Crosslinking Collagen with a Novel Cross linker Based on Oligopolylactide

Wen Chung Chang and Yng Jiin Wang*

Institute of Biomedical Engineering, National Yang Ming University, Shi Pei, Taipei, Taiwan, ROC.

* To whom correspondence should be addressed
Y.J. Wang: Institute of Biomedical Engineering, National Yang Ming University Taipei, Taiwan, Republic of China.
Tel: 886-2-28267019
Fax: 886-2-28210847
E-mail: wang@bme.ym.edu.tw
ABSTRACT:
A novel crosslinker based on oligopolylactide was fabricated and then utilized to crosslink collagen in the study. Various specific molecular weights (350-10,000 Da) of terminal carboxylic acid group oligopolylactide were synthesized by ring-open reacting of DL-lactide with succinic acid at a different molar feed ratio in the presence of Sn(II)octoate catalyst and these oligomers have function group that can be chemically activated with N-hydroxysuccinimide to form macro crosslinkers. Gel permeation chromatography and nuclear magnetic resonance were used in order to characterize these oligopolylactide and crosslinkers. The application of this end-group activated oligopolylactide was demonstrated by its cross linking function with suspension collagen. Fibrillar collagen treated with oligopolylactide demonstrated significant decreases in neural solubility, increases in fixation index as compared to noncrosslinked controls, and a similar transition was revealed in the electrophoresis analyses of fibrillar collagen. In addition, the crosslinked collagen fibers have a slight increased in density of collagen fibers and forms arrange in order by transmission electron microscopy. These data suggest that collagen fiber can be crosslinked by the end-group activated oligopolylactide. In this case the whole scaffold is combining the specific properties pertaining to these two different kinds of polymers, one might be able to develop excellent biomaterials for medical and pharmaceutical applications.

Key words: collagen; crosslinker; oligopolylactide; biodegradable

Update references; 2000-
INTRODUCTION

Because of the excellent biocompatibility and its specific amino sequences[1-3], collagen is an ideal biomedical material for tissue engineering using. But collagen is usually lack of mechanical strength to form a rigid 3-D matrix. In order to be the tissue-engineering scaffold, collagen must strengthen its structures by treated with physical methods or modifying with chemical crosslinkers[1-3]. Physical method such as drying, heating, ultraviolet irradiation, gamma ray and microwave [4-6], although they don’t have chemicals agent toxic but they make material denatured and have undefined side-effects [7]. In addition, chemical crosslinkers using for modifying collagen fibers include aldehydes [8]、epoxy compounds [9-11]、hexamethylenediisocyanate (HDC) [12]、polyurethane-diisocyanate [13]、acyl azide [14,15]、carbodiimides (EDC) [16-18], and Genipin [19,20]. Of these crosslinkers, glutaldehyde is the most commonly used as chemical fixation reacting on the primary amino groups of collagen molecules, but the toxic metabolite and undesirable calcify of glutaldehyde fixation has been observed in vivo. [21-24]. In addition, EDC is deficient the fixed distance of space effects when EDC acts as activation intermediate, whereas, genipin could supply the appropriate space distance for crosslinking collagen molecules. Other crosslinkers are either non-degradable or strong inflammatory reaction after degradation.

On the other hand, the synthetic hydrophobic polymers such as poly(lactide), poly(glycolide) and their copolymers are also well biocompatible and degradable into small molecules that enter the metabolic pathway are widely used in biomedical applications [25-27]. Although these synthetic polymers provide certain mechanical strength, they do not have any molecular segment specific for cell recognition and signal transduction. For promote the hydrophile of these synthetic polymers and
provided with function groups. I. Arvanitoyannis [28], S. H. Kim [29] and H. Korhonen [30] have synthesized short oilglycolactide terminal end with OH [31] and these oligomers possess function groups could be activated and then used for conjugate biologically active agents or modify natural biopolymers. Thus, by combining the specific properties pertaining to these two different kinds of polymers, one might be able to develop excellent biomaterials for clinical applications.

The aim of this study is to overcome some of the limitations associated with glutaraldehyde and other fixation approaches used in the art. We synthesize hydrophobic, short-chain poly(lactic acid) with specific molecular weight and especially with their both ends bearing –COOH groups. The terminal groups of these polymers will be chemically activated, and then utilized to cross link collagen. Because these cross linkers are biodegradable, they are expected to resorb after implanted in the host. The natural polymeric chain of the crosslinked biomaterial could be digested by enzymes. The resulting small molecules along with the hydrolyzing products of the cross linker could be further metabolized in the host. These cross-linked biomaterials should have excellent biocompatibility.

MATERIALS AND METHODS

Materials

DL-lactides were purchased from Bio Invigor corporation (Taiwan, Republic of China.). N-hydroxysuccinimide and 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Merck-Schuchardt (Germany). Succinic acid and Sn(II) octanoate were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of reagent grade.
Synthesis of NHS activated carboxy-end oligolactide

Short-chain poly(DL-lactide) (abbreviated as oligolactide) with two carboxyl terminals was synthesized by ring-opening reaction of DL-lactide with succinic acid (Fig. 1). Briefly, DL-Lactide was purified by recrystallization from ethylene acetate and then dried overnight in vacuum at room temperature. This purified DL-lactide was mixed with various amounts of succinic acid and Sn(II) octanoate catalyst (0.02% mole) in a flask. The reaction mixture was heated and maintained at 180°C for 8 hours under nitrogen atmosphere to obtain oligolactide with two carboxy-ends. The reaction product was dissolved in acetone and then precipitated in water. The procedure was repeated twice and the resulted carboxy-end oligolactide was dried in vacuum at ambient temperature.

The carboxylic acid ends of the oligolactides were activated with N-hydroxysuccinimide (Fig. 2) by the following procedure. Oligolactides derived from different ratios of lactide and succinic acid were mixed with NHS and dissolved in DMSO (Table 1). EDC was then added to the DMSO solution and the mixture was stirred at 37°C for 20 hours under nitrogen atmosphere. The reaction mixture was poured into water to precipitate the NHS-activated oligolactide. The precipitate was washed twice with water and dried in vacuum at ambient temperature.

Characterizations of oligolactides

The molecular weight distributions were determined by using gel permeation chromatography (Jasco LC system) coupled with a refraction index detector. Polymer samples were dissolved in tetrahydrofuran at a concentration of 0.2 wt% and THF as eluent with a flow rate of 1.0 ml/min through the PL gel column.

The composition ratios of succinic to lactide of the carboxy-end oligolactides were determined from the results of nuclear magnetic resonance spectroscopy.
(^{1}H-NMR and ^{13}C-NMR). The polymer was dissolved in chloroform-d$_{1}$ at a concentration of 1wt % and the NMR spectra were recorded on a Bruker DMX-500 MHz FT-NMR.

**Cross linking of fibrous collagen with NHS-activated carboxy-end oligolactide**

Type I collagen was prepared from bovine skin according to the procedures described previously [32] and stored in 0.5M acetic acid at 4°C until use. The concentration of collagen was determined by a modified Lowry’s method [33]. Collagen (6mg/ml in 0.5M acetic acid) was reconstituted by adjusting pH to 7.3-7.4 with phosphate buffered saline (PBS) at 18-22°C to obtain fibrous collagen. The fibrillar collagen were harvested by centrifugation (g force?), and the supernatant solution were replaced by % ethanol? a series of aqueous alcohol solutions with increasing ethanol concentration of up 95%(v/v), and finally suspended in 30ml of ethanol solution (95%,v/v) at a collagen concentration of approximately 1mg/ml. The NHS-activated carboxy-end oligolactide-20 was added to the suspension of fibrillar collagen to yield a final oligolactide concentrations ranging from 0.00075mmole-0.0375mmole and the preparations were incubated for approximately 20hr at 18-22°C. Following the crosslinking reaction of oligolactride, collagen fiber were washed with alcohol to remove the residual oligolactide, centrifuged, and suspended in PBS.

**Characterization of the crosslinked collagen**

The crosslinked collagen was analyzed with its solubility at neutral pH, mobility in gel electrophoresis, fixation index based on ninhydrin and morphological observation under transmission electron microscope.

The neural solubility of collagen was determined by heating collagen solution at 45°C in PBS for 30 min, centrifuging the samples to remove insoluble materials, and
measuring the amounts of collagen remained in the supernatant with a modified Lowry’s method.

SDS-Polyacrylamide gel electrophoresis of the crosslinked collagen was performed according to the method of Studier [34] to modify. Collagen samples were dissolved to obtain a concentration of 2mg/ml in 50mM acetic acid. The acetic solution was mixed with sample buffer with 1:1 ratio, heated at 100°C for 2 minutes, and then centrifuged to remove insoluble materials. 10μl of supernatant solution was loaded into each upper well of the gel. After electrophoresis, the gel was stained with Coomassie Blue R250 for 20 minutes and destained with acetic acid.

The extent of cross linking could be assessed by determining the fixation index derived from ninhydrin test [35]. The fixation index is defined as the percentage of amino groups in collagen reacted with the crosslinking agent. For ninhydrin test, dry sample was heated with a ninhydrin solution for 20 min in dark, and then the optical absorbance of the solution was recorded at 570nm with UV/Vis spectrophotometer using glycine as the standard. The fixation index can be calculated by the following equation:

\[
\text{fixation index (\%) = \frac{[(\text{NHN reactive amine})_{\text{fresh}} - (\text{NHN reactive amine})_{\text{fixed}}]}{[(\text{NHN reactive amine})_{\text{fresh}}]}.}
\]

where (NHN reactive amine)_{fresh} is the amounts of total amino groups of collagen before crosslinking, and (NHN reactive amine)_{fixed} is the amounts of residual amino groups of collagen before collagen crosslinking.

For observation under transmission electron microscope, collagen samples were fixed with 2.5% glutaldehyde in PBS, followed by 1% osmium tetraoxide in the same buffer and dehydrated by suspending in ethanol. After dehydration, the samples
were then embedded in spur, cut into ultrathin sections (50-70nm), and stained with uranyl acetate and lead citrate.

RESULTS

Synthesis of oligolactide crosslinker

Caboxy-end oligolactides with various molecular weights (Mn = 850-8,000 Da) were synthesized from the ring-open reaction of DL-lactide with succinic acid by adjusting the molar ratio (5/1 to 80/1) of lactide and succinic acid in the initial feedstock.

Figure 4 shows the $^1$H-NMR spectra of oligolactide-10 (DL-lactide/ succinic acid = 10/1) in chloroform-d$_1$. $^1$H-NMR were used to determine the copolymer ratio and molecular weight of the SAL from the integration ratio of resonances at 5.11 ppm originated from -CH of the oligopolyolactide and -CH$_2$CH$_2$- of the succinic acid residue at 2.7 ppm. The molecular weight of the polymer can be calculated from the ratio(ex: DL-lactide/ succinic acid = 11/1, Mw=1,704). In addition, in the $^{13}$C-NMR spectra of SAL10 (Fig. 5), the peak at 16.64, 20.31 ppm corresponding to the -CH$_3$ of oligopoly(lactide), the peak at 29.25 ppm corresponding to the –CH$_2$ of succinic acid, the peak at 66.6 and 68.7 ppm corresponding to the OCH of oligopoly(lactide), the peak at 169.3 ppm corresponding to the –COO of oligopoly(lactide), the peak at 171.5 ppm corresponding to the –COO of succinic acid and the peak at 174.97 ppm corresponding to the –COO of both end groups.

The results of the SAL copolymer synthesis are summarized in Table1. The molecular weight of the SAL can be controlled by changing the feed ratio of DL-lactide and succinic acid and polymer yields were found the succinic acid contents are always associated with higher polymer yields. In Addition, the molecular weight of the SAL was found to be inversely proportional to the amount of
succinic acid added in the range of feed ratio applied as shown in Fig. 6. By increasing the feed ratio of DL-lactide and succinic acid from 5/1 to 80/1, the molecular weight of the SAL increase from 850 to 8,000Da, respectively.

Fig. 7 shows the $^1$H-NMR spectra of SAL20-NHS (DL-lactide/ succinic acid = 20/1). The esterification extent of SAL20-NHS was calculated from the integral ratio of the -CH$_2$ in the succinic acid ($\delta$=2.7 ppm) to the -CH$_2$ in the NHS ($\delta$=2.8 ppm) derived from the NMR spectra. Based on the integration ratio (7.18/3.68=1.95$\pm$2), the both ends bearing –COOH groups of SAL have been activated with N-hydroxysuccinimide and to form a macro crosslinker (SAL-NHS).

**Characterization of cross-linked collagen**

Cross-linked collagen with SAL-NHS had a significant impact on the physicochemical characteristics. The neural solubility in PBS at 45°C was significant decreased from 100% for nontreated control to less than 0.0375 mmole SAL20-NHS treated samples (shown in Fig. 8) and a similar transition was revealed in the SDS polyacrylamide gel electrophoresis analyses of fibrillar collagen (shown in Fig. 9). The results of these experiments showed that these crosslinked collagen fibrils were essentially insoluble, even under the extreme conditions, heating the samples for several minutes at 95°C in detergent-containing buffer. These data suggest that treatment of suspension collagen with SAL20-NHS resulted in intermolecular crosslinking which stabilized fibrils structure to make them highly resistant to disruption and solubilization. In opposition, the fixation index of SAL20-NHS fixed collagen had a significant increase by increasing the concentration of SAL20-NHS which was recorded in Fig. 10. The data suggest that N-hydroxysuccinimide groups of SAL20-NHS have reacted with the amino acid side groups present on the collagen molecules.
In order to investigate the morphology of the noncross-linked and cross-linked collagen fibers, collagen samples were examined under transmission electron microscopy. Fig. 11(a)-(b) shows the TEM monograph of collagen fibers reconstituted by neutralization with a PBS at 18-22°C. As can be seen in Fig 7(a), at a high magnification, the noncross-linked fibrillar reconstituted collagen fibers exhibited a regular banding pattern with a cross-striation of 50~60 nm in width, which is typical of collagen fibers found in animal tissues. Suspension collagen was treated with SAL20-NHS(0.015 mmole) forms interconnecting fibers and arrange in order, and crosslinked collagen fibers have a slight increased in density of collagen fibers, as shown in Fig. 7(b).

Degradation of cross linked collagen

DISCUSSION

A ideal crosslinker that should possess non-toxic, biocompatible and biodegradable for medical and pharmaceutical application. For to overcome some of the limitations associated with glutaraldehyde and other crosslink agents approaches used in the art. There are many synthetic polymer such as polyethylene glycol(PEG), polyethylene oxide (PEO), polyvinyl alcohol (PVA), extran and the like were research as crosslinkers [36-37]. For example, various derivatives of polyethylene glycol have been used as crosslinker to conjugate collagen [38-40]. Although these biocompatible crosslinked polymer have biologically inert and water soluble cores, but can not decompose completely in the host. Therefore, preferably with well biodegradable linkages such as polylactide that has greatly potential to develop as biodegradable crosslinkers.
Fibrillar suspension of collagen been utilized as model compound to demonstrate the application of this oligopolylactide in this study. These collagen molecules assemble to form microfibrils, which in turn assemble into fibrils by neutralization with a PBS at 18-22°C, and the distance of fibrillar collagen ranges between 1.3-1.7nm. In addition, the amino acids which make up the collagen molecules contain side groups, including amine (NH$_2$), acid (COOH) and hydroxyl (OH) groups, all of the polymer backbone are sites for potential chemical reaction on these molecules. On the other hand, the synthetic polylactide is a linear polymer that has a hydroxyl and carboxyl group at the both terminal of polylactic acid, so do not normally react with other functional groups simultaneously. For to combine these two different kinds of polymers, a oligomer that possess both ends function group at least, with specific molecular chain length, and can react with the functional groups on the collagen molecules was devised. First, we successfully synthesize SAL with both terminal carboxylic acid groups by ring-open reacting of DL-lactide with succinic and change the feed ratio of DL-lactide and succinic acid to control the molecular weight of the oligomer range between 850 and 8,000Da (about 5.3nm ~ 42.1nm). These SALs have function group that can be chemically activated to form macro crosslinkers and can to provide various fixed distance for spacer effects. As an alternative, these oligomers may be used to deliver biologically active agents or drug compounds.

In general, functional groups such as alcohols or carboxylic acids do not normally react with amines under physiological conditions. However, such functional groups can be made more reactive by using an activating group such as N-hydroxysuccinimide. Several methods for activating such functional groups are known in the art [41]. Preferred activating groups include carbonyldiimidazole,
sulfonyl chloride, aryl halides, sulfosuccinimidyl esters, N-hydroxysuccinimidyl ester, succinimidyl ester, epoxide, aldehyde, maleimides, imidoesters and the like. The N-hydroxysuccinimide esters or N-hydroxysulfosuccinimide groups are the most preferred groups for crosslinking of proteins or amine functionalized polymers. Therefore, N-hydroxysuccinimide been selected as an activating group and form a N-hydroxysuccinimide-based electrophilic groups to react with the nucleophilic amino acid side groups present on the collagen molecules. In addition, although H$_2$O are weaker nucleophilic groups but great quantity water will compete with amine to react with electrophilic groups of the NHS-SAL due to mass law and effect the crosslink reaction. Therefore, organic solvent such as alcohol and DMSO be utilized to substitute for aqueous media and prevent the loss of crosslinking ability due to water-reactive. From the results of neural solubility, electrophoresis and fixation index indicate that the activated oligopoly lactide having reactive groups capable of forming irreversible and stable chemical bond formation with the reactive amino acid side groups present on the collagen molecules.

The molecular chain length of the oligomeric crosslinker (4.43~41.28nm) are greater than the distance of fibrillar collagen(1.3~1.7nm), which possess capable of proceeding interfibrillar crosslink with the reactive amino acid side groups present on the collagen molecules and form interlock structure. However, these oligomeric crosslinker are linear polymer and weaker of flex, so it is difficult to proceed intrahelical and interhelical crosslink near collagen molecules. As can be seen in TEM monograph of collagen fibers suggest that the collagen forms interconnecting fibers and arrange in order regional. Therefore, these macro crosslinker can complement each other with natural crosslinker such as Genipin(chain length $\cong$ 0.8~1.25nm) [42-43] to provide more crosslink spacer effects.
In conclusion, we have developed a novel method of preparing activated oligopolylactide thus obtained have a potential as macro crosslinkers for modify natural biopolymers such as collagen and hyaluronic acid. In this case the whole scaffold is combining the specific properties pertaining to these two different kinds of polymers, one might be able to develop excellent biomaterials for medical and pharmaceutical applications. In addition, the various specific molecular weight of oligopolylactides that possess multi-arm function groups (-COOH or-OH), the interaction between collagen molecular and the crosslinker, the in vivo biocompatibility and degradability of these crosslinkers or crosslinked collagen are underway in our laboratory.

Acknowledgements

This work was supported in part by grant from NSC (National Science Council), ROC.

REFERENCE

6. Visser CE, Voute AB, Oosting J, Boon ME, Kok LP. Microwave irradiation and


38. Pathak; C. P., Moore, Mark A. Cross-linking tissue with a compound having a C.sub.8 to C.sub.40 aliphatic chain. United States Patent 6,156,531
**Figure and Table Legend**

Figure 1  Reaction scheme of DL-lactide / succinic acid ring-open polymerization

Figure 2  Reaction scheme of the NHS-actived SAL

Figure 3  Schematic illustration of cross-linking collagen with NHS-actived SAL

Figure 4  $^1$H-NMR spectra of SAL10(10/1, molar ratio)

Figure 5  $^{13}$C-NMR spectra of SAL(10/1, molar ratio)

Figure 6  Molecular weight (Mp) of SAL depended on the feed ratio of dl-lactide to succinic acid

Figure 7  $^1$H-NMR spectra of SAL20-NHS(20/1, molar ratio)

Figure 8  Neutral solubility of collagen crosslinked by SAL20-NHS

Figure 9  SDS-Polyacrylamide gel electrophoretic analyses of noncrosslinked (Lane1) and crosslinked fibrillar collagens. Lanes2-7 crosslinked with 0.00075, 0015, 0.00375, 0.0075, 0.015 and 0.0375 mmole NHS-SAL20, respectively.

Figure 10  Fixation index of collagen crosslinked by SAL20-NHS

Figure 11  Transmission electron microscopic analyses of fibrillar suspensions of collagen (a) noncross-linked fibrillar collagen (b) cross-linked fibrillar collagen with SAL20-NHS

Table 1  Ring-open polymerization of DL-lactide with succinic acid at a different molar feed ratio in the presence of Sn(II)octoate catalyst. These
oligopolymer were carried out at 180°C for 8 hour.

Figure 1 Reaction scheme of DL-lactide / succinic acid ring-opening polymerization

Figure 2 Reaction scheme of the NHS-activated SAL
Figure 3  Schematic illustration of cross-linking collagen with NHS-activated SAL

Figure 4  $^1$H-NMR spectra of SAL10(10/1, molar ratio)
Figure 5: $^{13}$C-NMR spectra of SAL(10/1, molar ratio)
Figure 6  Molecular weight (Mp) of SAL depended on the feed ratio of dl-lactide to succinic acid

Figure 7  $^1$H-NMR spectra of SAL20-NHS(20/1, molar ratio)
Figure 8 Neutral solubility of collagen crosslinked by SAL20-NHS

Figure 9 SDS-Polyacrylamide gel electrophoretic analyses of noncrosslinked (Lane 1) and crosslinked fibrillar collagens. Lanes 2-7 crosslinked with 0.00075, 0.0015, 0.00375, 0.0075, 0.015 and 0.0375 mmole NHS-SAL20, respectively.
Figure 10  Fixation index of collagen crosslinked by SAL20-NHS

Figure 11(a)
Figure 11(b)

Figure 11 Transmission electron microscopic analyses of fibrillar suspensions of collagen: (a) noncross-linked fibrillar collagen and (b) cross-linked fibrillar collagen with SAL20-NHS (0.015 mmole)

Table 1 Ring-open polymerization of DL-lactide with succinic acid at a different molar feed ratio in the presence of Sn(II)octoate catalyst. These oligopolymers were carried out at 180°C for 8 hours.

<table>
<thead>
<tr>
<th>Run</th>
<th>LA/SA (feed molar ratio)</th>
<th>LA/SA (1H-NMR)</th>
<th>Mw(^a)</th>
<th>Mw(^b)</th>
<th>Mp(^c)</th>
<th>Mn(^c)</th>
<th>Mw(^c)</th>
<th>Mw/Mn</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5/1</td>
<td>5.7/1</td>
<td>840</td>
<td>941</td>
<td>934</td>
<td>856</td>
<td>985</td>
<td>1.15</td>
<td>76.8</td>
</tr>
<tr>
<td>2</td>
<td>10/1</td>
<td>11/1</td>
<td>1,560</td>
<td>1,704</td>
<td>1,648</td>
<td>1,505</td>
<td>1,791</td>
<td>1.19</td>
<td>75.4</td>
</tr>
<tr>
<td>3</td>
<td>20/1</td>
<td>24/1</td>
<td>3,000</td>
<td>3,576</td>
<td>3,622</td>
<td>3,034</td>
<td>4,114</td>
<td>1.35</td>
<td>73.2</td>
</tr>
<tr>
<td>4</td>
<td>40/1</td>
<td>48/1</td>
<td>5,885</td>
<td>7,032</td>
<td>5,648</td>
<td>4,578</td>
<td>8,115</td>
<td>1.77</td>
<td>65.3</td>
</tr>
<tr>
<td>5</td>
<td>80/1</td>
<td>105/1</td>
<td>11,640</td>
<td>15,240</td>
<td>11,840</td>
<td>7,963</td>
<td>14,765</td>
<td>1.85</td>
<td>40.2</td>
</tr>
</tbody>
</table>

\(a\). Determined by calculate
\(b\). Determined by 1H-NMR
\(c\). Determined by GPC