12.711	

Table3- 2 Raw data and the calculation of activated hydroxyl group in CDAP method by alkaline hydrolysis (Aqueous Washes, Section 2.3) - Effect of activation time. The sample is the same with those in Fig. 3-12 and the blank is the 10 mM NaOH solution used in alkaline hydrolysis.

	CDAP 0µL 1hr	CDAP 25 µL 1hr	CDAP 100 μL 1hr	CDAP 200 μL 1hr	CDAP 400 µL 1hr	Blank
Abs	-0.111	-0.085	0.373	0.883	2.395	0.185
Abs w/o background	-0.297	-0.270	0.187	0.697	2.210	0
Extinction Coefficient (M ⁻¹ cm ⁻¹)	14600	14600	14600	14600	14600	
Dilution factor	1	1	1	1	1	
Calculated concentration (µM)	-20.311	-18.505	12.833	47.750	151.356	
Volume (mL)	2	2	2	2	2	
total amount (nmole)	-40.623	-37.009	25.666	95.501	302.712	
calculated activated OH surface density (nmol/cm ²)	-10.107	-9.208	6.386	23.761	75.317	

Table3- 3 Raw data and the calculation of activated hydroxyl group in CDAP method by alkaline hydrolysis (Aqueous Washes, Section 2.3) - Effect of CDAP amount. The blank is the 10 mM NaOH solution used in alkaline hydrolysis. The formula for calculation is the same with Table 3-2.

3.4 X-ray photoelectron spectroscopy (XPS) characterization

With the test results of different grafting strategies (Section 3.1) and washing protocols (Section 3.2), it became more and more clear that the plasma EDC/NHS method create cell-adhesion promoting surface more efficiently and the Autoclave Washes protocol can remove most adsorbed peptides. However, there is no crucial evidence to prove the immobilized peptide is grafted rather than adsorbed. Therefore, XPS analysis was performed to characterize the surface bound status (grafted or adsorbed). In order to simplify the system, a model molecule 6F-Val was applied to be immobilized onto HA25 disk surface according to plasma-EDC/NHS method and Autoclave Washes (Section 2.3) protocol. Subsequently, the molecules RGD and FITC-RGD were tested with the same treatment.

3.4.1 6F-Val model molecule

The 4,4,4,4',4',4'-Hexafluoro-DL-valine (6F-Val) serves as a model molecule to verify if the covalent conjugation between target and the polymer surface exists and to examine the washing step can remove the physical adsorbed molecules onto the polymer surface. The full washing steps including overnight MilliQ water shaking, 1 hr sonication, and 10 cycles of autoclave were applied (Autoclave Washes, Section 2.3). The result shows the total N element percentage decrease along the wash steps progress, yet the amide (+C-CO)/C-C to amine /C-C ratio increase (Fig. 3-13). The phenomenon suggests that the adsorbed 6F-Val desorbed during the washing, and the remaining peptides are covalently conjugated. The desorption behavior of 6F-Val is also corresponding to the FITC-RGD samples in Fig. 3-9 and Fig. 3-10. The stepwise release of fluorescence during the washing decreases the surface adsorbed peptides. Therefore, the ratio of grafted to adsorbed peptides increases as the trend we observed in Fig. 3-13.



Fig.3- 13 Quantification of each step of washing from XPS C1s peak deconvolution data, the amide to amine ratio increases (lower) along to the washing stages in which the total nitrogen element percentage decreases (upper)

3.4.2 RGD and FITC-RGD peptide

XPS was also employed to determine the surface chemical composition of surface of HA25 before and after RGD or FITC-RGD peptide grafting. The XPS spectra of the different status of chemical modifications were collected and their element compositions were calculated and shown in Fig. 3-14, Fig. 3-15, Fig. 3-16, and Table 3-4. The peak deconvolution results of the C1s spectra and the N1s spectra are shown in Fig. 3-14 and Fig. 3-15. The XPS data suggest that the RGD (or FITC-RGD) peptides, same as the model molecule 6F-Val, are grafted onto HA25 polymer. The detailed peak assignment and reasoning are written in the discussion part (Section 4.4.2).



Fig.3- 14 Deconvoluted XPS N1s (left) and C1s (right) spectrums of the HA25 - RGD grafted (upper) and adsorbed (lower) sample



Fig.3- 15 Deconvoluted XPS N1s (left) and C1s (right) spectrums of the HA25 - FITC-RGD grafted (upper) and adsorbed (lower) sample



Fig.3- 16 Quantification of amide/C-C and (amine + C-CO)/C-C ratios for each surface from the C1s spectrums of Fig. 3-14 and Fig. 3-15

Atomic %	С%	0%	N%	Si%
HA25	70.8	25.2	0.4	3.6
HA25 - RGD ads	70.7	25.9	0.2	3.2
HA25 - RGD graft	70.0	26.2	1.0	2.8
HA25 - FITC-RGD ads	69.1	24.3	2.4	3.7
HA25 - FITC-RGD graft	70.0	23.5	3.1	3.0

Table3- 4 Experimental atomic composition obtained by XPS analysis

3.5 Cell morphology and EMT biomarker expression

The peptide surface functionalization aim at enhancing the adhesion of LECs on HA25 polymer. In addition, the adhered LECs have to exhibit no epithelial mesenchymal transition (EMT) feature. Therefore, characterization of the original LECs status is necessary. With the profile of cell morphology, spatial distribution, EMT biomarker expression built from conventional cell culture, the LECs growth on peptide functionalized surface can be compared. The porcine LEC cell line was previously prepared by colleagues and literature suggests the primary LEC culture undergo spontaneous EMT after several passages. Therefore, cell lines MCF7 and MDA-MB-231 were used as positive control and negative control of E-cadherin expression (Epithelial marker) to characterize the EMT status of the porcine LEC cell line. In addition, the commercial available human LEC cell line HLE-B3 is compared at the same time.

In Fig. 3-17, the cell morphology and the expression of E-Cadherin (epithelial marker) were tested in control and human or porcine lens epithelial cell lines. Mammary cancer derived MCF-7 cells, which are considered non-metastatic, express high levels of the epithelial marker E-Cadherin and low levels of the mesenchymal markers. The opposite expression profile is observed in MDA-MB-231 cells, which are considered to be metastatic breast cancer cells. The results shows HLE-B3 and LECp have the epithelial cell features such as clustering, cuboid shaped (Fig. 3-17). The expression level of E-Cadherin is not as high as the positive control MCF-7 naturally, but higher as the low level (taken as "negative") control MDA-MB-231. This assay suggests that the porcine LECs used in this research maintain epithelial cell type.



Fig.3- 17 Fluorescence (left) and phase contrast (right) images in 10X (upper, scale bar = 100 μ m) or 40X (lower, scale bar = 25 μ m) magnification of cultured cell lines. The samples are MCF-7, MDA-MB-231, HLE-B3, and LECp cells cultured in TCPS culture flask and stained with E-cadherin (red) and DAPI (blue).

3.6 Cell adhesion assay

As shown in the previous sections of this chapter, the grafting chemistry and surface cleaning method were established to functionalize peptides on HA25 polymer. In this section, the FITC-RGD peptide, as well as other bioactive peptide (or analogue), was immobilized on HA25 polymer surface via plasma EDC/HNS method and investigated for its cell adhesion promotion ability. The cell type is porcine lens epithelial cells (LECp) and the culture time is 72 hours (See Section 2.7 for material and methods).

Fig. 3-18 shows the relationship between cell coverage and the surface-immobilized peptide amount. Although there could be a considerable amount of physically adsorbed FITC-RGD peptide, however, it seems only the grafted peptide promotes the cell adhesion (also seen in Fig. 3-19). The plasma treatment itself does not promote cell adhesion onto the HA25 disk; the plasma activation combined with EDC/NHS crosslink to the peptides promotes the cell adhesion.

Fig. 3-19 shows different immobilized bioactive peptides and the combination effect to LECp cells. To verify if the RGD sequence promotes the cell adhesion ability, the nonfunctional analog RGE (Fig. 2-3) was tested. The result suggests that peptide sequence "RGD" itself promotes the LECp cell adhesion specifically (Fig. 3-19). In addition to RGD peptide sequence, another biological functional peptide WQPPRARI (Fig. 2-5) was tested at the same time. From the result, peptide WQPPRARI alone promotes cell adhesion as RGD, and the scramble of WQPPRARI with RGD (ie. 1 mM WQPPRARI and 1 mM RGD in the peptide solution during grafting reaction, Section 2.2.6) leads to a higher adhesion ratio than individual.

From the results of cell adhesion assays, neither peptides adsorbed on HA25 polymer, nor plasma treatment, solely promotes LEC adhesion. The bioactive peptide grafted surface shows cell adhesion promoting ability significantly. In addition, the RGD grafted surface exhibits similar cell coverage to the hydrophobic acrylic control material GF, suggesting that the surface functionalization strategy may successfully turn the HA25 material as bio-sticky material, which is considered limiting PCO.



Fig.3- 18 Fluorescence microscope images of cell adhesion assay. The samples are hydrophobic acrylic polymer (GF), HA25 virgin disk, oxygen plasma treatment control, and HA25 disks immobilized (adsorbed or grafted) with FITC-RGD. (Ultrasonication Washes, Section 2.3) Alpha-smooth muscle actin (α-SMA) is stained in red and FITC-RGD is in green. (Scale bar = 100 µm)



Fig.3- 19 Fluorescence microscope images of cell adhesion assay. The samples are hydrophobic acrylic polymer (GF), HA25 virgin disk, oxygen plasma treatment control, and HA25 disks immobilized (adsorbed or grafted) with RGD, RGE, WQPPRARI and scramble (RGD + WQPPRARI) (Autoclave Washes, Section 2.3) (magnification 10X, cytokeratin in red, tubulin in green, nucleus in blue, respectively.)

3.7 EMT biomarker assay

The biomarker involved in EMT process and the cell morphology, spatial distribution may help to identify the cellular status onto the polymer surface. The positive and negative controls are the same LECp onto the TCPS surface treated with TGF- β and Rapamycin, respectively. The mutual effect of TGF- β and Rapamycin to LEC was also evaluated because Rapamycin is not a direct antagonist to TGF- β in EMT pathway. It is also interesting to investigate the LEC response in a combined treatment. Rapamycin strongly inhibits whereas TGF- β slightly promotes LECp proliferation onto TCPS surface. In addition, free RGD peptides in culture medium may reduce cell coverage ratio because the peptide may compete integrin binding site of the cells (Fig. 1-22).

The result shown in Fig. 3-20 suggests that the treatment of TGF- β promotes LECp elongated whereas the treatment of Rapamycin keeps LECp clustered. The addition of free RGD peptide does not alter the morphology of the cells but reduces the coverage ratio. The same phenomenon can be observed in the other cell adhesion experiments (Fig. 3-21). The RGD grafted HA25 disk prepared by plasma EDC/NHS method shows epithelial feature clustering as the control GF material. In addition, the mesenchymal cell marker α -SMA is not up-regulated in the LECp onto the RGD immobilized surfaces.



Fig.3- 20 Fluorescence microscope images and the quantified coverage percentage of cell EMT biomarker assay. The samples are LECp onto TCPS surface treated with porcine TGF- β 10 or 20 ng/mL, Rapamycin 10 or 20 nM, and RGD 50 μ M (red: α -SMA, green: tubulin, blue: DAPI) (Magnification 10X, Scale bar = 100 μ m).



Fig.3- 21 Fluorescence microscope images of EMT induction assay. The controls are tissue culture grade polystyrene (TCPS), TCPS added 20 ng/µL porcine TGF- β (TCPS/TGF- β) or rapamycin 20 nM (TCPS/Rapamycin). The samples are hydrophobic acrylic polymer (GF), HA25 virgin disk (HA25), oxygen plasma –treated HA25 (HA25 - Plasma), and HA25 disks immobilized with RGD (HA25 –RGD ads and HA25- RGD graft). (Magnification: 10X, Scale bar = 100 µm, α -SMA stained in red, tubulin in green, nucleus in blue, respectively.) (Autoclave Washes, Section 2.3)

3.8 MTS cytotoxicity assay

The evaluation of the cytotoxicity of disk-conditioned medium on L929 cells was adapted from ISO 10993-5 (2009). The MTS compound is bio-reduced by cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product was measured by absorbance at 490 nm and related to viable cell population. The obtained values of absorbance were divided by that from the blank control sample, which is the L929 cells cultured in the normal medium, and transformed into percentage. The assay results by conditioned medium method (Section 2.9) shows the surface functionalized with RGD (or FITC-RGD) peptides via plasma-EDC/NHS (with Autoclave Washes protocol) (Fig. 3-22) method have no cytotoxicity potential. The viabilities of all groups are above 70%, which is considered as non-cytotoxic. The surface functionalized samples created by peptide grafting or adsorption are free of cytotoxicity.



Fig.3- 22 Cytotoxicity potential of the neat and modified disks (The peptide concentration during the coupling reaction is 1 mM.)

3.9 Optical properties tests

This part of the work was developed to evaluate the potential use of RGD grafted-IOLs in manufacture. This surface functionalized biomaterial should be homogenously transparent with no color deviation, and exhibit similar optical properties to fulfill the requirement of being an intraocular lens. Therefore, the light transmittance and the optical bench measurement were selected to evaluate the optical properties of the novel biomaterial.

3.9.1 Light transmittance assay

The light transmittances of the disks with different peptide surface immobilized statuses by plasma-EDC/NHS (Fig. 3-23) were measured. The disks were homogenously transparent with no color deviation, and presented a light transmittance of greater than 80% from green light to red light spectrum. The RGD immobilized samples (HA25 – RGD Ads and HA25 – RGD Graft) exhibit the same optical properties as the virgin HA25 polymer. The small thickness variance of polymer disks may cause a homogenous effect in the entire transmittance spectrum. The decrease in light transmittance in blue light rises from the "Blue Filtering" design of the bulk polymer disk.



Fig.3- 23 Light transmittance spectra of the neat and modified disks (No significant difference among 3 groups by ANOVA) (The peptide concentration during the coupling reaction is 1 mM)
3.9.2 Optical bench measurement

For this experiment, the real IOLs samples, rather than the disks, prepared by plasma EDC/NHS method and Ultrasonication Washes protocol are used. The concentration of the peptide solution in immobilization step is 1 mM. Both RGD graft and adsorption did not impact the optical power, expressed in diopters, and the contrast sensitivity, expressed by the MTF (modulation transfer function, an index of surface quality use in the industry), of the lenses (Table 3-5, Fig. 3-24). The commonly applied criterion that an IOL resolve in air at least 60% of the Rayleigh cutoff spatial frequency corresponded to a minimum requirement of 0.43 MTF units at 100 mm⁻¹ in a model eye (EN/ISO-11979-2) [89]. Current ISO and ANSI standards allow a tolerance of ± 0.50 D for IOLs of 15.00 D to 25.00 D [90]. These parameters remained within the pre-established industrial tolerances after IOL modification.

Sample name	MTF	Theoretical diopter, D	Measured diopter, D	
HA25 n° 1	0.64	20,50	20.35	
HA25 n° 2	0.65	20,50	20.78	
HA25 n° 3	0.65	21,00	21.08	
HA25 - RGD ads n°1	0.60	22,50	22.71	
HA25 - RGD ads n°2	0.65	22,50	22.23	
HA25 - RGD ads n°3	0.63	18,50	18.38	
HA25 - RGD graft n°1	0.65	22,50	22.62	
HA25 - RGD graft n°2	0.61	15,50	15.57	
HA25 - RGD graft n°3	0.65	21,00	21.10	
Acceptable tolerances: MTF > 0.43 : theoretical diopter $\pm 0.4D$				

Table3- 5 IOLs analyzed with an optical bench. The numbers indicate the serial number of the IOL in corresponding group.



Fig.3- 24 Images of neat and modified IOLs analyzed with an optical bench

3.10 Mechanical properties tests

For the following mechanical properties tests, the real IOLs samples, rather than the disks, prepared by plasma EDC/NHS method and Ultrasonication Washes protocol are used. The concentration of the peptide solution in immobilization step is 1 mM.

3.10.1 Haptic compression force

The haptic compression force is the force that haptics applies to the capsular bag. The haptics are designed to fix the IOL inside the capsule bag. Given the intrinsic deformability of hydrophobic and hydrophilic acrylic IOL materials, lens haptic compress in order to compensate capsular bag variations. The test result (Fig. 3-25) shows that the forces of different RGD immobilized IOL exhibit similar compression force to different test wells compared to the initial IOL (statistically non-significant by 1-way ANOVA test).



Fig.3-25 IOLs analyzed with haptic compression force

3.10.2 IOL injection force

The IOL injection force is the applied force to inject the IOL to the capsular bag from the injector. This force may change with a different injector, IOL geometrical design, buffer system, material water uptake nature, and surface friction. The higher the injection force, the more risk of damage in the IOL (scratches, haptic or optic cracks) or in the injection system (cartridge break). No damage was observed in both optic and haptic part of IOL and all forces are close to 14N, which is within the applied industrial criteria for this material and lens model. The test result shows that RGD immobilized IOL exhibit flexibility compared to the initial lens (Table 3-6).

Sample	Diopter	Injector	Viscoelastic gel	Optic damage	Haptic damage	Injection force (N)
HA25	19.0-	Accuject 2.2 –	Integral	Ν	N	13,87
IOL	22.0	1P				
RGD Ads	19.0- 22.0	Accuject 2.2 – 1P	Integral	Ν	Ν	14,41
RGD	19.0-	Accuject 2.2 –	Integral	N	Ν	13,64
Graft	22.0	1P				

Table3- 6 IOLs analyzed with injection force

3.11 Contact angle measurement by liquid-droplet method

To verify if the surface functionalization altered the hydrophilicity of the polymer, the water contact angles at the surface of the disks were measured after RGD peptide grafting. Samples of different RGD functionalized surface were tested by the water-droplet method (Fig. 3-26). The surfaces were hydrophilic and presented the contact angles between 61° to 54°. The contact angle of the peptide adsorbed samples and untreated HA25 disk were not statistically significant using ANOVA test, indicating that the surface hydrophilicity is approximately the same. The plasma treated surface, however, became more hydrophilic. In addition, the RGD peptide grafted surface became even more hydrophilic than the virgin or plasma treated surface. The contact angle measurement result suggests that the oxygen plasma-based peptide grafting method may slightly make the surface more hydrophilic. In addition, the hydrophilicity nature of the peptide may also contribute the surface hydrophilicity.



Fig.3- 26 The water contact angle of the neat and modified disks (The peptide concentration during the coupling reaction is 1 mM)

Chapter 4. Discussion

4.1 Peptide immobilization strategies

4.1.1 Selection of RGD peptide surface modification for bioadhesive IOL material to prevent PCO

The PCO incidence is high related with the selection of IOL materials. The hydrophobic acrylic IOL is reported superior than hydrophilic acrylic IOL in PCO prevention aspect [2]. Despite this advantage, the hydrophobic acrylic IOL suffers from a higher rate of glistening [1]. Glistening is induced by temperature fluctuations, sterilization techniques and packaging, which promote the condensation of excessive water in the microvoids of the bulk material on cooling [91]. Unfortunately, this physical phenomenon is hard to control and the malfunctioned IOLs are needed to be explanted and replaced. Recently, there are glistening-free products such as GF (PhysIOL, Liège, Belgium) [91] and enVista (Bausch & Lomb, Rochester, NY, USA) [92] available in market. However, it is still interesting to develop a strategy based from the hydrophilic acrylic materials because this material takes other advantages such as controlled unfolding and longer history [1].

The bioadhesive IOL, shown in Fig. 1-21, is proposed according to some facts and the sandwich model theory. Firstly, the hydrophobic acrylic polymers attract more cell adhesion [17]. Secondly, intraocular lenses made from the hydrophobic acrylic material are reported low incidence of PCO [1-3, 15]. The sandwich model suggests that the IOL and the capsular bag form a closed system in which the LECs do not migrate (Fig. 1-16). From abovementioned description, two types of bioadhesive IOL can be designed: the adhesion between IOL and capsular bag is direct or indirect (via LECs).

For the direct adhesion design, the key factor is to increase the IOL's adhesion force to the lens capsular bag. Since the capsular bag is mainly composed by collagen IV [10], a high affinity molecule is needed to be presented onto the IOL surface. It is known that integrin [93], laminin [94], TGF-β1 [95]...etc. may bind to collagen IV. However, as introduced in Section 1.7, use of proteins is a challenging task. In addition, during cataract surgery, it is difficult to remove all the LECs [38]. As the cells migrate to the interspace between the IOL and the capsular bag, if the cells cannot attach well onto the surfaces and form cell-cell junction, the cells can still over-proliferate, transdifferentiate, and provoke PCO.

For the indirect adhesion design, the key factor is to attract the LEC adhesion to the IOL surface. There are integrins onto cells surfaces. Therefore, the binding between integrins and collagen IV may help to form the adhesion force between the lens capsular bag and the LECs. Similarly, as we functionalize the RGD peptides, the binding between integrins and RGD peptides may help to form the adhesion force between the lens IOL and the LECs. Via the LECs, the IOL and the capsular bag could form a closed system to prevent cell migration. In addition, since the IOL surface promotes the cell adhesion, more LEC can attach well onto the surfaces and form cell-cell junction, and therefore inhibit EMT and control PCO.

There is another reason to adapt surface functionalization technology in PCO control: the surface modification strategy is superior to the pharmaceutical drug loading methods in the dose control perspective [96]. For the patients applying higher diopter, the IOL is thicker and the volume to surface ratio is higher. Therefore, the drug releasing kinetics and the overall drug loading dose may vary case by case, which is hard to standardize in manufacturing by industry and regulation by government. Therefore, we further convinced the surface functionalization of RGD peptide to create a bio-adhesive surface is a simple and safe way to PCO control.

As for the RGD peptide sequence selection, it is known that "RGD" is the minimal functional motif to promote cell adhesion [69]. However, it is also reported the sequence "GRGDSP" has higher activity by inhibition assay [97]. Therefore, this sequence was selected in the experimental design. On the other hand, as the need of

fluorescence to trace the peptide, residue G was replaced by K to conjugate the FITC fluorophore via the side chain (Fig. 2-4). In addition, for the potential grafting chemistry design and XPS characterization, the sulfhydryl group containing residue C was incorporated into the peptide sequence although this design was not used though this thesis study. In addition, this peptide sequence KRGDSPC is used in Dr. Durrieu's laboratory and showed good biological function to promote cell adhesion [98].

4.1.2 Efficiency comparison of different grafting methods

4.1.2.1 Tresyl chloride method

The use of tresyl chloride to activate the polymer surface to graft RGD peptides was proposed by Hubbell's team [79]. The peptide GRGD (Fig. 2-7) is covalently attached on surface of polymer including pHEMA and modified PET. The RGD functionalized polymer surfaces showed better cell spreading ability comparing untreated polymer surfaces. The peptide grafting chemistry involves two stages. First, the activation of hydroxyl group on the polymer surface by tresyl chloride: This stage requires dry ether to provide aqueous-free environment and triethylamine to neutralize the byproduct hydrochloric acid. Second, the conjugation by peptide solubilized in the sodium bicarbonate buffer: This stage begins with the rinse of sodium bicarbonate buffer to remove the organic solvent, and incubate the polymer film in the same buffer with 80 ng/mL (~0.2 μ M) GRGD peptide for 20 hr. The amine group of the peptide reacts with the activated hydroxyl group and forms a covalent linkage without spacer (Fig. 2-7). In our case, the peptide concentrations were increased from 10 μ M to 20 μ M (Fig. 3-1, Fig. 3-2).

In order to know if this method functions, fluorescein amine (Fig. 2-8) was selected as a model molecule because this compound has an amine group similar to the peptide. In addition, this compound is the building unit of the FITC-RGD peptide. If samples modified by this compound can be observed under fluorescence microscope, the FITC-RGD modified samples might be the same, too.

From the pre-test result (Fig. 3-1), the increase of fluorescence background in fluorescein amine treated suggests that the tresyl chloride method may work. The polymer disks remain transparent and colorless after the grafting reaction (Fig. 3-2, upper).

4.1.2.2 Permanganate oxidation - EDC/NHS method

In 1996, Boxus et al. proposed the permanganate oxidation method to covalent coupling of adhesive proteins on PET membranes developed as supports for cell cultivation [99]. This method was further modified in Durrieu's team for the surface functionalization of cell adhesive peptides on PET films [100-102]. The scopes of the studies on these PET films include osteoblast differentiation (orthopedics application) [101, 103] and endothelium adhesion (vascular research) [100].

This coupling method involves three stages. First, the oxidation of hydroxyl group on the polymer surface to carboxyl group by KMnO₄: sample is incubated in the solution of KMnO₄ dissolved in H_2SO_4 at 60°C, and further washed by 6N HCl and deionized water at 60°C. Second, the activation of carboxyl group by EDC and NHS: the sample is incubated in the EDC/NHS at 4°C for 24 hours followed by deionized water rinse. Third, the conjugation by peptide solubilized in deionized water: the sample is incubated in peptide solubilized in deionized water.

Different from the tresyl chloride method, this permanganate oxidation – EDC/NHS method transforms the hydroxyl into carboxyl group, and use the coupling reagent EDC and NHS to create amide linkage between peptide and the polymer. This method uses harsh condition (strong oxidant, high concentration of acid, and heating) to prepare the polymer.

From the test result in Fig. 3-3, with FITC-RGD as model molecule, the increase of fluorescence background implies possible graft by this method. However, the opacification of the polymer was observed during the 6N HCl acidification step (Fig. 3-4).

This phenomenon limits the ophthalmological intention of the polymer because the opacification will significantly reduce spectral transmittance, resolving power (modulation transfer function), contrast sensitivity, and block the vision.

With the knowledge of chemistry, two possibilities are proposed. Firstly, the internal ester bonding (Fig. 2-1) was also hydrolyzed during the 1 hr oxidation step in H_2SO_4 at 60 °C. This may lead the polymer surface loss of homogeneity and become opaque. Secondly, the acidification of the polymer with 6N HCl reaction may be too rapid to burst chloride gas formation, and trapped into the internal space of the polymer as bubbles with the following reaction:

$$2KMnO_4 + 16HCl = 2KCl + 2MnCl_2 + 5Cl_2 \uparrow +8H_2O$$

To avoid chloride gas formation, an extra washing step before 6N HCl acidification can be introduced. Incubate the KMnO₄ oxidized sample with deionized water at 60 °C for at least 1 hour might be able to remove the excess KMnO₄. According to the equation, the chloride gas will not be generated if there is no KMnO₄.

Since the PET films are thin and the transparency is not a problem for the study in orthopedics application and vascular research, the protocol is maintained and works well in Dr. Durrieu's team. However, with our thick sample requiring transparency, this method is not suitable for the HA25 polymer.

4.1.2.3 CDAP method

CDAP (1-cyano-4-dimethylaminopyridinium tetrafluoroborate) is known to cyanylate hydroxyl [104] or sulfhydryl [105] group to form an activate intermediate. This activate intermediate can further react with primary amine and release DMAP (4-Dimethylaminopyridine) (Fig. 4-1, path C) [81]. The ability of cyanylation of sulfhydryl group makes CDAP a unique cysteine-labeling reagent. CDAP has been reported to be reactive between pH 2 and pH 8 with a maximum labeling efficiency at pH 5 [106]. This activity under acid condition avoids potential thiol-disulfide exchange and provides an advantage over other sulfhydryl labeling reagents to investigate protein structure.



Fig.4- 1 Principles of membrane modifications with 1-cyano-4-dimethylaminopyridinium (CDAP) [81]

On the other hand, the ability of cyanylation of hydroxyl group makes CDAP a good crosslinking reagent. CDAP has been reported to conjugate various proteins to polysaccharides including glucose oxidase [81, 107], BSA [108-110], and flagellin [68]. CDAP is reactive between *p*H 7 and *p*H 9 with a maximum activation efficiency at *p*H 9 [109]. The amino group of the proteins reacts with the CDAP activated hydroxyl groups of the polysaccharides and forms isourea bond linkage (Fig. 2-10). Moreover, this

coupling reagent is also reported to immobilize glucose oxidase onto plasma treated poly(etherurethaneurea) [107]. This study suggests that the biomolecules can be conjugated onto the artificial polymer surface by CDAP.

The advantage of CDAP method is that, unlike tresyl chloride, the reaction works in aqueous system (Fig. 2-10). Being an aqueous system, there is less possibility for the polymer material shrinking/ swelling or polymer surface been damaged by solvent [70]. Nevertheless, the activation level of CDAP can be evaluated by alkaline hydrolysis (Fig. 4-1, Path B) [81]. Combining with the result of peptide surface density determination methods, the turnover ratio (final immobilized amount / activated intermediate amount) can be calculated and be used to optimize the peptide grafting reaction. However, the byproduct DMAP is highly toxic and is particularly dangerous because of its ability to be absorbed through the skin (Material Safety Data Sheet). Therefore, a dedicated wash protocol is needed to reduce the potential hazardous effect. In addition, DMAP is also known produce undesirable levels of racemization (ie. generation of enantiomers from the amino acid of the peptide), which may lead loss of biological activity of the peptide [111].

From the fluorescence imaging results (Fig. 3-5, Fig. 3-6), the increased fluorescence after CDAP/FITC-RGD treatment suggests that this method can immobilize peptide. Use of enhanced fluorescence level (for example, CDAP (+)/CDAP (-) comparing to HOBt (+)/HOBt (-) in Fig. 3-6) as a non-quantitative indicator, it is suggested that that the ranking of the efficiency in peptide immobilization is Plasma-EDC/NHS > CDAP > HOBt.

On the other hand, from the cell adhesion results (Fig. 3-6), the increased amount of attached LEC on the CDAP/FITC-RGD treated HA25 disk suggests that the immobilized peptide is biologically functional. Take the number attached LEC as an indicator, it is suggested that the ranking of the efficiency in active peptide immobilization is Plasma-EDC/NHS> CDAP > HOBt.

In Fig. 3-6, the amount of attached LEC on CDAP sample is obviously lower than the Plasma-EDC/NHS sample. In addition, from the green channel, the fluorescence signals from FITC-RGD also exhibit the amount of immobilized peptides on CDAP sample is obviously lower than the Plasma-EDC/NHS sample. These results suggest the CDAP efficiency is low. In order to enhance the efficiency, there are several means from the literature to be considered: increase ligand (peptide, in our case) or CDAP concentration, increase activation and coupling time, change activation buffer system and *p*H [109].

Adapted from the published protocol [81], two factors were tested during the study: CDAP concentration and activation time (Table 3-3 and Table 3-2). The results indicate increasing of CDAP concentration increases the amount of activated hydroxyl group (Table. 3-3) and increasing of CDAP activation time slightly increase the immobilized peptide (Table. 3-2). The results fit the trend predicted from the literature (Fig. 4-2, Fig. 4-3) [81, 109]. However, even with near the best condition (200 µL CDAP, 24hr activation time) of the tested conditions, the efficiency is still far lower than Plasma-EDC/NHS method (Fig. 3-6).



Fig.4- 2 Effect of increasing CDAP concentration on protein BSA and dextran coupling efficiency [109]. (DEX: dextran)



Fig.4- 3 Kinetics of direct coupling of BSA to CDAP activated-dextran [109].

4.1.2.4 HOBt method

HOBt (Hydroxybenzotriazole), together with EDC (1-Ethyl-3-(3dimethylaminopropyl)carbodiimide), is able to create ester from hydroxyl group and carboxyl group [112]. As a result, HOBt is a common crosslinking reagent in peptide coupling reactions [111]. Moreover, this method is also applied to immobilize HA (hyaluronic acid) onto PCL (poly(ε-caprolactone)) surface [113], suggesting that this method is able to conjugate biomolecules onto the artificial polymer surface. In addition, this method is used to immobilize IgG to a hydroxyl-bearing polymer [82].

Similar to CDAP method, the HOBt method works in aqueous environment. The feature of HOBt is that the reaction activates the carboxyl group of the peptide first, and then the activated peptides couple with the hydroxyl group of the polymer and form covalent bond (Fig. 2-11). Carbodiimide activation of amino acid derivatives often causes a partial racemization of the amino acid. Therefore, adding an equivalent of 1hydroxybenzotriazole (HOBt) is proposed to minimize this problem [114]. From the fluorescence imaging results (Fig. 3-5, Fig. 3-6), the increased fluorescence after HOBt/FITC-RGD treatment suggests that this method can immobilize peptide. However, the enhanced fluorescence level is relatively low. The results suggest that the ranking of the efficiency in peptide immobilization is Plasma-EDC/NHS > CDAP > HOBt. On the other hand, from the cell adhesion result (Fig. 3-6), the increased amount of attached LEC on the HOBt/FITC-RGD treated HA25 disk also suggests that the immobilized peptide is biologically functional. However, the attached cell number is low. The result suggests that the ranking of the efficiency in active peptide immobilization is Plasma-EDC/NHS > CDAP > HOBt.

4.1.2.5 Plasma-EDC/NHS method

Considering the low performance of the aqueous crosslinker systems mentioned above, the oxygen plasma, or radio frequency glow discharging (RFGD) method was applied. Using plasma treatment to modify polymer surfaces has several advantages such as enhanced surface wettability [115], improved biocompatibility [116], surface functionalization by molecular immobilization [117], and promotion of cell adhesion [62]. Moreover, by adjusting the parameters of plasma treatment (time, temperature, power) and the type of gas (N₂, O₂, Ar), the status (wettability, chemical structure, biocompatibility) of the polymer surface can be improved. This technique is particularly attractive because this technique could induce specific functional groups on the polymer surface to fulfill the biomaterial engineering needs [54].

The plasma treatment can modify polymer surfaces in different ways: etching, cleaning, activation, cross-linking, and implantation, which specifically means incorporating elements (in this case: oxygen) in the chemical structure of the polymer [118]. The advantages of applying plasma treatment in the surface modification are reliability, reproducibility, relative cost-effectiveness, and applicability to different sample geometries as well as different materials such as metals, polymers, ceramics, and composites [112]. In the ophthalmological field, the plasma treatment has been shown to be able to graft molecules such as Ti, TiN, and PEGMEM (Poly(ethylene glycol) methyl

ether methacrylate) to prevent LEC adhesion [56, 59]. Nevertheless, the oxidization of acrylic IOL by UV/O_3 could also increase LEC adhesion, inhibit LEC proliferation, and therefore possibly control PCO in rabbit model [119].

Theoretically, oxygen plasma imparts oxygen containing functional groups to polymer surfaces (Fig. 2-13) [120]. In addition to oxygen plasma, CO₂ plasma is used to introduce carboxyl groups on different polymers such as PP, PS and PE [76, 121, 122]. Moreover, the air plasma is reported to oxidize PMMA [123]. As for the grafting chemistry designed here, EDC/NHS form the amide linkage between amine and carboxyl groups. It is also possible to introduce amine group on polymer by NH₃ or N₂ plasma [76, 124]. The EDC/NHS activates the amino group of the N₂ plasma treated surface and conjugates with the carboxyl group of the peptides (Fig. 2-2, Fig. 2-3, Fig. 2-4, and Fig. 2-5) or the model molecule 6F-Val (Fig. 2-6). Therefore, there is more than one gas can be used in the plasma treatment process.

However, the carboxyl group from HA25 polymer cannot react with the amine group from the peptide at room temperature in theory. Therefore, in order to promote covalent amide bond formation, the aqueous EDC/NHS coupling reaction is introduced. The EDC/NHS coupling method is widely applied in biology research and the detailed reaction mechanism is known. The conjugation can be made from the amide bonding formation between the amine group of the RGD peptide and the carboxylic acid group of the oxygen plasma treated polymer [80, 100-103, 125-130]. Therefore, the stepwise process is applied to our sample preparation method to graft RGD peptide on HA25 surface (Fig. 2-13).

The test of plasma-EDC/NHS suggests that the reaction increases the fluorescence signal onto the HA25 polymer disks (Fig. 3-6, Fig. 3-7). From the fluorescence imaging result in Fig. 3-6 (green channel), the increased fluorescence after plasma-EDC/NHS treatment suggests that this method can immobilize peptide. In addition, the enhanced

fluorescence level is relatively high. The results suggest that the ranking of the efficiency in peptide immobilization is Plasma-EDC/NHS > CDAP > HOBt.

On the other hand, from the cell adhesion result in Fig. 3-6 (Blue/Red channel), the increased amount of attached LEC on the Plasma-EDC/NHS/FITC-RGD treated HA25 disk also suggests that the immobilized peptide is biologically active. In addition, the attached cell number is high. The result suggests that the ranking of the efficiency in active peptide immobilization is the same to above-mentioned.

4.1.2.6 Summary of efficiency comparison of different grafting methods

The results and the evaluation of all the tested methods are shown in Table 4-1. To summarize, the plasma-EDC/HNS method seems to be most efficient from the results. The peptide immobilized surfaces remain transparency and homogenous. The cell adhesion assays also illustrate that LEC are evenly distributed onto the surface. Therefore, the plasma-EDC/HNS method was chosen for further investigation.

Method	Fluorescence	Cell	Light	Evaluation
	Intensity	adhesion	transmission	
TsCl	+	Not tested	transparent	Non-aqueous system
KMnO ₄ / EDC-	+	Not tested	opaque	Not able to use
NHS				
HOBt	(+/-)	(+/-)	transparent	Low efficiency
CDAP	++	+	transparent	Low efficiency
plasma/EDC-	++++	+++	transparent	High efficiency;
NHS				selected for further
				test

Table4-1 Summary of the test results and the evaluation of peptide grafting methods

4.2 Disk cleaning method

Protein adsorption on surface and its interaction are major concerns in numerous fields such as biology, medicine, biotechnology, and food processing. The important factors of the field of design of biocompatible materials include adsorbed amount and selectivity for adsorption [131]. The adsorbed amount is affected by various factors, for example, properties of proteins (charge, size, structure stability, amino acid composition, steric conformation), properties of the solid substrate surface (charge, chemical composition, hydrophilicity, roughness, porosity), and environmental conditions (pH, temperature, ionic strength) [40, 131, 132]. Since peptide and protein share the same amino acid building units, the peptide adsorption could be understood in a similar way.

From the literature, the most common clean method after peptide surface functionalization is rinsing shortly with deionized water, PBS, or conjugation buffer [83, 86, 133-136], which is similar to Aqueous Washes protocol (Section 2.3). Since the designed peptide functionalized HA25 is designed for implantable medical device IOL, desorption of bioactive peptides into body circulation may potentially cause deleterious reaction. As a result, adsorbed peptides have to be removed as possible as we can. A more stringent method has to be added into the disk cleaning method. Literature shows the change in *p*H and ionic strength could possibly facilitate protein desorption [137]. However, in our case, use other chemical substance to remove the peptide may also lead another contamination. Therefore, use of the deionized water combining other physical method is preferred.

Ultrasonication and elongation of rinsing time were then applied into the protocol. Ultrasonication is often used to reduce the extraction time. The advantages of ultrasonic extraction include wide solvent selecting range, rapid and reproducible, cost-effective, low risk for hydrolysis breakdown, and least traumatic [138]. This method has been applied to extract substance from polymers [139]. On the other hand, increase extraction time is also a common method to enhance extraction [139]. Elongation of rinsing time (up to weeks) to extract peptides from polymers has been proposed [101, 126, 140]. However, even combining these two factors, the adsorbed peptides remain considerable quantity on the surface of HA25 (Fig. 3-9).

As a result, a more stringent protocol by applying autoclave is further proposed. Autoclave is typically used to sterilize equipment and supplies by subjecting them to high pressure saturated steam at 121 °C for around 15–20 minutes. To promote the efficiency of peptide extraction, the duration time is elongated to 45 min (maximum time of the instrument). In the field of surface functionalization studies, RGD peptide has been reported resistant to autoclave sterilization [70, 83]. From Fig. 3-10, a great amount of peptide is removed from the HA25 polymer. In Fig. 3-11, after the multiple autoclaving, the peptide grafted surface retains cell adhesion promoting ability. Combining together, it is suggested that both polymer and peptides resist to, in a certain level, hydrolysis breakdown potentially caused by heating.

The immobilized RGD peptides remaining active after autoclaving suggests that the surface functionalized HA25 material is able to be sterilized by autoclave. Concerning the industrial sterilization of IOL, the gamma irradiation could lead to partial oxidative degradation or to form carcinogenic compounds [91] within the material of IOL or of its packaging, and the ethylene oxide (EO) sterilization is associated with a risk for highly toxic residual contaminants, such as EO or epichlorhydrine. It seems that the autoclaving method is superior for IOL sterilization because of material-friendly and long history in the medical field [91]. The fact that the immobilized RGD peptides remain active after autoclaving suggests that the surface functionalized HA25 disk is able to be sterilized by autoclave.

Considering the industrial utilization in future, it will be better if the procedures of manufacturing surface functionalized IOLs can be automated. Based on this result of autoclave-assisted extraction, it could be further improved by soxhlet extraction, which is widely used for solid sample extraction. The principle of soxhlet extraction is continuous extraction of a solid by repeated boiling-condensation cycles of a solvent. The extraction fluid is therefore continuously refreshed. Therefore, applying soxhlet extraction could take advantages of low cost, large sample size, no reverse diffusion back to polymer, unattended operation, and automation [139].

On the other hand, applying consecutive autoclaving for extraction in disk cleaning step also challenges the peptide grafted surface. Although report indicated the sterilization by autoclaving might render breakdown of the peptide bond [141], however, from our experimental data, the disks after 10 cycles of autoclave challenge showed decrease in the amount of eluted peptides but retain the ability to promote cell adhesion (Fig. 3-11 and Fig. 3-19). This challenge would provide an opportunity to examine not only the stability of the biological function of the peptide but also the robustness of the peptide immobilized surface in harsh condition. The observation that the disks remain clear after raising temperature to 121°C and back to room temperature provides a hint to further experimental design in medical device industry. Firstly, from ISO 11975-5 (2006), the test for hydrolytic stability requires at least five years or using a simulated exposure time by multiplying the actual study time with the factor $2.0^{(Ta-To)}/10$, where Ta is the accelerated temperature and To is the temperature of the inside of the eye (35°C). In addition, from ISO 11979-6 (2006), the real-time shelf-life is calculated by multiplying the studied time period with the factor of $1.8^{(Ta-To)}/10$, where Ta is the accelerated temperature and To is the typical storage temperature (usually room temperature). Therefore, the heat compatibility of the RGD immobilized HA25 polymer enables the accelerated study in R&D process in IOL industry.

4.3 Surface density determination

In this section, the peptide surface density determination methods (ninhydrin, sulfo-SDTB) and the CDAP activated hydroxyl group quantification by alkaline hydrolysis are discussed.

4.3.1 Ninhydrin method

Ninhydrin (2,2-Dihydroxyindane-1,3-dione) is a chemical used to detect ammonia or primary and secondary amines. When reacting with these free amines, a deep blue or purple color known as Ruhemann's purple is produced (Fig. 2-14). Ninhydrin is commonly used to detect fingerprints. The terminal amines of the peptides and proteins, which are sloughed off in fingerprints, could react with ninhydrin. Ninhydrin assay has been proposed to characterize the aminated poly(D,L-lactic acid) (PLA) [142], aminated polycaprolactone (PCL) [135], RGD grafted polyethersulfone (PES) [143] or poly(dimethylsiloxane) (PDMS) [83]. In our case, the peptides immobilized onto the HA25 polymer were hydrolyzed to alpha amino acids in an acidic environment. The hydrolyzed alpha amino acids contain primary amines which can react with ninhydrin. Then the extracted solutions were mixed with the ninhydrin reagent to precede the coloring reactions.

With the simplest cleaning method (Aqueous Washes), the ninhydrin reagent mixture can change the color after heating. This method was success to quantify the peptide surface density (Fig. 3-12). From the result calculated, the surface density of RGD peptide falls into the range around 10 nmole/cm² (60 molecules/nm²). Considering the average size of the peptides, diameter of the RGD peptide is estimated 1.3 nm and that of the FITC-RGD peptide is around 1.5 nm. In this context, the calculation suggests that the peptide covered area is more than surface area, which can be explained the grafted or sorbed peptides have penetrated into the inner space of the polymer. From the literature, the plasma treatment can penetrate around 10 nm in depth [122, 144] and the wet-chemistry method could go further. Therefore, the calculated peptide surface density can be considered as the available amount of peptide near the surface.

On the other hand, as long with the cleaning procedure improvement (Ultrasonication Washes and Autoclave Washes), the method cannot react with sufficient α -amino acids and make color change. Therefore, it is speculated that the quantity of the surface immobilized peptides has reached the limit of detection of ninhydrin.

The limit of detection of ninhydrin may vary from different amino acids. The overall limit of detection falls in sub-microgram range (Table 4-2) [66]. Take 0.25 μ g as minimal detection limit of the RGD peptide and transform into surface density, the minimal detectable surface density is 0.082 nmol/cm². As obtained previously (Fig. 3-12), the surface density of simple washed (rich in physically adsorbed peptides) samples is around 100 fold to the detection limit, which is able to be quantified (ie. 25 μ g RGD peptide been reacted with ninhydrin). As extraction step applied (Fig. 3-9), the majority of the peptide was extracted out (around 25 μ mole FITC-RGD, equal to 31.6 mg) and the final amount of the surface bound peptides is lower than the minimal detection limit, leading the failure of coloration reaction of ninhydrin.

Amino acids	Detection limit	Amino acids	Detection limit
Alanine	0.009	Histidine	0.05
Valine	0.01	Arginine	0.01
Leucine	0.01	Phenyl alanine	0.05
Isoleucine	0.2	Tyrosine	0.03
Serine	0.008	Tryptophan	0.05
Threonine	0.05	Cysteine	0.02
Aspartic acid	0.1	Cystine	0.01
Asparagine	0.1	Methionine	0.01
Glutamic acid	0.04	Proline	0.1
Glutamine	0.1	Hydroxy proline	0.05
Lysine	0.005	Glycine	0.001

Table4- 2 Detection limit (μ g)of ninhydrin to each amino acid [66]

4.3.2 Sulfo-SDTB Method

The sulfo-SDTB method is proposed to quantify the amine groups on polymers, for example, aminated polyetheretherketone (PEEK) [145], aminated polytetrafluoroethylene (PTFE) [86], and aminated silicone [146]. Sulfo-SDTB can react with the primary amine and form 4,4'-dimethoxytrityl cation with a high extinction coefficient of 70000. The detectable range of this product was found to be from 0.33 to 20.5 nmol/mL [46]. The typical calibration curve from the literature (Fig. 4-4) [46] illustrates that the values of absorbance at 489 nm are from 1 to 0.01. In addition, it is reported that, quantified by sulfo-SDTB, the amino group surface density of aminated PEEK surface is about 0.6 ±0.2 nmol/cm² [145]. Other report applying this method to detect the surface APTES ((3-Aminopropyl)triethoxysilane) density to immobilize RGD peptide shows the detection limit of surface density is around 0.1 nmol/cm² [54].



Fig.4- 4 Standard curve for sulfo-SDTB reaction [46]

As for the experimental design, since the extinction coefficient of 4,4'-dimethoxytrityl cation has been demonstrated and the analysis protocol has been reported [86], the samples prepared by CDAP method were taken to quantify the grafted RGD surface density without the generation of standard calibration curve. As shown in Table 3-1, the raw absorbance values are too low and fall out of the reliable range between 1 and 0.01. In addition, according to these values, the result of calculation indicates that the surface

densities of amine group onto different samples are around 0.02 to 0.06 nmole/cm^{2}, which is lower than the reported detection limit of 0.1 nmol/cm^{2}.

The negative Sulfo-SDTB data may be resulted from the failure of grafting or Sulfo-SDTB itself. Therefore, with the help of colleagues, a sample of RGD peptide grafted onto Si surface by APDMS (3-aminopropyldimethylethoxysilane) and SMP (3-succinimidyl-3-maleimido-propionate) with density of 0.13±0.07/nm2 (0.02±0.01 nmol/cm2) [147] was applied as a control. This control sample shows good cell adhesion promotion ability. Therefore, it is a good (and the only available) positive control to compare with the RGD immobilized HA25 disks. Despite the design of the positive control in this assay, the experiment again shows negative result because the surface density of this control is still lower than the detection limit.

4.3.3 Alkaline hydrolysis method

As discussed in 4.1.2.3, CDAP is able to cyanylate hydroxyl group to form an activate intermediate [104]. The activate intermediate can further react with primary amine to graft the peptide and release DMAP (Fig. 4-1, path C) [81]. However, this activate intermediate can also release DMAP in alkaline environment (Fig. 4-1, path B) [81]. This unique feature among all the test peptide immobilization method provides a chance to evaluate the efficiency of the first step of the peptide grafting process.

According to the literature, the released small molecule DMAP has high extinction coefficient of 14,600 M^{-1} cm⁻¹ at 282 nm [70]. A publication of conjugating enzyme glucose oxidase (160 KDa) onto the cellulose membrane studied the effects of activation time and CDAP amount. For the activation time, the plateau of activation level is reached around 120 nmol/cm² with the reagent-to-surface ratio of 13.5 µmol CDAP/cm² while the activation time is elevated to 2 min. As for the CDAP amount effect, the plateau of activation level is reached around 250 nmol/cm² with 2 min of activation time while the reagent-to-surface ratio is elevated to around 40 to 50 µmol CDAP/cm² [81]. This research suggests that the maximum activation level of hydroxyl group can be reached by increase of CDAP amount and activation time.

The abovementioned two factors are similar to the factors discussed in Section 3.2.1.4. However, the activation level of hydroxyl group and the coupling level (Fig. 4-2, Fig. 4-3) are different concepts. The coupling reaction of CDAP comprises the activation of hydroxyl groups and the conjugation of amine containing molecules. The alkaline hydrolysis method provides the information of first stage whereas the ninhydrin method provides the information of overall reactions.

The raw data of absorbance of alkaline hydrolysis in Table 3-2 falls into the linear range of spectrophotometry with the negligible low value of blank. Therefore, the data is qualified for further calculation. For the reagent to surface ratio, we use 10.6 μ mol CDAP/cm², which is comparable to the value of 13.5 μ mol CDAP/cm² from the literature. The activation levels are found to be 113 and 235 nmol/cm² for the samples of 1 hr and 24 hr activation, respectively, indicating the activation level is comparable to the literature, but can be even improved by elongating activation time.

On the other hand, some raw data of absorbance of alkaline hydrolysis in Table 3-3 falls out the linear range of spectrophotometry and the value of blank is not negligible. Therefore, the data are less reliable than those reported in Table 3-2. However, it is worthy to note the activation level of hydroxyl group increases along the increase of CDAP amount, which is corresponding to the literature [81]. On the other hand, it is suggested that the extinction coefficient of DMAP is known clearly, and the quantification protocol has been reported without utilization of calibration curve [81]. However, from the obvious difference of surface density between two independent experiments (Table 3-2, Table 3-3, CDAP 200 µL 1hr sample), the calibration curve should generated in parallel. Considering the turnover ratio (ie. the conversion ratio of CDAP activated intermediate to target-conjugated product), the authors use 1,8-diamino-2,6-dioxaoctane as a model molecule and obtained 92 nmol/cm² surface amino group by TNBS method (Fig. 4-1, path D). The turnover ratio is around 77%. As for our experimental design, we directly conjugated with RGD peptide without applying such model molecule. The surface density of the peptide was determined by ninhydrin and the turnover ratio is 13% and 7% for the samples of 1 hr and 24 hr activation, respectively. In addition, as we discussed previously (3.2 and 3.3.1), the major surface bound status of detected peptide is adsorbed rather than grafted. Therefore, the real turnover ratio may even less than 0.01 fold to what we have now.

The concentration of reactant might be able to explain this. The authors used large molar excess of 1 M 1,8-diamino-2,6-dioxaoctane in 1 M carbonate buffer (pH 9.5) overnight at room temperature to react with the surfaces. To us, it is impossible for precipitation formation over 10 mM in concentration. In addition, with the real target enzyme glucose oxidase (Fig. 4-1, path C), the authors obtained a value of 1.74 μ g/cm² (equal to 0.011 nmol/cm²) determined by enzyme activity. Although the amount of the real grafted enzyme must be higher, the turnover ratio is around 0.009%, which is even lower than what we have with the RGD peptide.

The alkaline hydrolysis method serves as a window to observe the activation level during the CDAP mediated grafting reaction. This protocol reported a normal range as literature obtained. However, the turnover ratio is quite low for biomolecules from the literature and our experimental data, which limit the application of CDAP method.

4.3.4 Summary of surface density determination methods

A summary of surface density determination discussed in this section is presented in Table 4-3. Among all the methods to quantify the molecules onto the HA25 surface, none of them is applicable for the too low surface density that below the detection limit of the methods. Despite the method of alkaline hydrolysis of CDAP works well in our study, the method is linked with CDAP method which has even lower grafting efficiency as we discussed in Section 4.1. In order to quantify the low surface density, a more sensitive method (around pmol/cm²) should be applied.

Alternatively, time-of-flight secondary ion mass spectrometry (ToF-SIMS) has been developed to quantify the surface density of functional groups or molecules on polymer [122, 124, 148]. The detection limit is known around the order of 0.01 nmol/cm² [149, 150]. Typically, ToF-SIMS measurement probes to ~1 nm depth, whereas the plasma treatment activates ~10 nm depth of the polymer [122, 144]. In addition, the general mass range of ToF-SIMS technique is between 0 – 10000 atomic mass units [151], whereas the molecules in our project including the surface carboxyl group (45 Da), the model molecule 6F-Val for XPS (225 Da), and the targeting peptides (762 - 1264 Da) fall into this range. In addition, applying ToF-SIMS to quantify surface density may help to characterize the spices of Si pollution and therefore provide more detailed information of the surface [122]. Although some researchers argue ToF-SIMS not a perfect tool for quantification [151, 152], this technique offers the complementary information to the conventional XPS surface analysis and the quantification results could serve as an estimation of the activation or grafting efficiency among different methods [76].

From the report, ToF-SIMS is used to quantify the carboxyl group of the CO₂ plasma treated polyolefin surface [122]. The carboxyl groups on the surfaces are pre-quantified by another UV-Vis spectrophotometry-based thionine acetate titration method and then subjected to the ToF-SIMS. The quantified carboxyl surface densities are around 0.5 nmol/cm². This report is particularly interesting because the polymer surface is functionalized with carboxyl group by plasma. In addition, the thionine acetate titration method is applicable in our system. Hence, the carboxyl group generated by plasma could be possibly quantified. Moreover, the authors estimate this method could detect down to 0.067 nmol/cm², which is close to what we are looking for.

In addition to ToF-SIMS, fluorescence dye labeling methods is proposed to determine the functional group on polymer surface [150]. Different functional groups can be quantified by applying different fluorescence dyes. For example, thionin acetate (THA), acriflavine (AFN), and ethidium bromide (EB) can be used for quantify carboxyl group by ion exchange reaction. Owing to the high sensibility, the detection limit by fluorescence dye can reach to pmol/cm² [144] or even fmol/cm² [149]. In addition, the conjugating of fluorogenic compound (for example o-Phthalaldehyde) on peptide might be also possible to quantify the amount of peptides as low as pmole range [153]. Moreover, it is also possible to design a cleavable dye labeled on the RGD peptide. The cleavage of the dye can be triggered and the dye can be quantified after grafting. For example, design a fluorenyl-methoxycarbonyl (Fmoc) group at the N-terminus and a cleavage site of elastase (Fmoc-A \downarrow ARGD) in the peptide sequence. When the Fmoc-A moiety released by enzyme digestion, the surface density can be calculated from UV absorbance in HPLC [134].

Method	Target	Detection	Result	Comment
		limit		
Ninhydrin	Amine group	~0.1	Able to quantify simply	Not suitable to use
		nmol/cm ²	washed samples but not able	in our system
			to quantify well washed	(detection limit)
			samples	
Sulfo-SDTB	Amine group	~0.1	Not able to quantify well	Not suitable to use
		nmol/cm ²	washed samples	in our system
				(detection limit)
Alkanline	Activated	~10	Able to quantify the CDAP	Not suitable to use
hydrolysis of	hydroxyl	nmol/cm ²	activated HA25 polymer	in our system (low
CDAP	group		(approaching detection limit)	grafting rate)

Table4- 3 Summary of the surface density determination methods

4.4 X-ray photoelectron spectroscopy (XPS) characterization

XPS analysis was performed to characterize the surface bound status (grafted or adsorbed) of the selected molecules or peptides and to verify the effectiveness of the sample preparation procedure. Typically, XPS measurement probes to around 10 nm depth, which is close to that of a functionalized layer created by plasma treatment [122, 144]. The XPS detection limit is estimated to be 0.01 pmol/cm² [154].

Unlike surface coating or grafting onto a thin-layered material [126, 155], our sample is a real intraocular lens or an about 1 mm thick, coin-sized disk. The measurement of ATR-FTIR and Raman spectrometry detects only the bulk polymer rather than the peptide (data not shown). However, the XPS characterization results in 6F-Val and RGD peptide experiments conclude that the Plasma EDC/NHS (Section 2.2.6) conjugation and Autoclave Washes (Section 2.3) washing step in our sample preparation procedure are critical and effective. In our system, the physical-chemical proof of surface biomolecule grafting is only available by XPS method.

4.4.1 Model molecule 6F-Val

The 4,4,4,4',4',4'-Hexafluoro-DL-valine (6F-Val) serves as a model molecule to verify if the covalent conjugation between target and the polymer surface exists and to examine the washing step can remove the physically adsorbed molecules onto the polymer surface. The model molecule 6F-Val is strategically selected for three crucial advantages. Firstly, fluorine contained compound is widely used in Chemical Derivatization XPS (CD-XPS) to quantify the functional groups which might be ambiguous in XPS spectrum (ether or alcohol for example) [156-158]. The higher content of fluorine made this molecule sensitive in XPS spectrum detection because fluorine is not normally present in the polymer. Additionally, this molecule is an alpha amino acid without active functional group on its side chain. If the grafting chemistry works in this model molecule, it should be transferable to the desired RGD sequence since this peptide is also made by common alpha amino acids. The third advantage is that this molecule contains only amine functional group. If the amide signal is detected in the system, the direct evidence of conjugation is obtained.

Data of the 6F-Val grafted surfaces in different washing stages illustrates the amide signal, which supports the successful conjugation (Fig. 3-13). The full washing steps include overnight in MilliQ water shaking, 1 hr sonication, and 10 cycles of autoclaving (Autoclave Washes, Section 2.3). As for the fluorine spectrum, the experimental data suggests the fluorine content is close to the detection limit and not able to be used as a quantitative index (data not shown).

As amide signal found in all steps of the samples, the conclusion of conjugation is made. In addition, the decrease of total nitrogen element percentage, the increase of amide/C-C and the decrease of (amine + C-CO)/C-C ratio along the washing stages imply the grafted 6F-Val become predominant (remove of adsorbed molecules with washing).

4.4.2 RGD and FITC-RGD peptide

XPS analysis was performed to characterize the surface bound status (grafted or adsorbed) of the RGD peptides and to verify the effectiveness of the sample preparation procedure. Biomimetic grafting was performed by means of a three-step reaction procedure: creation of COOH functions onto the HA25 surface using plasma treatment, grafting of the coupling agent, and conjugation of the RGD peptides. As shown in Fig. 2-13, this grafting method between the polymer surface and RGD or FITC-RGD resulted in an amide linkage.

The theoretical atomic compositions of RGD and FITC-RGD peptides are 56% C, 21% N, 21% O, 2% S and 64% C, 15% N, 19% O, 2% S, respectively (Table 2-1). On the other hand, RGD and FITC-RGD-grafted HA25 surfaces exhibited increased nitrogen element (Table 3-4) in comparison with virgin HA25 and RGD-adsorbed HA25.

As expected, the amount of nitrogen is higher in the case of FITC-RGD (grafted or adsorbed) than in the case of RGD (grafted or adsorbed). Each surface exhibits the expected elements with, additionally, a non-negligible pollution of Si, which has no impact on components including nitrogen. On the other hand, the virgin HA25 surface shows an N pollution (Table 3-4), which probably originates from additives (polymerization initiators or compounds used for blue filtering) [1], impurities, or contaminants upon storage and transportation [159].

The fitting components of C1s spectra and the N1s spectra are shown in Fig. 3-14 and 3-15. The C1s components were assigned according to the literature: C-C, 284.9 eV; C-CO + amine, 285.8 eV; C-O, 286.6 eV, amide, 287.6 eV; COOR, 288.9 eV [93, 160, 161]. As for N1s, the assignment is amine, 400 eV; amide 402.5 eV [160, 162, 163].

The peak fittings components of C1s spectra reflect the increased amide linkage between the peptides and the polymer (Fig. 2-13, Fig. 3-14, and Fig. 3-15).

Moreover, the amide/amine increases in the case of HA25 - RGD graft (0.219) in comparison with HA25 - RGD ads (0.143) (Fig. 3-16). The same statement is also applicable in the case of FITC-RGD peptide. The grafted sample exhibit higher amide/amine ratio (0.385) than the adsorbed one (0.345), further illustrating that the conjugation occurs between the polymer and the targeting molecules (Fig. 3-14, Fig. 3-15).

The N1s fitting peak also shows a more defined peak in the case of HA25 - RGD graft (in comparison with HA25 - RGD ads) with one amide and one amine components (Fig. 3-14). With FITC-RGD peptides grafted onto HA25, the amide component increases in comparison with HA25 - FITC-RGD ads (Fig. 3-15). The N1s peak of HA25 FITC-RGD ads is well defined considering that the peptide has an additional nitrogen percentage.

For the RGD and FITC-RGD data set, although existence of the amide peak (at 402 eV) cannot conclude conjugation, we still observe the amide peak increased in the grafted sample (Fig, 3-14, Fig. 3-15), which is also a complementary supporting evidence of conjugation as we have discussed in C1s fits. By combining C1s and N1s fitting results, we conclude that the RGD grafting process is effective to make the RGD surface grafted, rather than adsorbed, HA25 polymer.

4.5 Cell morphology and EMT biomarker expression of the culture cell lines

In vivo, cuboidal lens epithelial cells form a monolayer which overlies the fibers and is subjacent to the lens capsule. The cells are in the G_0 phase of the cell cycle after birth (Fig. 1-6) [35]. However, they also maintain the ability to replicate throughout life if stimulated by wounding or cell culture. Cultured mouse lens epithelial cells have a finite and well-characterized population doubling level, maintain a diploid set of chromosomes, are contact inhibited and grow as a monolayer [164].

During cataract surgery, the structure of the lens is broken and the residual LECs can become active in proliferation and migrate into the space between the posterior capsule and the IOL, which can lead to the secondary cataract or post capsular opacification. The LECs further undergo Epithelial-Mesenchymal Transition (EMT) and transdifferentiate to fibroblasts. The later cells express α -smooth muscle actin and secrete collagen I, III, V and VI which are not normally present in the lens. In addition to the gained mesenchymal biomarkers such as fibronectin, N-cadherin, and α -smooth muscle actin, the expression of epithelial biomarker are attenuated in the cells, such as E-cadherin, ZO-1, and cytokeratin [81]. In addition to fibrosis, EMT is also involved in invasive cancer metastasis process [165].

In order to verify the starting cells is epithelium and the antibody is specific, the lens epithelial cell lines were culture onto cell culture grade polystyrene surface compared with the epithelium MCF-7 cell and the mesenchymal MDA-MB-231 cell. The cells were fixed and stained with anti-E cadherin antibody (ab15148 or BD 610181, Table 2-3) and DAPI. The phase contrast images show HLE-B3 and LECp have the epithelial cell features such as clustering, cuboid shaped (Fig. 3-17).

During the study, the attempt of immunofluorescence staining against anti-E cadherin antibody (ab15148) shows no signal (data not shown) whereas the staining of anti-E

cadherin antibody (BD 610181) shows clear image (Fig. 3-17). This phenomenon may be resulted from the primary antibody (BD 610181) (e.g. unable to recognize antigen) or the secondary antibody (62270) (e.g. unable to recognize primary antibody or perished fluorescence). In order to clarify the source of this problem with the reagents currently available in the laboratory, another test on fluorescence with two anti-rabbit secondary antibodies (62270 and A-10039) was performed. The result shows strong fluorescence of both the tested secondary antibodies, suggesting that the anti-E cadherin antibody (ab15148) could be the reason of immunofluorescence failure despite we do not have a positive control of rabbit antibody to verify the effectiveness of the secondary antibodies (ie. able to recognize the primary antibody).

From the fluorescence images (Fig. 3-17), the E-cadherin located at cytoplasmic part and focused in the cell-cell junction region in the positive control of MCF-7. In the negative control of MDA-MB-231 cells, the expression of E-cadherin was lower and aggregated as points in the cytoplasmic part. The expression levels of E-cadherin of HLE-B3 and LECp are found not as high as the positive control MCF-7 naturally. In addition, research illustrates the possible recovery of E-Cadherin expression of cancer cell found in distant metastasis [166]. This phenomenon is explained by the revert process of EMT. Therefore, E-cadherin might be not an appropriate indicator to trace EMT in our system. As a result, E-cadherin is excluded from the experimental design of the EMT assay.

On the other hand, EMT is a reversible switch from an epithelial-like to a mesenchymallike phenotype, and the protein markers increase/decrease along the process. The finding of the lower E-cadherin expression in HLE-B3 and LECp suggests that the cells undergo EMT spontaneously during cell culture as reported [79]. In addition, the heterogeneity expression of E-cadherin in individual MDA-MB-231 cell is also reported in the literature [167]. It is shown that in the majority of human carcinoma cell populations, the loss of E-cadherin appears to be heterogeneous [168-170]. In addition to the loss of epithelial marker E-Cadherin, the gain of mesenchymal markers such as vimentin, N-Cadherin, and Snail in cancer cells has been reported heterogeneously [171,

172]. Therefore, the inhomogeneous fluorescence intensity of individual HLE-B3 and LECp cells in Fig. 3-17 could be explained by the heterogeneous E-cadherin expression phenomenon. In addition, the inhomogeneous immunofluorescence stain of cytokeratin in Fig. 3-19, alpha-SMA in Fig. 3-20 and Fig. 3-21 could be explained by the same way.

In this section, the standard cell morphology and EMT biomarker expression of the culture cell lines is built. This information is used as a reference to examine effect of different types of polymer disks and peptides, different types of polymer disk-peptide interaction, and different molecules that control the EMT process. These results are discussed in Section 4.7.
4.6 Cell adhesion assay

During cataract surgery, the overall protein concentration and cell amount in the aqueous humor are increased due to the blood-aqueous barrier (BAB) breakdown. The protein adsorption of the IOL occurs immediately after implantation. *In vitro* cell adhesion and spreading process is dependent on culture environment including cell types, culture medium, adsorbed proteins, and underlying substrate. Anchoring proteins (fibronectin, collagen, vitronectin for example), secreted or coming from the surrounding medium, mediate cell adhesion and spreading on the culture surface. These proteins will be adsorbed on the biomaterial, allowing the adhesion of the LECs via specific receptors (integrins) able to bind to the adhesion proteins [17]. As a result, the ability of IOL to adsorb protein, or anchoring proteins more specifically, determines its ability of cell adhesion.

The process of integrin mediated cell adhesion on surface includes four steps [65]. At first step, cell adhesion occurs as cell contacts the surface and some ligand binding are formed which allows the cell to withstand gentle shear forces. At second step, cell spreading occurs as cell body begins to flatten and its plasma membrane spreads over the substratum. At third step, organization of actin cytoskeleton occurs inside the cells which leads to actin organization into microfilament bundles, referred to as stress fibers. At fourth step, the formation of focal adhesions (cell-matrix adhesion) occurs, which links the external molecules ECM to the internal molecules such as actin cytoskeleton.

Focal adhesion is well-organized zones of the cellular membrane of about 7 μm in diameter comprising a great concentration of adhesion proteins attached to cytoskeleton [82]. Molecular biology studies show focal adhesion consists of clustered integrins and more than fifty other transmembrane, membrane associated, and other cytosolic molecules including tetraspanins, growth factors receptors, syndecans, lipids, tensin, talin, vinculin, paxillin, and focal adhesion kinase (FAK) [65]. Reorganization of focal adhesion is involved in cell morphology, contractility, and motility [173, 174]. As introduced in Section 1.6 (Fig. 1-21), our proposal is to use RGD peptide to create a bio-adhesive IOL material and to examine the LEC response to evaluate the PCO development risk indicators *in vitro*. Therefore, it is necessary to have controls of high and low PCO incidence. Therefore, the commercial available hydrophobic IOL material (GF) for low PCO level reference and the starting material hydrophilic IOL HA25 (virgin polymer) for high PCO level reference were taken. In addition, in order to know the normal LEC status *in vitro*, the tissue culture grade polystyrene (TCPS) is also taken for the maximum growth control.

The *in vitro* LEC adhesion assay of the FITC-RGD-immobilized surfaces (Fig. 3-18) showed that the LEC adhesion was only promoted in FITC-RGD-grafted sample. The fluorescence backgrounds of the control samples without fluorescent peptide treatment (GF, HA25, and HA25 plasma) were significantly lower than the samples with fluorescent peptide treatment (FITC-RGD ads and FITC-RGD graft), suggesting that fluorescence is an appropriate tool to trace the peptides. The difference in fluorescence backgrounds between GF and HA25 may have been a result of the difference in their chemical compositions. The green fluorescence intensity of the sample reflected the amount of the FITC-RGD peptide present. In the case of the RGD-adsorbed sample, the high fluorescence may have resulted from the sorption phenomenon (i.e. molecules penetrated into the inner space of the hydrophilic acrylic polymer), which can be seen in Fig. 3-7. Although the extreme condition of autoclaving had been applied to remove the surface-adsorbed peptides, a large amount of FITC-RGD peptides was "sorbed" inside the polymer [175], which could explain the low cell coverage of the FITC-RGD-adsorbed surface. In addition, RGD-adsorbed surfaces have been reported to have a lower ability to promote cell adhesion because of the low stability, unstable links, and uncontrolled desorption of biomolecules in physiological environments [77].

Moreover, by image quantification (Fig. 3-18), the cell coverage percentage of the FITC-RGD grafted sample (HA25 – RGD Graft) increases to the level of hydrophobic material

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(GF), whereas the plasma treatment (HA25 Plasma) and the adsorbed (HA25 - RGD Ads) samples share the same LEC adhesion level with the virgin polymer (HA25). This phenomenon suggests that the grafted, not the adsorbed, peptide are sufficient to convert LEC adhesion from the level of high PCO risk material HA25 to the level of low PCO risk material GF.

However, from the result in Fig. 3-18, it is still a concern of specificity: the graft itself promotes cell adhesion rather than via biological function of RGD. To rule out this uncertainty, it is suggested to apply inactive RGE peptide as control [65, 100, 134, 176]. Cells attached on RGE functionalized surfaces do not spread and form focal adhesion [100, 134]. Therefore, the RGE peptide (Fig. 2-3) was immobilized to HA25 surface and tested for cell adhesion in parallel. From the result in Fig. 3-19, the RGD sequence, rather than the integrin-non interacting RGE sequence, promotes LEC adhesion. Therefore, this result proves that adhesion observed in the case of RGD graft is due to the specific interaction between RGD and cell integrins.

Similar to Fig. 3-18, the result in Fig. 3-19 shows that the spreading of LECs can be seen in RGD grafted sample and GF sample, illustrating the proper adhesion and focal adhesion formation. In contrast, the LEC onto the virgin polymer (HA25) or plasmatreated (HA25 Plasma) polymer showed a rounded shape, suggesting loose attachment. Again, the similarity of LEC coverage level between the RGD grafted hydrophilic material (HA25 – RGD Graft) and the hydrophobic material (GF) (Fig. 3-19) further confirmed the result obtained form FITC-RGD treated samples in Fig. 3-20. In addition, this consistency of cell coverage ratio among the FITC-RGD Graft, RGD Graft, and GF samples suggests that the FITC moiety may not obstruct the integrin binding affinity (Fig. 2-4). Moreover, it would imply similar capsule-IOL adhesive force mediated by LECs, and therefore similar low incidence of PCO [17].

Sequence WQPPRARI locates at the C-terminal heparin binding domain of fibronectin [177]. This sequence was reported directly promoting the adhesion, spreading, and

migration of rabbit corneal epithelial cells in a concentration-dependent manner [178]. The major activity of this peptide resides in the sequence PRARI. From rat embryo fibroblasts study [179], both the cell-binding domain (RGD) and the heparin-binding domains (WQPPRARI) of fibronectin are essential for cell survival, implying these two peptides work in synergy.

The combination of RGD and WQPPRARI was tested by coating onto the poly(vinyl amine) surface. The report showed that human pulmonary artery endothelial cells can adhere and spread equally, form similar stress fiber and focal adhesion onto the WQPPRARI (with or without RGD) coated surfaces and onto the fibronectin positive control surface. However, the WQPPRARI alone was unable to support long-term cell growth and survival. Cells showed a loss of focal adhesions and cytoskeletal disorganization after 24 hours from seeding the cells. With the addition of RGD, the surfaces behaved similarly to the positive control fibronectin in cell density. These results indicate that the WQPPRARI promote initial cell adhesion and spreading, but not long-term survival [180].

In addition to poly(vinyl amine), the combination of WQPPRARI and RGD peptide has been introduced on PTFE and PET surfaces. The results shows that the cell attached more in the combined sample than in each alone samples [181] and the micropatterned distribution of WQPPRARI and RGD works better than the homogenous scramble [140]. However, researchers did not the extend WQPPRARI study to the field of LEC adhesion or explored its role in PCO control.

In order to know the effect of WQPPRARI peptide to LEC adhesion on HA25 polymer, we made WQPPRARI and the scramble of WQPPRARI and RGD treated onto HA25 disks polymers. From the result in Fig. 3-19, the graft of WQPPRARI alone significantly promotes cell adhesion. The adhesion level of RGD alone, as well as WQPPRARI alone, are similar to the PCO negative control GF, suggesting that each peptide can be a good

candidate for PCO prevention. Moreover, the cell adhesion level of scramble is significantly higher than each peptide alone, implying that the synergic action of RGD and WQPPRARI promotes LEC adhesion and spreading. Therefore, the graft of combination of RGD and WQPPRARI onto HA25 may even perform better than hydrophobic acrylic polymers in PCO prevention.

4.7 EMT biomarker assay

EMT is a cellular process in which epithelial cells change their phenotype and acquire mesenchymal properties. The two key changes occurring in epithelial cells undergoing EMT are: (i) detachment from neighboring epithelial cells and (ii) the migration into the interstitium (i.e. the interspace between the original epithelium layer and nearby tissue) where they may start producing matrix, shown in Fig. 4-5 [182]. Since PCO is involved in tissue repairing and EMT process, the model can be referred tissue damage induced fibrosis. Originally, quiescent epithelial cells are tightly cohesive to the basement membrane. After subjected to noxious stimuli like cataract surgery, some lens epithelial cells could become primed in EMT and engaged in the molecular pathways rendering degradation of the basement membrane, loss of cell-cell cohesion, increase of motility, change in cell shape, and migration into the space between IOL and posterior lens capsule. Then, as long as the mesenchymal phenotype is fully developed, these EMT-derived de-differentiated cells could further transdifferentiate into fibroblast/myofibroblast and might participate in the production of the fibrotic tissue, which leads to the fibrosis type PCO [182].

To investigate the EMT status of LEC on surface functionalized polymer *in vitro*, it is necessary to build a positive control exhibiting mesenchymal phenotype and a negative control exhibiting epithelial phenotype. Therefore, the cells on the surface can be compared to the references and their relative EMT status can be determined. From the literature, the compound rapamycin is suggested to prevent PCO by inhibiting EMT [47, 48]. Rapamycin inhibits cell proliferation, growth, motility and survival by inhibiting its cellular receptor mTOR (Mammalian Target Of Rapamycin) [48]. On the other hand, TGF- β is known for inducing EMT [39, 165] and PCO is induced by TGF- β /Smad signaling pathway in EMT progression [3, 37, 183]. In addition, it is suggested a convenient way to induce/inhibit EMT by directly adding TGF- β /rapamycin to epithelial cells in culture [39, 48]. As a result, porcine TGF- β and rapamycin treated LECs onto TCPS surface can be taken into our experiment as the PCO-positive (EMT-positive) and PCO-negative (EMTnegative) control, respectively.

With the EMT positive and negative controls generated TGF- β and rapamycin, the cells attached onto RGD immobilized surface were subjected into EMT analysis. The analysis comprises cell coverage, cell morphology, spatial distribution, and the detection of specific EMT molecular markers. In addition, as built the standard cell morphology and EMT biomarker expression of the porcine lens epithelial cell line in Section 4.5, in this section, the further abovementioned analysis were made and reported in Fig. 3-18, Fig. 3-19, Fig. 3-20 and Fig. 3-21.

For the cell coverage, as discussed in Section 4.6, the cell adhesion is promoted by grafted RGD peptides. Although it is known that TGF- β induced EMT is involved in PCO pathogenesis [3, 10, 62], however, the effect of TGF- β in cell proliferation were reported controversial. TGF- β exhibits little or no effect to human LEC [62], inhibits rabbit [184] or bovine [185] LEC proliferation, or stimulates proliferation of fibroblast which is inducted via TGF- β induced EMT [39]. Therefore, it is worthy to investigate the TGF- β effect to porcine LEC coverage.

In Fig. 3-20, the cell coverage slightly increases along the elevated TGF- β concentration in culture medium. In addition, the cell coverage significantly decreases along the elevated rapamycin concentration in culture medium. The combined effect is also tested and shows rapamycin has a higher effect than TGF- β in cell coverage. From this test, we confirm that the TGF- β treatment may have slight effect to promote LEC coverage. In addition, when treated in combination, the inhibition effect by rapamycin dominates the LEC coverage. Therefore, the cell adhesion is reduced by the proliferation-inhibiting effect of rapamycin.

As for the cell morphology and spatial distribution, during EMT, cells experience drastic shape changes during transition from a cuboidal, cobblestone morphology characteristic of epithelial cells to an elongated, spindle-like shape typical of mesenchymal cells [165].

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In addition, along EMT progression, cells lose epithelial cell polarity, separate into individual cells, and subsequently disperse after the acquisition of cell motility [186]. Therefore, the cell morphology and spatial distribution can serve as indices to evaluate the extent of EMT.

In Fig. 3-20, the mutual effect and dose-dependent model of rapamycin, TGF- β to the LEC have been tested in advance. Although the native status of LEC is cuboid like with evident cell-cell adhesions [187] while confluent, the cells reported here are far from confluence by shortened incubation time. The purpose of this treatment is to see each individual cell clearly. Stimulated by TGF- β , the cells become fibroblastoid spindle-shaped. The concentration of 10 ng/mL seems to be sufficient to trigger morphology change. Inhibited by rapamycin, the LEC become cuboid-shaped cells.

From Fig. 3-21, with the longer incubation time, the cells are allowed to form cell-cell interaction and can be further examined for the cell spatial distribution. The TGF- β treated cells disintegrate the clustered structure, whereas the rapamycin treated cells become more clustered than the non-treated cells (TCPS). For the polymer disk samples, cells on HA25, HA25-plasma and HA25-RGD Ads disks show low cell density and are able to form cell-cell interaction. For the HA25-RGD Graft and GF samples, the cells show similar focal adhesion and start to form clustered structure. On the other hand, the same to Fig. 3-20, the cells treated by TGF- β become fibroblastoid spindle-shaped whereas the cells treated by rapamycin become more cuboid-shaped. Although the porcine LECs used in this assay are slightly spindle-shaped naturally [91], the LECs onto RGD grafted surface are not further elongated comparing to the TCPS sample. In addition, the cell morphology is similar between the RGD grafted and GF samples. Combining these two indices, the RGD grafted HA25 disk, as well as the PCO negative control GF disk, show no sign in EMT progress.

For the EMT biomarker expression level, as discussed in Section 4.5, EMT is involved in PCO development and the progress of EMT can be detected with protein biomarkers including acquired marker α -SMA and attenuated marker cytokeratin [188]. In addition, the background expression of α -SMA is observed in porcine LEC as reported in other mammals [189]. Here, by the immunofluorescence images, the expression of these two markers are compared among peptides surface functionalized samples, the PCO positive control HA25, and PCO negative control GF.

Comparing to the hydrophobic sample or the TCPS sample, the LEC onto the RGD grafted hydrophilic sample do not acquire more α -SMA (Fig. 3-18). On the other hand, the expression of cytokeratin does not attenuate in the RGD grafted sample compared with the controls (Fig. 3-19). Therefore, the biomarker assays of EMT illustrate no difference between RGD grafted hydrophilic material and the hydrophobic material, which would imply a low chance of undergoing EMT and induce PCO.

Although it is generally accepted that the RGD promotes cell adhesion, however, the relationship between RGD peptide and EMT remains unclear from the literature. For the epithelium interacting with soluble RGD peptides in perspective of EMT, contradictory results have been reported. One study suggests that EMT is favored by finding that TGFβ1 activated proteolysis of the L1 cell adhesion molecule (L1CAM) inducing its RGDmotif binding to integrin and triggering the EMT pathways [190]. However, in another report, blockage of integrin by soluble RGD peptide inhibits the human hepatic epithelial carcinoma acquiring mesenchymal phenotype and protein marker vimentin, in a system of co-culture with mesenchymal stem cells [191]. In addition, studies on associating EMT with the integrin expression level of LEC are controversial [192, 193]. Furthermore, the soluble RGD peptides block the TGF-β1 induced EMT in mouse mammary gland cells [194]. As for the grafted RGD peptides, there is no precedent to predict the EMT effect. Therefore, direct assessment of EMT of the LEC response to immobilized or soluble RGD peptide is needed. From our preliminary experimental result (Fig. 3-18, Fig. 3-19, Fig. 3-21), the RGD immobilized samples shows no significant effect in biomarker expression and change in morphology. On the other hand, with the presence of high concentration of free RGD peptides (Fig. 3-20), the cell morphology does not alter. Therefore, neither surface bounded RGD nor free desorbed RGD has a measurable effect in EMT, at least under present experimental conditions.

Potentially the adsorbed RGD peptides may desorb during the cell adhesion assay. These soluble RGD peptides may compete against immobilized ones for the cell surface integrin. As a result, the cell adhesion to the RGD functionalized surface may be obstructed by soluble RGD. Researches show that a higher concentration of the soluble RGD peptide inhibits cell adhesion. In the human dermal fibroblast model, 0.77 µM of free tri-peptide RGD do not inhibit adhesion and viability whereas 770 µM of RGD inhibits cell adhesion by reducing 70 to 75% cell density [195]. In another model of 3T3 Swiss fibroblast, 0.1 µM of free penta-peptide GRGDS do not inhibit adhesion whereas 1 mM of RGD inhibits cell adhesion by reducing around 95% cell number [196].

In our case, the inhibit effect of the desorbed RGD peptides is examined (Fig. 3-20). The 50 μ M free RGD peptide slightly reduces LEC coverage ratio by 18% (TGF- β 10, Rapa 0 sample compare to TGF- β 10 + RGD 50, Rapa 0 sample), whereas around 50% of inhibition is reported from literature [196]. In addition, the concentration of potential desorbed RGD peptide is lower than 10 μ M (Fig. 3-10), which is reported around 30% inhibition [196]. Considering in body environment, the potential desorption rate of RGD should be far lower than the autoclaving condition. In addition, the actual size of IOL is much smaller than disk and the aqueous humor is circulating and refreshing continuously. Therefore, soluble RGD peptide must be far lower than 10 μ M in concentration and exhibit no inhibit effect in the body.

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On the other hand, because RGD is recognized by numerous integrins in various cell types, different types of cells may non-discriminatorily attach to RGD functionalized surfaces [197]. The lack of biological specificity renders the RGD-based strategies not fully optimized for controlling more integrated processes, for example cell differentiation [198]. To our knowledge, there is no simple peptide sequence that could specifically promote LECs adhesion. Therefore, it is possible that macrophages and fibroblastic cells, which migrate from uveal tissues into the capsular bag [199], could also attach to the RGD grafted HA25 surface as BAB breaks down during the surgery. However, LECs could take advantage of bind to the RGD peptide temporally and spatially: these cells originated outside from capsular bag are found inside maximized around 1 to 3 months postoperatively [10] whereas literature suggests that the cell adhesion promoted by RGD becomes irreversible after 24 hours of incubation [200]. The pre-existing LECs, which are agitated during cataract surgery, would be the predominant cell type in the capsular bag which interact with the grafted RGD peptide on HA25 polymer and occupy most of the available RGD sites on the surface theoretically. Moreover, BAB alteration can be minimized by combining the clear corneal incision surgical method [201], which involves an incision in the plane of the cornea without altering uveal tissue [202]. Therefore, the risk of adhesion of non-epithelial cell types on RGD functionalized surface can be reduced. However, the deduction is based on the in vitro literature and needed to be verified by in vivo experimental data. Adhesion of macrophages could be studied in vitro, as well as the cell reaction after phacoemulsification and IOL implants in rabbits.



Fig.4- 5 Cellular changes occurring in EMT in a hypothetical context of tissue damage leading to fibrosis. [182]

4.8 MTS cytotoxicity assay

Non-cytotoxic is a general biocompatibility requirement for all medical implants. The MTS assay is widely applied to evaluate the cell viability by relating the intracellular dehydrogenases activity to the living cell population [83, 135, 180]. In our case, since the virgin polymer itself does not attract cell adhesion, it will lead to a low viability value although it is proven to be non-cytotoxic. Alternatively, indirect cytocompatibility study by conditioned medium is suggested in ISO 10993-5:2009.

The use of L929 cell line to determine the cytotoxicity of medical devices is demonstrated in ISO 10993-5 and is also applied by biomaterial researches on IOL and other medical devices [117, 203, 204]. The use of the L929 is preferred because it is established and obtained from recognized repositories (ISO 10993-5.5).

The surfaces prepared by plasma-EDC/NHS method (Fig. 3-26) were subjected to MTS assay with the conditioned medium method. Our data reveals that all the RGD peptide immobilized samples have cell viability values greater than 70%, indicating no cytotoxicity potential by the ISO definition (>70%). In addition, the values are also comparable to those of the virgin polymer, which was proven to be non-toxic in clinical cases.

4.9 Optical properties tests

As a candidate of new biomaterial in ophthalmic implant, the peptide immobilized polymer should provide appropriate optical properties, mechanical properties and cytocompatibility. Since the starting material, HA25, is a conventional biomaterial used in IOL, it is appropriate to use it as a control to investigate the impact of the peptide surface functionalization process.

4.9.1 Light transmittance assay

Surface modification on IOL by ion beam or plasma method has been proposed to improve the surface hydrophobicity or biocompatibility [57, 117]. However, it is still risky to have color deviation after coating [56]. Therefore, the light transmittances of the disks with different peptide surface immobilized status by plasma-EDC/NHS (Fig.3-23) were measured.

From our data, the grafting of RGD peptide onto IOL surface does not change the light transmittance spectrum partially or globally (Fig. 3-23), which illustrates no color deviation and high transparency as the starting material.

The transmission spectrum in the 550 nm to 999 nm is greater than 90%, which is similar to the literature [62]. The transmittance drop in the range between 350 nm and 550 nm is due to the presence of "Blue Filtering" chromophore copolymerized within the IOL material and aiming to prevent the retina from the harmful blue light [205]. In addition, UV-light filter is also typically copolymerized filtering the light up to 350 nm. The present data demonstrate that the surface functionalization procedure of plasma treatment and RGD peptide grafting does not change the light transmittance of the bulk material.

4.9.2 Optical bench measurement

An intraocular lens is intended to restore the vision of the patient and its optical parameters such as diopter are being calculated prior to implantation. Therefore, any

kind of surface modification of the lens should not be detrimental for its optical performance. The optical power of an IOL is expressed in diopters and the industrial tolerances, inspired by the ISO 11979-2, should be respected. The data from the optical bench measurement demonstrate that the experimental optical power of the neat and modified lenses was preserved and remained within the tolerances ($D_{experimental} = D_{theoretical} \pm 0.34D$), suggesting no IOL curvature or refractive power deviation as result of the modification (Table 3-5, Fig. 3-24).

Indirectly, the preservation of the contrast sensitivity of the optic, expressed by the MTF, argues for good surface quality, i.e. homogeneous, low roughness, the latter parameters being most frequently associated with contrast sensitivity deviations [206].

4.10 Mechanical properties tests

Although plasma treatment and surface coating have been applied to improve surface function in biomaterial studies, there are still evidences showing these modifications may alter the mechanical properties of the bulk media [115, 207]. As for the IOL study, this aspect becomes important because the change in mechanical properties may lead implantation failure during (failure of injection system, unfolding) or after cataract surgery (dislocation of IOL, damage of IOL or lens capsule). Therefore, the mechanical properties tests aim at verifying whether the RGD grafted IOL is still suitable for ophthalmological implantation. As a result, the unmodified HA25 IOL is used as positive control.

4.10.1 Haptic compression force

The measurement of haptic compression is also a part of the standardized testing of IOL performance to simulate its behavior in *vivo*. The haptic compression force should neither be too high to damage the capsule bag nor too low to unfix the IOL. Reports show that the maximum force loading of lens capsule is between 400 to 800 mg, compared with 23 to 131 mg in our RGD grafted IOL (Fig. 3-29) [208]. This low force range ensures the mechanical safety to lens capsule. Additionally, the haptics should exert steady force to different size of capsular bag for different patients. On the other hand, the striae formation (caused from high extension forces) in posterior capsular right after surgery may confer a chance to LEC migration, and leading PCO formation. The lower compression force may lead lower circumferential pressure on the posterior capsule, which minimized the striae formation [209].

4.10.2 IOL injection force

The measurement of IOL injection forces is a part of the standardized testing to ensure the safety of the device during the implantation. From our data, the injection forces of the test groups are close to 14N (Table 3-6), falling to the normal range of the hydrophilic IOL [87].

4.11 Contact angle measurement by liquid-droplet method

It is known that the surface hydrophobicity could affect cell adhesion behavior. If the surface is extremely hydrophobic, the adsorbed proteins will be denatured [210]. The receptors on the cellular membrane can hardly recognize the denatured proteins and no cell can adhere. On the other hand, if the surface is extremely hydrophilic, no protein is adsorbed, which again leads to no cell adhesion. Previous studies have shown that the optimized hydrophobicity for cell adhesion falls in the range of 45° to 75° of aqueous contact angle [210] (Fig. 4-6).

From the result (Fig. 3-26), all the samples are located in the optimized region. The RGD and FITC-RGD peptide adsorbed samples show similar contact angles to the HA25 control sample, suggesting that the surface-adsorbed peptides are mostly removed during the wash process and have no effect on the contact angle. In addition, the oxygen plasma treated samples became more hydrophilic significantly, which corresponds to the literature [211]. The phenomenon of RGD grafted surfaces exhibiting even lower contact angles can be possibly explained by the "Hydrophobic Recovery" effect of the material and hydrophilicity nature of the RGD peptide. Plasma treated materials have been reported having a "Hydrophobic Recovery" effect that polymer partially restores the original hydrophobic surface to the extent that it adapts their composition to the interfacial force [211]. The mechanism of this effect is considered as reorientation of nonpolar groups from the bulk to the surface or reorientation of polar groups from the surface to the bulk phase [212]. Since the grafting reaction conjugates peptides to the carboxyl group at the surface, the volume of the peptide would inhibit the reorientation of the carboxyl group into the bulk. In addition, the outer space occupied by the peptide may also be a hindrance to inhibit the reorientation of nonpolar groups from the bulk to the surface. Overall, the grafting of peptides may lead a reduced hydrophobic recovery effect [213]. On the other hand, the RGD peptide is composed mainly by hydrophilic amino acid residues. The FITC-RGD peptide grafted

surfaces, however, showed higher contact angle than RGD grafted surfaces and exhibit no significant difference to the plasma treated surfaces, presumably because of the relative hydrophobic fluorescein moiety of the FITC-RGD peptide (Fig. 2-2 and Fig. 2-4). Therefore, RGD grafted surfaces exhibit more hydrophilic than plasma treated or untreated HA25 surfaces with small contact angle differences.



Fig.4- 6 Cell adhesion as a function of the water droplet contact angle of polymer substrates. The symbols represent different cell lines. [210]

Chapter 5. Conclusion and Prospect

"Intraocular Lenses with Functionalized Surfaces by Biomolecules in Relation with Lens Epithelial Cell Adhesion" is the title of this thesis work. This study comprises a proposal of secondary cataract (posterior capsular opacification, PCO) control by biomaterial engineering method, a design of bioadhesive intraocular lens by surface peptide functionalization, a series of preparation and verification methods to realize the design, and a series of biological, physical and chemical tests to validate the proposal and the design is effective and safe.

A proposal of cataract secondary control is proposed: use of biomolecule to functionalize the hydrophilic intraocular lens surface to the attract cell adhesion reaching the level that hydrophobic material has. This proposal is based on the following facts:

- 1. The hydrophobic acrylic polymers attract more lens epithelial cell (LEC) adhesion than the hydrophilic acrylic polymers.
- 2. Intraocular lenses made from the hydrophobic acrylic material are reported low incidence of secondary cataract.

In the proposal, the surface functionalized hydrophilic acrylic intraocular lens exhibits a bio-adhesive surface attracting thin cell layer formation and could tightly bind to the capsular bag via the cells, which diminishes the space for potential over-proliferation of cells leading to secondary cataract.

A design of bioadhesive intraocular lens by surface peptide functionalization is further depicted from literature search. The RGD peptide, well-known for its ability to attract cell adhesion by interacting with integrins on cell surface, is selected to functionalize the hydrophilic acrylic polymer surface. In addition, in order to maximize the cell adhesion effect of the peptide, the covalent coupling of polymer and peptide by grafting is considered. Therefore, different methods for sample preparation, including grafting/immobilization, washing, characterization are proposed.

For the grafting/immobilization step in sample preparation, among all the methods tested, oxygen plasma with EDC/NHS coupling method performs best. The advantages of plasma are reliability, reproducibility, relative cost-effectiveness, and applicability to

different sample geometries. In addition, the aqueous system of EDC/NHS coupling better preserve the activity of the targeting biomolecule. Moreover, by change the gas of plasma treatment, different functional groups such carboxyl or amine group can be introduced to the polymer surface. Therefore, this method is transferable to other cases such as grafting functional peptides/proteins to any bulk polymer surface.

For the washing step in sample preparation, the consecutive extraction by autoclaving can effectively reduce the reversibly adsorbed peptides on the surface. This finding illustrates that the grafted peptides preserve high activity to attract cell adhesion and can resist high temperature challenge. On the other hand, the thermo-stability of this bioadhesive polymer enables the hydrolytic stability evaluation and shelf-life evaluation by accelerating heating method. In addition, it also offers an alternative way for industrial sterilization.

For the characterization step, XPS data of the model molecule 6F-Val and the real sample RGD and FITC-RGD all shows the covalent binding between targeting molecules and the polymer, validating the oxygen plasma with EDC/NHS coupling an effective way to graft. On the other hand, the colorimetric methods such as Sulfo-SDTB and ninhydrin were introduced to quantify the surface density. The peptide surface density of well-washed sample is below 0.1 nmol/cm², which seems to be the detection limit for most colorimetric methods. For further investigation in the future, the more sensitive method such as fluorescence dye labeling (lower than 1 pmol/ cm²) or ToF-SIMS (around 0.01 nmol/cm²) can be considered.

The designed bioadhesive polymer is effective to attract LEC adhesion. The cell adhesion level is comparable to the low level secondary cataract control. In addition, the cells exhibits normal epithelial cell biomarker, spatial distribution, and morphology, which indicate the design does not exhibit the epithelial-mesenchymal transition (EMT) adverse effects in the experimental tested conditions. In addition, LEC exhibit a higher response to rapamycin (EMT inhibitor) than TGF- β (EMT promoter), but show no EMT-promoting effect to immobilized or soluble RGD peptides. The mutual interaction of these players to LEC is studied with no precedent. However, a more quantitative method such as RT-PCR (Reverse Transcription Polymerase Chain Reaction) or western-blot should be introduced to validate these findings.

Furthermore, tests of the surface hydrophobicity, cytotoxicity, optical and mechanical properties required for intraocular lens illustrate no potential risk with this design. Therefore, this design is worthy to be further examined in *ex-vivo* (capsular bag) or *in-vivo* animal system (rabbit) to explore its effectiveness and safety.

Other biomolecule, peptide WQPPRARI, has been grafted into the surface alone or scramble with the RGD peptide. The WQPPRARI grafted surface shows similar ability to attract cell adhesion as RGD grafted and hydrophobic acrylic control surface, whereas the scramble grafted surface shows even higher ability than previous three. This finding may lead a direction to further design optimization if we could confirm a higher cell adhesion leads a low PCO incidence in animal model.

To conclude, this proposal brings new insight to the secondary cataract control by material engineering and the results support the design feasible. Nor cell-RGD peptide interaction or polymer surface functionalization is novel in the field of biomaterial engineering. However, the combination of these two technologies into the field of ophthalmological disease control is original and the study result is significant. Therefore, it will be potentially beneficial to cataract patients and ophthalmologists in the future.

Chapter 6. References

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