THE EVALUATION OF THERMAL PROPERTIES AND IN VITRO TEST OF CARBODIIMIDE OR GLUTARALDEHYDE CROSS-LINKED GELATIN FOR PC 12 CELLS CULTURE

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ABSTRACT

The thermal and degradable properties of carbodiimide (EDC) or glutaraldehyde (GTA) cross-linked gelatin membranes have been investigated in order to evaluate the effects of different concentrations of two kinds of cross-linking reagent on the stability of membranes. In the thermogram recorded from a gelatin membrane cross-linked with EDC solution, the endothermic peak of 0.8% EDC cross-linking gelatin was centered at about 61°C that was higher than other samples treated with EDC solutions. Denaturation temperature (Td) of gelatin samples increased on increasing EDC concentration (0.2% to 0.8%), in agreement with the simultaneous increased of the extent of cross-linking. But increasing GTA concentration from 0.05% to 0.6%, the Td values of gelatin samples were decreased from 66.2°C to 56.3°C. In addition, two endothermic peaks were observed in 0.4% and 0.6% GTA cross-linking groups because of the GTA concentration was too high to complete cross-linking reaction. Therefore, partial of gelatin membrane was cross-linked completely but others were not. In the thermogravimetric analysis, the proportion of cracking endothermic peak of 0.6% GTA cross-linking gelatin (g15G0.6) was higher than the peak of 0.6% EDC cross-linking gelatin (g15C0.6). Therefore, g15G0.6 cracked to smaller molecules has to absorb more calorific capacity than g15C0.6. The increase in the strength of covalent binding on increasing the proportion of endothermic peak was evident. The results of degradable rate were in agreement with the lower concentration of cross-linked reagent the faster degraded rate of gelatin membrane. The MTT assay showed that 15% gelatin cross-linked by 0.8% EDC has the least cytotoxicity, and cell activity of this group was similar to control group (blank dish). As the concentration of GTA in gelatin membranes was down to 0.05% or 0.1% the cell viability was returned to approach the value of control group.

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1. INTRODUCTION

As results of mature neurons do not replicate and axons cannot regenerate over gaps, neurons would lose the function of transmitting signal once injured. However, there is now considerable evidence that

peripheral nerves have the potential to regenerate if an appropriate microenvironment is provided, and eventually reestablishing functional contacts. Peripheral nerve injuries that result in long gaps require surgical implantation of a bridge or guidance channel between the proximal nerve end and the distal stump in order to restore full function and organ reinnervation [1]. In clinical practice, direct end-to-end suturing techniques are suggested for a short nerve injury. When large gaps remain between the ends of injured peripheral nerves, autografts, allografts, or xenografts have been used for the nerve repair but there were several problems troubled the researchers, such as limited supply of available nerve autografts, certain donor site morbidity and immune rejection [2-3].

A wide range of materials has been developed for use as nerve guidance channel which were divided into three groups [4-9]. The first was undegradable nerve graft that silicone tubes were one of the most common materials because of its inert and elastic properties. However, silicone tubes often lead to long-term complications including chronic nerve compression and fibrosis [10]. It's consequently to remove the conduits with a second surgical procedure. The second group was biodegradable materials, such as polylactic acid (PLA), poly(lactic-co-glycolic) acid (PLGA), but the degraded products of PLA and PLGA would lead to lower local pH value and could be detrimental to surrounding cells and tissue [11,12]. The third group of the materials used as nerve guidance channel was autologous blood vessel or denatured muscle tissue. These kinds of materials have also been used as nerve conduits with varying levels of success, but the supply still could not obtain extensively.

In our study, gelatin was used for nerve regeneration. Thermal denaturation or physical and chemical degradation of collagen involves the breaking of the triple-helix structure to give gelatin, a biodegradable, biocompatible and nonimmunogenic product, suitable for medical applications [13]. In addition, gelatin is relatively cheap and is available in a pyrogen-free form and it has the potential for use with a variety of medicinal agents [14]. The degraded products of gelatin were amino acids that were harmless substances for humans. However, the mechanical properties of gelatin were not enough for surgical manipulation. That was the reason we adding two different cross-linking reagents to enhance the mechanical strength. The ratio of gelatin and cross-linked reagents could be utilized to regulate the cross-linked degree and degradable rate. Glutaraldehyde (GTA) is the most successful chemical agent and is the only commercially viable process that has received widespread acceptance [15]. No new method has yet been able to replace it effectively. However, the toxic effect of GTA was its disadvantage as well known. The

reagent 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) offers another method for generating cross-links between corresponding reaction sites, without itself being incorporated [15]. That is to say, it might not cause cytotoxicity if the gelatin membrane was washed thoroughly. The cross-linking reactions of gelatin samples cross-linked by GTA or EDC were shown in Fig. 1, Fig. 2 respectively. The purpose of this study was to evaluate the feasibility of the different concentration of two cross-linking reagents in gelatin membrane by thermal analysis. Finally, this polymer has putative bioadhesive properties which could culture with neuron or Schwann cells to advance nerve axon regeneration. Therefore, the PC12 cell which was one kind of the rat adrenal gland pheochromocytoma cells would be cultured with gelatin membrane to evaluate the effect on the biocompatibility of cross-linked gelatin.

2. MATERIALS AND METHODS

2.1 Preparation of Gelatin Membrane

Type A gelatin (300 Bloom, Sigma Chemical Co., St. Louis, MO, USA) from porcine skin and differential concentration of cross-linked reagents were used to prepare gelatin membrane. 15% gelatin solution was well mixed by magnetic stirrer at the temperature of 70°C, and then air cooling to temperature of 40°C. Glutaraldehyde solution or carbodiimide solution were added to occur reaction of cross-link. The mixture was poured down into a petri dish and heat dried in an oven. The developed materials were divided to two groups. One was control group that included blank dishes and 15% gelatin membrane. Another was experimental group. We prepared several concentrations of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (Sigma Chemical Co., St. Louis, MO, USA) (EDC) to cross-link 15% gelatin respectively, there were 0.2%, 0.4%, 0.6%, 0.8%, 1%, 5% and 10%. At the same time, there were 0.2%, 0.4%, 0.6% glutaraldehyde (Sigma Chemical Co., St. Louis, MO, USA) solutions (GTA) to cross-link 15% gelatin respectively. All of experiment groups were described in Table I, every sample of test has repeated ten times (n=10).

2.2 Thermal Analysis of Gelatin Membrane

The thermal properties of cross-linked gelatin membranes were analysed by using DSC (Differential scanning calorimetry). Calorimetric measurements were performed using a DSC (TA Instrument DSC 2010). The calibration of temperature and enthalpy was performed by using high-purity standards. The membrane samples (4~5mg) were sealed in aluminum

pans hermetically to prevent any loss of liquid during measurements. Heating was carried out at $20^{\circ}\text{C}/\text{min}$ in the temperature range from 35°C to 150°C . Denaturation temperature (T_d) of gelatin was determined in the endothermic phenomenon.

2.3 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis was carried out on dried samples using a TGA (TA Instruments, Inc., 109 Lukens Drive, New Castle, DE 19720). Heating was performed in an alumina crucible in an air flow ($20\text{ cm}^3/\text{min}$) at a rate of $5^{\circ}\text{C}/\text{min}$ up to 450°C . The samples weights were in the range of 9~10 mg.

2.4 Degradation of Gelatin Membrane

The in vitro gelatin degradation from cross-linked membranes was determined by UV/VIS spectrophotometer (Perkin elmer, Norwalk, CT06859). Dissolution experiments were conducted at the temperature of 37°C . The samples of gelatin membranes were about 2.5g (diameter was 3cm and thickness was 0.1cm). Gelatin membranes were placed

Fig. 1 Proposed mechanism of monomeric GTA reaction with amine groups on gelatin to form cross-links.

Fig. 2 The sequence of reactions for EDC cross-linking gelatin.

in 80ml PBS (phosphate buffer solution, pH 7.4) and at predetermined time intervals, 3ml PBS solution of the released solution were drawn in the colorimetric tubes. The absorbance of each solution at 275nm was measured by an UV/VIS spectrophotometer. And then the drawn out PBS solution of released solution were replaced. The gelatin concentration was calculated by reference to previously constructed calibration curve.

2.5 In Vitro Experiments

The method of cell culture was briefly described as follows. The PC 12 cells (Food Industry Research and Development Institute) were used to evaluate proliferation of cells on the developed gelatin membrane. The culture medium of PC 12 cells was RPMI 1640 medium (LIFE) with 10% HS (horse serum, LIFE) 5% FBS (fetal bovin serum, LIFE) and 1% penicillin streptomycin (LIFE). They were cultured at 37°C , 5% CO_2 in incubator. After 3 days, the cells were added 5ml PBS to wash two times, and added 4ml trypsin solution to react 1 min at 37°C . Then observing cells by microscope whether cells have been come off dishes. After PC 12 cells was suspension, the cultured medium was added to stop the reaction. PC 12 cells were seeded on the 24-well culture plates when each well had been put a gelatin membrane. The suitable concentration of cells was 2.5×10^4 cells/well. Finally, to culture the PC12 cells with cross-linked gelatin for 1, 3 and 7 days was evaluated by MTT assay (Thiazolyl blue, Sigma Chemical Co., St. Louis, MO, USA) respectively [16].

3. RESULTS AND DISCUSSION

3.1 Evaluation of Cross-Linking by DSC Measurement

In DSC results, a typical thermogram recorded from the uncross-linked and cross-linked gelatin samples were reported in Fig. 3. It displayed an endothermic peak at about 45.7°C associated to the helix-coil transition of gelatin, which was the denaturation temperature (T_d) [17]. In theory, cross-linking degree increases the thermal stability of gelatin membranes, as shown by the shift of the T_d to higher values. In the thermogram recorded from a gelatin membrane cross-linked with EDC solution, the endothermic peak of 0.8% EDC cross-linking gelatin was centered at about 61°C that was higher value than the T_d of other samples treated with EDC solutions. The values of denaturation temperature obtained for gelatin membranes cross-linked with EDC and GTA solutions at different concentrations are reported in

Table 1. experiment groups

Group	gelatin concentration	cross-linking reagent	cross-linker concentration	Group	gelatin concentration	cross-linking reagent	cross-linker concentration
gelatin15	15%	na	na	g15C0.2	15%	EDC	0.2%
g15G0.05	15%	GTA	0.05%	g15C0.4	15%	EDC	0.4%
g15G0.1	15%	GTA	0.1%	g15C0.6	15%	EDC	0.6%
g15G0.2	15%	GTA	0.2%	g15C0.8	15%	EDC	0.8%
g15G0.4	15%	GTA	0.4%	g15C1.0	15%	EDC	1.0%
g15G0.6	15%	GTA	0.6%	g15C5.0	15%	EDC	5.0%
				g15C10	15%	EDC	10%

Table II. Denaturation temperature of gelatin samples increased on increasing EDC concentration (0.2% to 0.8%), in agreement with the simultaneous increased of the extent of cross-linking. But increasing GTA concentration from 0.05% to 0.6%, the Td values of gelatin samples were decreased from 66.2°C to 56.3°C. In addition, two endothermic peaks were observed in 0.4% and 0.6% GTA cross-linking groups because of the GTA concentration was too high to complete cross-

linking reaction. Therefore, partial of gelatin membrane was cross-linked completely but others were not. It is generally accepted that the endothermic process present in the DSC thermogram of collagenous materials involves rupture of hydrogen bonds and a rearrangement of the triple helix into a random configuration [18-19]. On the other hand, the increased thermal stability and decreased endothermic heat change exhibited by collagen after air-drying, have been ascribed to the presence of covalent bonds, such as cross-links, which break exothermically [20-21]. Likewise, the increased thermal stability of collagenous materials with aging has been related to the production of covalent cross-links [22]. Thus it can be seen that 0.05% GTA treated gelatin membrane shown the best degree of covalent cross-links in all experimented groups. In g15C0.6, the thermal stability was not as good as it of g15G0.05 because of the feature of EDC produced the relatively short length of the cross-links. The cross-link was two atoms long (Fig.2). This limits the spread of the cross-links to adjacent gelatin molecules, unlike GTA, where the length can be quite long [15].

Fig. 3 DSC curves of uncross-linked gelatin membrane, gelatin membranes cross-linked with 0.6%, 0.8%, 1.0% EDC, respectively. gelatin membranes cross-linked with 0.4%, 0.6% GTA, respectively.

3.2 Thermogravimetric Analysis

The values of melting temperature and cracking temperature obtained for the gelatin samples cross-linked by two different reagents of the same concentration were shown in Fig. 4 determined by thermogravimetric analysis. As previous investigation reported that the melting temperature of gelatin films was centered in 91.5°C, corresponded to the results in this study [23]. The melting temperature of gelatin15, g15C0.6 and g15G0.6 were 85.9°C, 88.2°C and 91.3°C respectively. However, water content greatly affects the melting temperature of the helix-coil transition [21]. Besides, the melting temperatures of water and gelatin sample in this experiment were overlapping in thermogram of thermogravimetric analysis, thus the

melting temperatures could not be directly compared. The cracking temperature of gelatin15, g15C0.6 and g15G0.6 were centered in 315.6°C, 328.3°C and 327.5°C respectively. The results of g15C0.6 and g15G0.6 approximated to each other, but the proportion of endothermic peak of g15G0.6 was higher than the peak of g15C0.6. Therefore, g15G0.6 cracked to smaller molecules has to absorb more calorific capacity than g15C0.6. The increase in the strength of covalent binding on increasing the proportion of endothermic peak was evident. Responded to the results of DSC, GTA reagent produced the relatively long length of gelatin molecules after cross-linking, and the cross-linking degree was greater than EDC produced.

3.3 Release and Degradation of Gelatin

The amount of degraded gelatin released in PBS was analyzed by UV/VIS spectrophotometer and the test extended for 168 hours. This experiments were

Table 2. Denaturation temperature (Td) of cross-linked gelatin membranes displayed as follows. Each value was the mean of 6 determinations and reported with its standard deviation.

Group	Td (°C)	Group	Td (°C)
gelatin15	45.7±1	g15C0.2	47.6±2
g15G0.05	66.2±1	g15C0.4	50.5±1
g15G0.1	63.6±1	g15C0.6	56.5±1
g15G0.2	56.7±1	g15C0.8	61.0±1
g15G0.4	55.4±2	g15C1.0	56.5±2
g15G0.6	56.3±2

Fig.4 The weight loss curves and the temperature difference curves by DTA/TGA of 15% gelatin, 0.6% carbodiimide and 0.6% glutaraldehyde cross-linked gelatin

performed at 37°C in order to simulate the typical conditions of dissolution *in vivo*. Fig.5 shows the kinetic degradation of gelatin membranes cross-linked by 0.4%, 0.6%, 0.8% and 1.0% EDC. The burst releasing happened in the first 8 hours about 2~3% in accumulative percentage. At the end of 24 hours, the amount of degraded gelatin of g15C0.4, g15C0.6, g15C0.8 and g15C1.0 were 2.3%, 3.1%, 3.3% and 4.8% in average. After 72 hours, g15C0.4 have degraded 8.2% of gelatin in PBS that was more than other groups. The rate of gelatin degradation was slow down thereafter. The results of Fig.5 were in agreement with the lower concentration of cross-linked reagent the faster degraded rate of gelatin membrane.

3.4 In Vitro Experiments

The GTA cross-linking influence was more violent than the EDC cross-linking, as previously reported. But the violent cross-linking reagent, GTA, was verified that it was cytotoxic for cells and tissues. Fig. 6 shows the MTT assay for the nine test groups of EDC cross-linking after cultured for 1, 3 and 7 days. The results indicated that 15% gelatin cross-linked by 0.8% EDC has the least cytotoxicity, and cell activity of this group was similar to control group (blank dish). And the 0.6% EDC cross-linking gelatin membrane has shown good for cells survival, too. Fig. 2 shows the sequence of events in a typical reaction. The EDC is first protonated and reacts with the carboxyl function on gelatin to form the o-isoacylurea [24-25]. This is followed by nucleophilic attack of the amine functions on the adjacent gelatin, generating the cross-link and simultaneously releasing the urea derivative of the EDC reagent. Therefore, properly washed steps to remove the activating reagent gave a membrane with no residual chemicals and no harm for cells or tissues. Nevertheless, the cell activity in low concentration of EDC groups (0.2% and 0.4%) were not satisfied, because of the degradation of gelatin membranes was faster than cell adhesion and cell differentiation. On the other hand, the decrease of cell viability followed the increase of concentration of EDC solution, when the EDC concentration was over 1%. As the results of Fig. 6, 5% and 10% EDC cross-linking gelatin membranes would lead cell activity decreasing, it might cause of the concentration of EDC in gelatin membrane was too high and brought about partial EDC cross-linked incompletely.

Fig. 7 shows the MTT assay for the different concentration of GTA cross-linking gelatin after PC 12 cells cultured for 1, 3 and 7 days. The higher concentrations such as 0.2%, 0.4% and 0.6% of GTA cross-linking gelatin revealed intense toxicity for PC 12 cells. However, when the concentration of GTA in

Fig. 5 Gelatin degraded profile from 0.4%, 0.6%, 0.8% and 1.0% EDC cross-linked gelatin membranes.

Fig. 6 The result of MTT test in each concentration of carbodiimide cross-linked gelatin membranes.

Fig. 7 The result of MTT test in each concentration of glutaraldehyde cross-linked gelatin membranes.

gelatin membranes was down to 0.05% or 0.1% the cell viability was returned to approach the value of control group. As the observation of optical microscope, the morphology of cells in EDC groups maintained its phenotype without transformation. However, the higher concentration GTA cross-linking gelatin was demonstrated that it was more cytotoxic to PC 12 cells, much cells suspended in culture medium.

Nonetheless, the lower concentration GTA cross-linking gelatin could avoid this problem. In the cross-linking reaction, the aldehyde is able to interact chemically with amino groups on gelatin to form chemical bonds, as depicted in Fig. 1 [15]. Since it is bifunctional, it links two different gelatin molecules, hence its mode of cross-linking. However, lower concentrations have been found to be better in bulk materials cross-linking compared to higher concentrations because of homogeneous cross-linking [26]. The excess GTA or uncross-linked aldehyde group in gelatin membrane might cause cytotoxicity to cells death.

4. CONCLUSION

In this study, the Td value of g15C0.8 was centered at 61°C that was higher than the Td of other samples treated with EDC solution. Increasing EDC concentration 0.2% to 0.8% the cross-linking degree of gelatin samples increased, but the inference in GTA cross-linking gelatin membranes were disagreement. The Td value of g15G0.05 was centered at 66.2°C the highest value of all experiment samples. After cross-linking, GTA reagent produced the relatively long length of gelatin molecules, stronger covalent binding and well cross-linking degree compared with EDC produced gelatin membranes as the results of DSC and TGA analysis. However, the results of MTT assay were shown that the excess GTA residue revealed intense toxicity for PC12 cells. Finally, the optimum concentrations of GTA and EDC used in cross-linking gelatin membranes for PC12 cells culture were 0.05% and 0.8% respectively. In the future application, the cross-linked gelatin could be used to copolymerize with other polymer or immobilize with neurotrophic factor for the nerve injury repair.

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