IN SITU ENZYMATIC SYNTHESIS OF COLLAGEN-CHITOSAN HYDROGEL USING ULTRASONIC PRETREATED COLLAGEN

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by

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LIST OF ABBREVIATIONS

ρHP	3-(4-Hydroxyphenyl)-propionic acid
AA	Antibiotic-antimycotic
DCC	Dicyclohexylcarbodiimide
DIC	N,N'-diisopropylcarbodiimide
DPBS	Dulbecco's phosphate buffered saline
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
F12: DMEM	F12: Dulbecco's Modified Eagles's Medium
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscope
HDF	Human dermal fibroblast
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLC	Human-like collagen
HRP	Horseradish peroxidase
MES	Morpholinoethanesulfonic acid
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
PCL	Poly(ε-caprolactone)
PGA	Poly(glycolic acid)
PEG	Poly(ethylene glycol)
PEGDA	Poly(ethylene-glycol)-diacrylate

PEGDMA	Poly(ethylene glycol)-dimethacrylate
PLA	Poly(lactic acid)
PLGA	Poly(lactic acid-*co*-glycolic acid)
PNIPAM	Poly(N-isopropylacrylamide)
SBF	Simulated body fluid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMOs	Sulfamethazine oligomers
Sulfo-NHS	N-hydroxysulfosuccinimide
TEM	Transmission electron microscope
UV-Vis	Ultraviolet-visible
2D	Two dimensional
3D	Three dimensional

LIST OF SYMBOLS

A_{sample}	Absorbance value of cell attached to conjugate hydrogels
A_{2D}	Absorbance value of two dimensional polystyrene surfaces
M_1	Mass of hydrogel before immersion in ethanol
M_2	Mass of hydrogel after immersion in ethanol
P	Density of absolute ethanol
V	Volume of hydrogel
W_b	Water-swollen weight of hydrogel
W_a	Initial weight of hydrogel
W_1	Initial weight of hydrogel
W_2	Final weight of hydrogel

SINTESIS IN SITU SECARA ENZIMATIK BAGI HIDROGEL KOLAGEN-KITOSAN MENGGUNAKAN KOLAGEN DIPRARAWAT DENGAN ULTRASONIK

ABSTRAK

Beberapa tahun kebelakangan ini, hidrogel suntik telah menunjukkan potensi yang besar untuk aplikasi bioperubatan kerana mempunyai ciri-ciri yang jelas berbeza, iaitu penggelan *in situ*. Dalam kajian ini, rawatan ultrasonik diperkenalkan untuk merawat kolagen daripada tendon untuk mengurangkan saiz fibril dan mencegah agregasi fibril. Kolagen (Kol) yang diperawat dengan ultrasonik telah digabungkan dengan kitosan (Kit) untuk meningkatkan sifat fizikal hidrogel. Komposit hidrogel dibentuk oleh gabungan oksidatif kumpulan fenolik hidroksil (Ph) dalam rantaian polimer dengan menggunakan peroksidase lobak kuda (HRP) dan hidrogen peroksida (H_2O_2). Kol-Kit-Ph yang terhasil dapat membentuk hidrogel yang cepat melalui tindak balas sambung silang yang dimangkin oleh peroksidase. Konsentrasi HRP dan H_2O_2 didapati mempengaruhi masa penggelan Kol-Kit-Ph. Selain itu, nisbah Kolagen kepada Kitosan (Kol:Kit) didapati mempengaruhi ciri fizikal, *in vitro* dan mekanikal hidrogel komposit dengan ketara. Hidrogel dengan komposisi chitosan yang lebih tinggi mempunyai penggelan yang lebih cepat, kekuatan mekanikal yang lebih tinggi, dan degradabiliti yang lebih rendah, tetapi menghasilkan keliangan matriks hidrogel yang lebih rendah. Keputusan yang diperolehi dalam kajian ini menunjukkan kepentingan nisbah kitosan kepada komposit hidrogel suntikan Kol-Kit-Ph. Keputusan yang paling ketara diperolehi dalam konjugat hidrogel dengan nisbah Kol:Khit 3:2, yang mempunyai kadar

pertumbuhan sel tertinggi berbanding yang lain selepas 5 hari inkubasi. Oleh itu, hidrogel ini mempunyai potensi untuk digunakan dalam bidang bioperubatan kerana ia stabil dari segi mekanikal dan menyokong fungsi selular.

IN SITU ENZYMATIC SYNTHESIS OF COLLAGEN-CHITOSAN HYDROGEL USING ULTRASONIC PRETREATED COLLAGEN

ABSTRACT

In recent years, injectable hydrogel has shown great potential for biomedical applications owing to its distinct properties of *in situ* gelation. In this research, ultrasonication was introduced to pretreat tendon collagen used in the project to reduce the size of fibrils and prevent fibril aggregation. The pretreated collagen (Col) was combined with chitosan (Chit) to improve the physical properties of hydrogels. Composite hydrogel was formed by oxidative coupling of the phenolic hydroxyl (Ph) groups in polymer chains using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). The resulting Col-Chit-Ph solutions were able to form rapid hydrogel via peroxidase-catalyzed crosslinking reaction. The concentration of HRP and H_2O_2 affected the gelation time of Col-Chit-Ph hydrogels. Besides, the Col to Chit (Col:Chit) ratio also significantly influenced the physical, *in vitro* and mechanical characteristics of composite hydrogels. The hydrogel with a higher composition of chitosan has faster gelation, higher mechanical strength, and lower degradability, but resulted in lower porosity of the hydrogel matrix. The results obtained in this study reveal the importance of the chitosan ratio to the Col-Chit-Ph injectable hydrogel composite. The most significant results were obtained in the conjugate hydrogel with Col:Chit ratio of 3:2, which has the highest cell growth rate compared to others after 5 days of incubations. Hence, this hydrogel has the potential for use in the biomedical fields as it is mechanically stable and supports cellular functions.

CHAPTER ONE

INTRODUCTION

1.1 Development of injectable hydrogels

Injectable hydrogel, which is also known as *in situ* forming hydrogel, has attracted growing interest in biomedical fields recently. Numerous studies on adapting injectable hydrogels as biomaterials have been reported owing to its ability to undergo an *in situ* solution to gel transition when administrated into the body (Liu et al., 2016). When compared to preformed hydrogels transplantation, injectable systems are more desirable as they allow accurate filling of irregular-shaped defects by simple injection of the hydrogel precursor solution to defect site. Injectable hydrogels can be fabricated by several crosslinking methods, mainly classified into physical and chemical crosslinking. These conventional crosslinking methods generally exhibit limitation of operational complexity, low stability, inefficiency coupling reactions and potential cytotoxic effects. Instead, enzymatic crosslinking method is considered as an effective route for development of injectable *in situ* forming hydrogels due to its high site specificity, rapid gelation and relatively mild reaction conditions that are suitable for living cells (Liu et al., 2017). Besides, undesirable side effects to cells can be prevented owing to the substrate specificity of the enzyme. In this study, an injectable enzymatically crosslinked hydrogels was synthesized via horseradish peroxidase (HRP) catalyzed crosslinking reaction in the presence of hydrogen peroxide (H_2O_2).

In addition to the crosslinking method, the biomaterials used for developing injectable hydrogels are of great importance (Heydarkhan-Hagvall et al., 2008). A

wide range of biomaterials, either naturally or synthetically-derived, have been exploited for biomedical uses. Naturally-derived biomaterials are the most common biomaterials used for synthesis of injectable hydrogels due to its intrinsic characteristic that promotes a better interaction with cells (Dhandayuthapani et al., 2011). The natural materials used as injectable hydrogels include collagen, gelatin, chitosan, fibrin, alginate, heparin, hyaluronic acid and chondroitin sulphate (Liu et al., 2017). Among these natural biomaterials, collagen is well-known for diverse biomedical applications owing to its biocompatibility, biodegradability and weak antigenic properties. It has been widely investigated for potential use as surgical suture, hemostatic agents, wound dressings and injectable biomaterials (Miller et al., 1964, Cameron, 1978, Ruszczak, 2003, Kuo et al., 2015).

In this study, collagen-based injectable hydrogel was prepared by integrating collagen with chitosan. Chitosan is a biomaterial known for being non-toxic, contains antibacterial properties and has structural similarity to glucosaminoglycans (GAGs) of the extracellular matrix. Previous studies confirmed that the combination of chitosan with collagen could improve the physical properties of injectable hydrogels (Chen et al., 2005). Nevertheless, there is no other research reported about the fabrication of this collagen (Col)-chitosan (Chit) composite hydrogel conjugated with phenolic hydroxyl (Ph) groups. The injectable Col-Chit conjugate hydrogels fabricated in this work may offer unique mechanical and biological properties for potential use in biomedical applications.

1.2 Problem Statement

Collagen has been considered as the most popular biomaterials in biomedical fields due to its excellent biocompatibility, biodegradability and weakly antigenic properties. The advantages of collagen have been evidenced by extensive investigation, mainly as wound dressings, tissue engineered scaffolds or matrices for drug delivery (Ruszczak, 2003, Lee et al., 2001, Wallace & Rosenblatt, 2003). In spite of the many advantages of collagen, this natural material has poor physical characteristic and rapid degradation rate that limit their practical use. Its mechanical strength is insufficient to maintain the structural stability of scaffolding materials, such as collagen hydrogels (Tangsadthakun et al., 2007). The composite of collagen with chitosan could overcome the limitations of hydrogels fabricated with collagen alone (Lee et al., 2001). However, collagen-chitosan composites do not exist together as blends in nature. The composite hydrogel from these two materials could resemble the main features of ECM, where collagen fibrils provide an excellent environment for cell activity while chitosan improves the mechanical properties of the hydrogel.

The collagen used in this study was extracted from ovine tendon, which is typically discarded as waste. Collagen extracted from ovine tendon is considered as cheap source of raw materials and large quantities of collagen can be isolated and purified for research purposes. However, compared to epidermal collagen fibrils which was proposed by Kuo et al. (2015) for the synthesis of injectable collagen-phenolic hydroxyl (collagen-Ph) hydrogel, tendon collagen fibrils were found to have limitations in terms of having large fibrils and aggregation of fibrils (Gathercole et al., 1987). The large collagen fibrils limit modifications that could be done to it, such as the conjugation of phenolic hydroxyl groups conducted in the current study.

Preliminary work on the present study showed that untreated ovine tendon collagen not only did not mix homogenously with chitosan but also failed to gel *in situ*. Thus, an ultrasonic pretreatment was introduced to reduce the fibrils diameter and prevent collagen fibrils aggregation. Furthermore, the composite of ultrasonic pretreated collagen and chitosan, conjugated with phenolic hydroxyl (Ph) groups is novel for fabrication of *in situ* enzymatically crosslinked hydrogels. This ultrasonic pretreatment could be applied in biomedical applications when using collagen extracted from sources that will yield collagen with large fibrils.

1.3 Objectives

The primary aim of this study is to develop a novel *in situ* forming collagen-chitosan conjugates (Col-Chit-Ph) hydrogel through HRP-catalyzed crosslinking reaction.

The specific objectives are:

1) To study the effect of ultrasonication on the diameter and distribution of collagen fibrils
2) To study the effect of horseradish peroxidase and H_2O_2 concentrations on the *in situ* gelation of collagen-chitosan composite
3) To evaluate the physical, *in vitro* and mechanical characteristics of composite hydrogels at different Col:Chit ratio