Harvard-MIT Division of Health Sciences and Technology
HST.535: Principles and Practice of Tissue Engineering
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Physical and chemical modification and evaluation of chitosan nerve conduit material

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Background

1. In China, the number of patients with nerve trauma caused by various injuries or diseases is 1-3 million per year.
2. Without proper therapy in time, these patients will loss corresponding physiological functions permanently.
3. Few of the patients can be treated properly: difficulties in technology and economy

Different choices of nerve recovery

1）Surgical suture end-to-end

Disadvantage: suitable only for small nerve gap

2）Graft as a guide for axon regeneration

Large gaps can be repaired with a graft inserted between the proximal and distal nerve stumps as a guide for the regenerating axon

- **Autograft**

nerve removed from another part of the body, blood vessels or muscle fibres

Disadvantages:

- **need for second surgical treatment;**
- **limited availability;**
- **denervation of the donor site**

- **Allograft,**

  nerve removed from other persons

Disadvantage:

- **immune rejection;**
- **low success rate;**
- **limited availability**

- **Heterograft**

nerve from animals such as pig

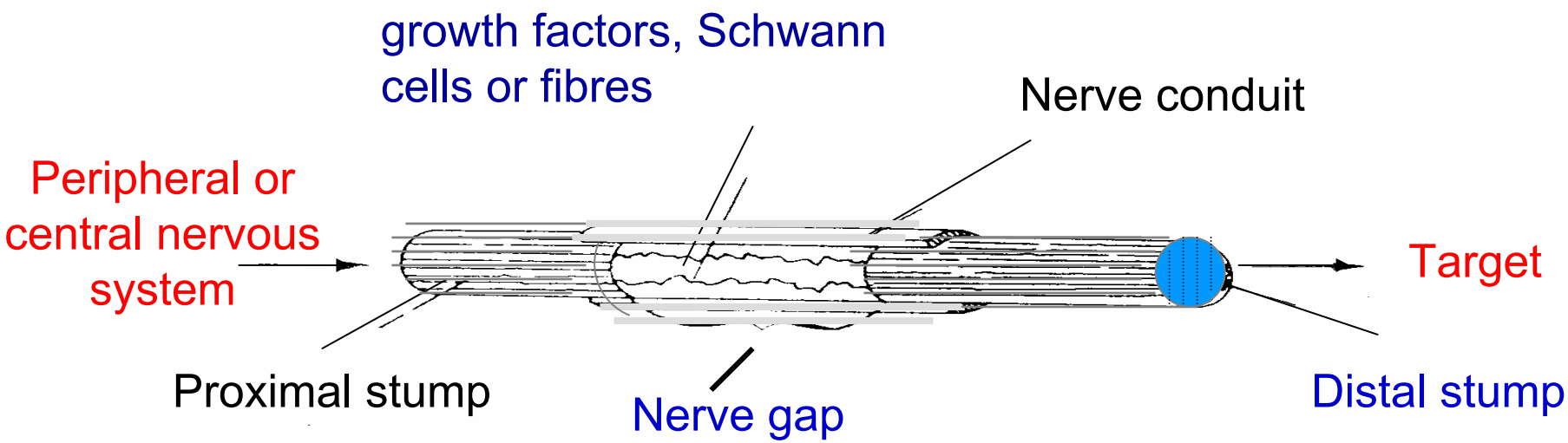
Disadvantage:

- **immune rejection;**
- **low success rate;**
- **some other risk**

•Artificial nerve conduit

Nerve conduit is an artificial graft that bridges the gap between the nerve stumps and directs and supports regenerating axon.

* **Hollow conduit;**
* **Conduit filled with growth factors, Schwann cells or fibres.**



The function of nerve conduit

Nerve conduit bridges the gap between the nerve stumps and forms an environment suitable for nerve regeneration

Advantages

- Concentrating neurotrophic factors;
- Reducing cellular invasion and scarring of the nerve;
- Providing guidance to prevent neuroma formation and excessive branching.

Nerve conduit biomaterials

1) Nonresorbable materials such as silicone rubber

Disadvantage： need for second surgical treatment

2) Biodegradable or resorbable biomaterials

- **Synthetic:**

∗**poly-lactic acid (PLA);**

∗**polyglycolic acid (PGA);**

...

Disadvantages：

∗**expensive;**

∗**acidic degradation product**

- **Natural:**

∗**chitosan;**

∗**collagen,**

...

Chitosan, the fully or partially deacetylated form of chitin, is a positively charged polymeric saccharide with (1,4)-linked D-glucosamine repeat units

Advantages:

- **biocompatible,**
- **biodegradable,**
- **plentiful in nature,**
- **cheap**

Disadvantages

- **Low mechanical strength and toughness;**
- **Low solubility;**
- **Difficulty in manufacturing and shaping;**

Work in our lab

- **Improvement of mechanical properties of chitosan;**
- **Improvement of chitosan biocompatibility;**
- **Control of chitosan biodegradability;**
- **Conduit making — chitosan shaping;**
- **Preliminary functional evaluation.**

Chitosan modification

- Blending or chemical linking with gelatin, collagen and polylysine;
- Surface coating with laminin, fibronectin, serum and polylysine;
- γ-ray irradiation;
- Alkylation;
- Crosslinking with different reagent;
- Modulation of deacetylation degree.

Evaluation of chitosan-derived materials

- Biocompatibility evaluation
- Biodegradability evaluation
- Physical property evaluation
- Functional evaluation

Biocompatibility evaluation

- **Contact angle**
- **Protein adsorption**
- **Cell affinity:**
  - **Attachement of cultured cells;**
  - **Proliferation: MTT measurement**
  - **Differentiation: neurite length and other morphological features**

- **Water contact angle**

➢ **The hydrophilicity of a biomaterial is a determinant of the material's biocompatibility.**

➢ **Hydrophilicity of a biomaterial is dependent on its surface contact angle.**

•Protein adsorption

➢The adsorption of proteins, such as some ECM molecules, onto material surface, is an important determinant of biocompatibility of biomaterials

➢The laminin and fibronectin adsorption on film surface was investigated using ELISA and desorption method.

- **Biodegradability evaluation**

- **Evaluation of other physical property**
  - **Solubility**
  - **Crystallinity**
  - **Mechanical properties**

Chitosan modification (1)

- **Blending or chemical linking with gelatin, collagen and polylysine;**
- **Surface coating with laminin, fibronectin, serum and polylysine**

Figures removed for copyright reasons.

See Fig. 1 through Fig. 8 in Gong, H. et al.
"Studies on nerve cell affinity of chitosan-derived materials."
J Biomed Mater Res 52 no. 2 (2000): 285-295.

Figures removed for copyright reasons.

See Fig. 1 through Fig. 5 in Cheng, M., et al.
"Studies on nerve cell affinity of biodegradable modified chitosan films."
J Biomater Sci Polymer Edn 14 no. 10 (2003): 1155-1167.

Effect of gelatin content on the biological and physicochemical properties of chitosan-gelatin composite

Figures removed for copyright reasons.

Figures 2, 4, 5, 8, 10 in Cheng, M., et al. "Study on physical properties and nerve cell affinity of composite films from chitosan and gelatin solutions."
*Biomaterials 2*4 no.17 (2003): 2871-2880.

Crystallinity and rupture strain maximum of chitosan-gelatin composite films

gelatin content (r)	crystallinity (Xc, %)	rupture intensity (MPa)	
		dry	wet
0. 0	19. 8	68. 6±2. 8	5. 50±0. 67
0. 2	14. 4	68. 2±3. 9	4. 20±0. 48
0. 4	6. 5	62. 4±3. 4	2. 80±0. 42
0. 6	4. 2	51. 6±2. 2	2. 30±0. 28
0. 8	0. 0	43. 3±2. 5	1. 40±0. 18
1. 0	0. 0	36. 7±1. 3	0. 0

Conclusion

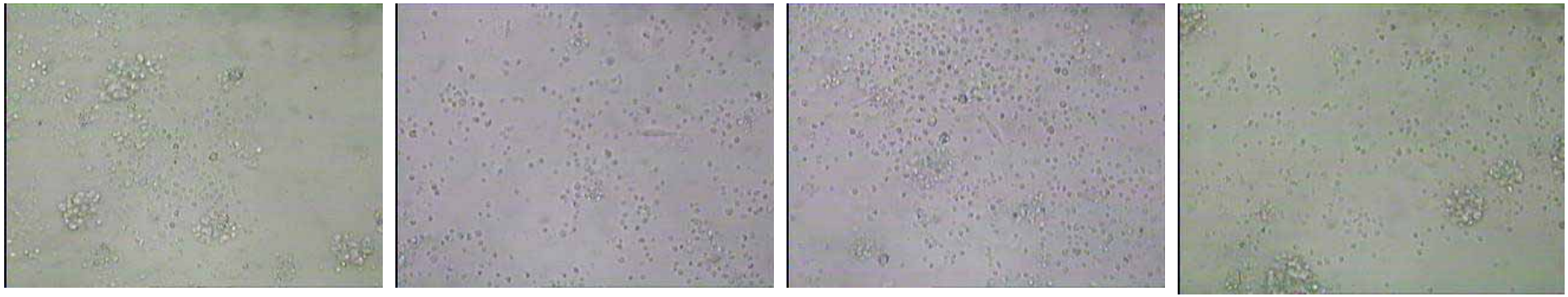
- Proper physical blending or chemical linking with gelatin, collagen and polylysine can improve the biocompatibility of chitosan and keep its physical properties reasonable.
- Even a simple coating with laminin, fibronectin, serum and polylysine is also of help for chitosan biocompatibility.

Chitosan modification (2)

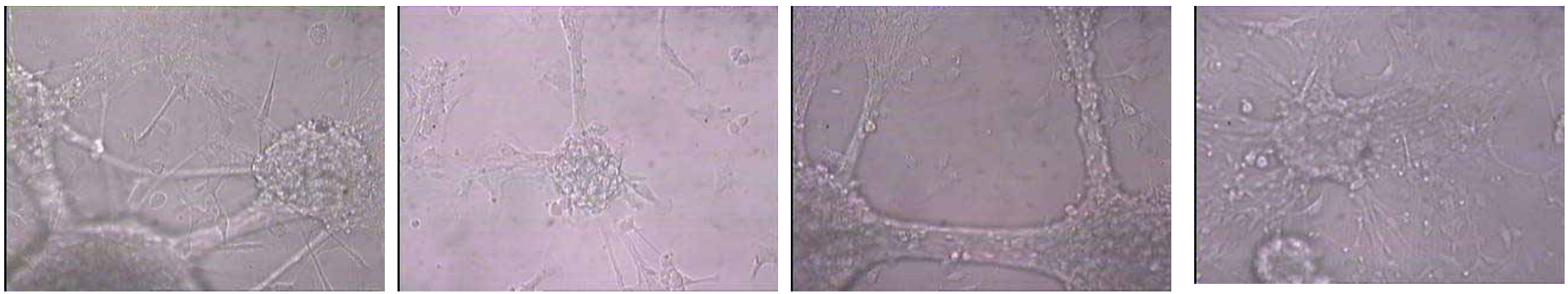
γ-ray irradiation

Figures removed for copyright reasons.

Yang, F. et al. "Performance Modification of Chitosan Membranes Induced by Gamma Irradiation ." Journal of Biomaterials Applications 16 no. 3 (2002): 215-226.



a1 b1 c1 d1



a6 b6 c6 d6

FMCC (fetal mouse cerebral cortex) cells on the chitosan membranes irradiated with different irradiation doses.

Culture time: a1, b1, c1, d1: 1 day; a6, b6, c6, d6: 6 days.
Irradiation dose: a—0 kGy，b—14 kGy，c—16 kGy，d—18 kGy；
magnification: ×200

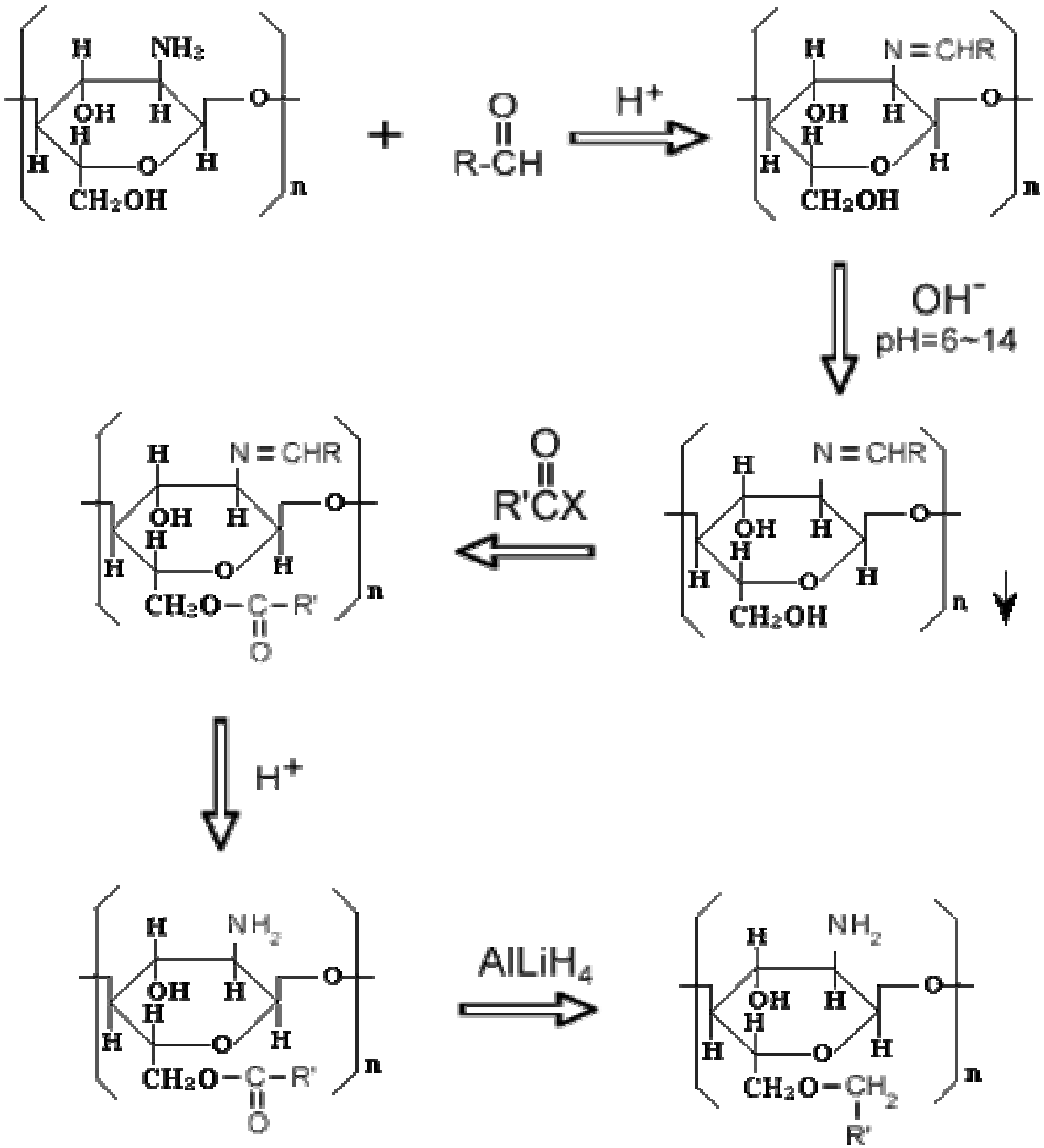
Yang F, et al. unpublished data

Conclusion

- **The mechanical properties (such as rupture intensity) of chitosan membrane was increased significantly after gamma-ray irradiation of proper doses(14-18 kGy).**
- **The gamma irradiation decreased the contact angles which indicates the increase of the hydrophilicity of the membrane.**
- **Fetal mouse cerebral cortex cell culture showed that the chitosan membranes with gamma-ray irradiation retained their good biocompatibility.**
- **The gamma-ray irradiation can improve the mechanical properties of chitosan conduit and maintain their good biocompatibility at the same time.**

Chitosan modification (3)

Alkylation



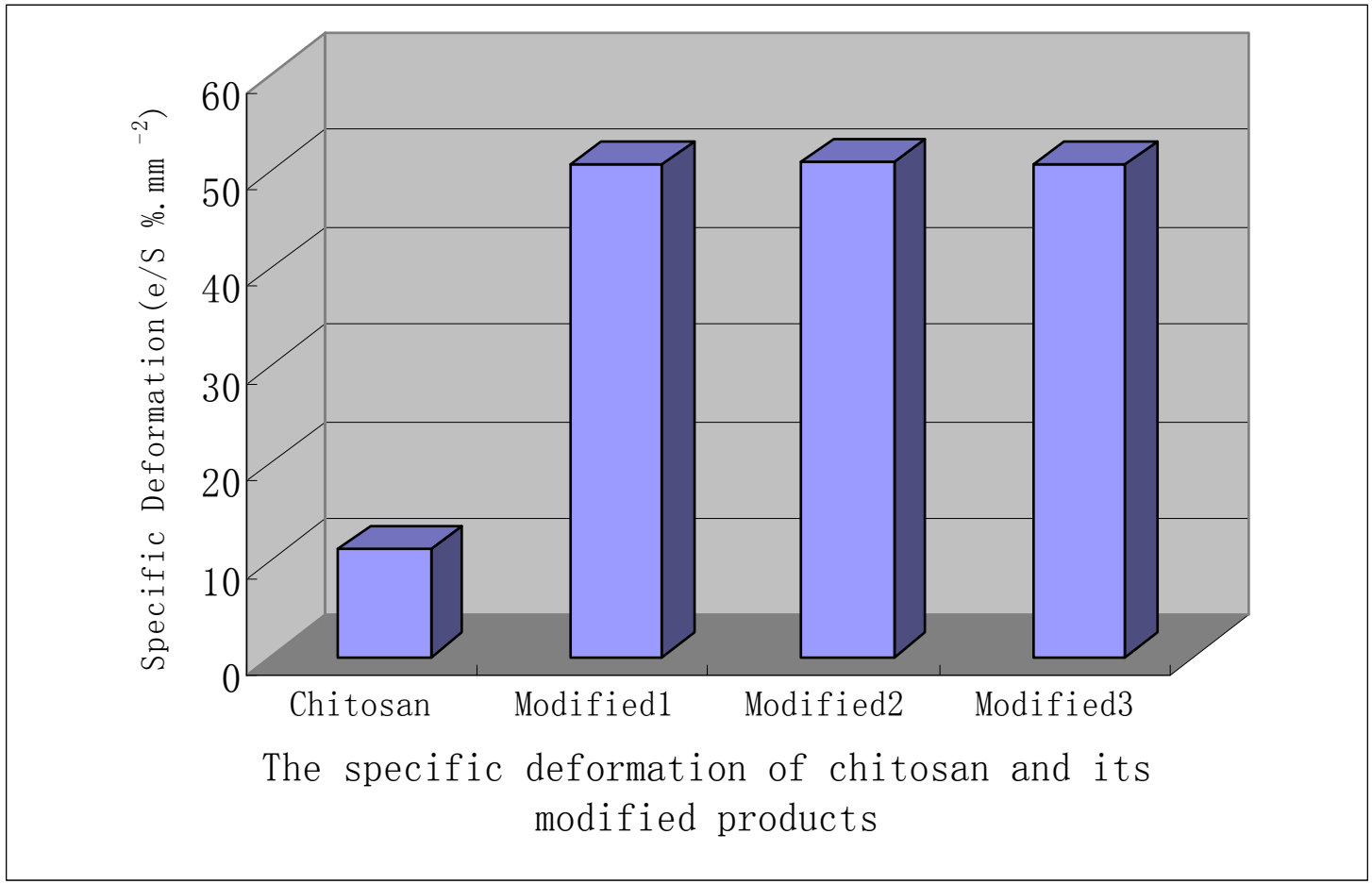
The diagram of chitosan O-alkylation

R is the group which links aldehyde groups. R´is long-chain alkyl (the number of carbon atoms is 8~15).

RCHO is benzaldehyde, the amino group protectant.

R´CXO is palmitoyl chloride, the reactant. $AlLiH_4$ is the reducer which reduces long-chain acyl producing the O-alkylat.

Deng JG, et al. unpublished data



The specific deformation of chitosan and alkylated chitosan

The specific deformation is an index of material toughness.

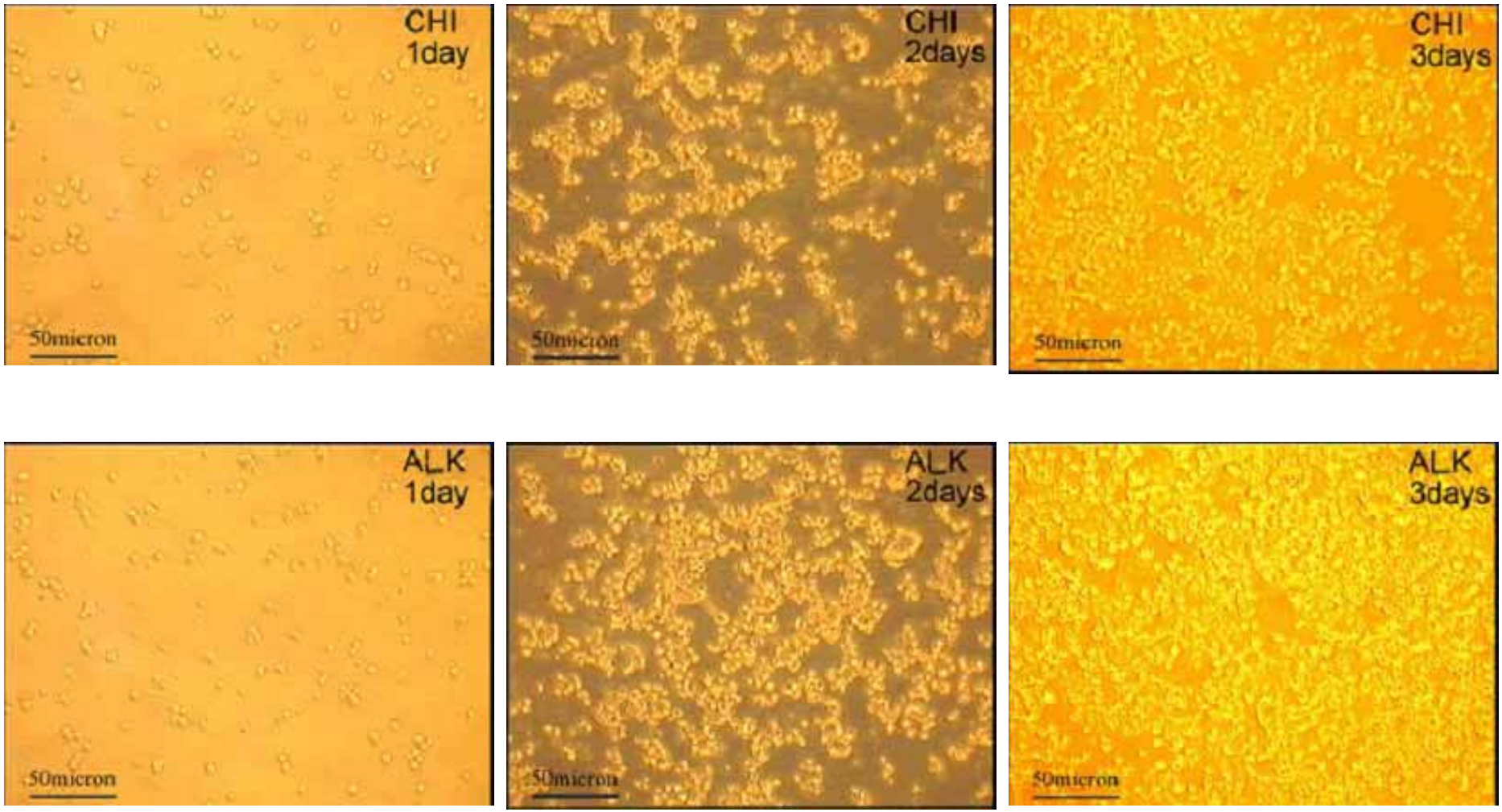
Deng JG, et al. unpublished data

Solubility of alkylated chitosan of different Deacetylation Degree (DD)

No.	Solubility *S* （%）	*DD*（%）
0	2.1	81.5
1	3.9	72.6
2	7.3	64.5
3	**9.8**	**50.4**
4	8.1	44.0
5	7.6	41.7
6	7.2	40.6

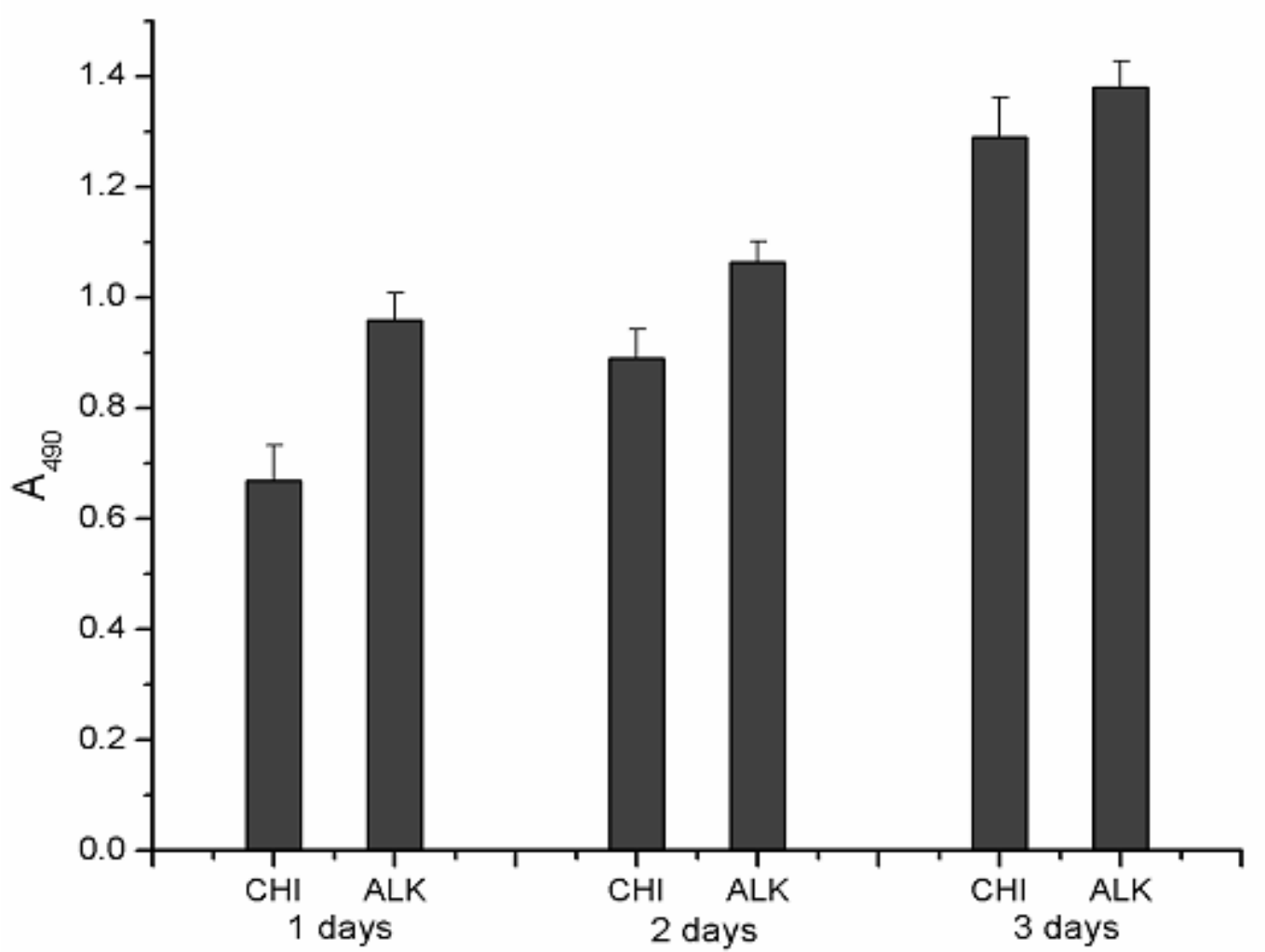
0: native chitosan;
1－6: alkylated chitosan of different DD

Deng JG, et al. unpublished data



PC12 cells cultured for 1, 2 and 3 days on native (CHI) and alkylated (ALK) chitosan

Deng JG, et al. unpublished data



Growth of PC12 cells on chitosan (CHI) and alkylated chitosan (ALK)

means±s.d. n=8

Deng JG, et al. unpublished data

Chitosan modification (4)

Crosslinking with different reagent

- Hexamethylene diisocyanate (HDI)
- Epichlorohydrin (ECH)
- Glutaraldehyde (GA)

Swelling index, water contact angle and mechanical properties of uncrosslinked and crosslinked chitosan films

Source: Cao, W.L., et al. *J Biomater Sci Polymer Edn* 16 no.6 (2005):791-807

Chitosan films	Swelling index (%)[a]	Water contact angle (°)[a]	Young's modulus (MPa)[a]	Tensile strength (MPa)[a]	Breaking elongation (%)[a]
CHI	158.1 ± 6.8	78.1 ± 1.6	15.7 ± 1.8	7.5 ± 0.8	47.9 ± 2.3
HDI-C	129.5 ± 3.1	67.3 ± 2.9	21.7 ± 2.8	9.3 ± 1.3	43.3 ± 1.9
ECH-C	79.0 ± 3.8	51.2 ± 3.2	27.9 ± 2.4	11.8 ± 1.2	42.3 ± 2.4
GA-C	126.0 ± 7.9	62.0 ± 0.9	30.4 ± 4.6	6.1 ± 0.5	20.2 ± 2.3

CHI, HDI-C, ECH-C and GA-C are chitosan films uncrosslinked and crosslinked with hexamethylene diisocyanate (HDI), epichlorohydrin (ECH) and glutaraldehyde (GA) as crosslinking agents, respectively.

a: n = 6, Mean ± SD

Quantification of fibronectin (A) and laminin (B) adsorption on chitosan films

Figures removed for copyright reasons.

Source: Cao, W.L., et al. ***J Biomater Sci Polymer Edn*** 16 no.6 (2005): 791-807.

CHI: chitosan film, control. HDI: hexamethylene diisocyanate; ECH: epichlorohydrin; GA: glutaraldehyde

Asterisks indicate a significant difference (* for $p<0.05$ and ** for $p<0.01$) compared with uncrosslinked chitosan films.

Data are plotted as mean $\pm$ SD (n = 6).

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Source: Cao, W.L., et al. ***J Biomater Sci Polymer Edn*** 16 no.6 (2005): 791-807.

Schwann cells culture on uncrosslinked and crosslinked chitosan films for 3 days

HDI-C films presented a better substratum for the adhesion and spreading of Schwann cells compared with the other films.

Proliferation of Schwann cells cultured on uncrosslinked and crosslinked chitosan films for 7 days

Photos removed for copyright reasons.

Source: Cao, W.L., et al. ***J Biomater Sci Polymer Edn*** 16 no.6 (2005): 791-807.

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Source: Cao, W.L., et al. ***J Biomater Sci Polymer Edn*** 16 no.6 (2005): 791-807.

SEM micrographs of the undegraded (A_0, B_0) and degraded chitosan films by lysozyme after 2 weeks (A_2, B_2), 4 weeks (A_4, B_4).

A: CHI; B: HDI-C;

After crosslinking, the degradation rate of chitosan films significantly decreased.

Photos removed for copyright reasons.

Source: Cao, W.L., et al. ***J Biomater Sci Polymer Edn*** 16 no.6 (2005): 791-807.

SEM micrographs of the degraded chitosan films by lysozyme after 6 weeks (A_6, B_6) and 8 weeks (A_8, B_8, C_8, D_8).

A: CHI; B: HDI-C; C: ECH-C; D: GA-C.

After crosslinking, the degradation rate of chitosan films significantly decreased.

Conclusion

- After crosslinking, the swelling index and the degradation rate of all the chitosan films decreased while their hydrophilicity and elastic modulus increased.
- The films crosslinked with epichlorohydrin and glutaraldehyde were not better substrata for the growth of Schwann cells than uncrosslinked chitosan films. The hexamethylene diisocyanate crosslinked films enhanced the spread and proliferation of Schwann cells.
- Surface crosslinking with hexamethylene diisocyanate is a promising way to modify chitosan nerve repair conduits.

Chitosan modification (5)

Effects of Deacetylation Degree (DD) on the Physicochemical and Biological Properties of Chitosan Films

Chitosans of different deacetylation degrees

Source: Cao, W.L., et al. *J Biomater Sci Polymer Edn* in press

Sample code [a]	DD (%) [b]	M_v	Preparation conditions [c]
CS-70	70.1±1.4	8.6×10^5	Reacetylation of CS-80, 0.05 M acetic anhydride in methanol, 25 ºC, 30 min
CS-76	76.0±1.2	8.6×10^5	Reacetylation of CS-80, 0.05 M acetic anhydride in methanol, 25 ºC, 15 min
CS-80	80.1±0.6	8.6×10^5	Acid hydrolysis, NM-CS, 1 M HCl, 50 ºC, 7 h
CS-85	85.4±0.7	8.7×10^5	Deacetylation of NM-CS, 40% NaOH, 100 ºC, 1 h
CS-91	91.4±0.6	8.4×10^5	Deacetylation of CS-85, 40% NaOH, 100 ºC, 1 h
CS-95	95.6±1.1	8.2×10^5	Deacetylation of CS-91, 40% NaOH, 100 ºC, 1 h
NM-CS	80.5±0.9	1.7×10^6	/

a. NM-CS: non-modified chitosan. The abbreviations for the other samples are as follows: CS refers to chitosan; the two numbers refer to the first two numbers of DD. For example, CS-70 denotes the chitosan sample with 70.1% DD.

b. n = 3, Mean ± SD.

c. The preparation conditions include the kind of reaction, raw material, reaction solution concentration, reaction temperature and reaction time.

Chitosan film	Contact angle (°) [a]	Laminin adsorption (% of control) [ab]	Fibronectin adsorption (% of control) [ab]
CS-70	77.1 ± 4.6	63.5 ± 1.4	61.4 ± 2.5
CS-76	74.4 ± 7.0	56.6 ± 2.5	55.8 ± 2.2
CS-80	80.6 ± 4.6	58.8 ± 2.1	55.2 ± 6.6
CS-85	78.1 ± 3.9	48.9 ± 3.1	50.0 ± 5.3
CS-91	74.0 ± 6.3	47.7 ± 1.6	54.8 ± 1.8
CS-95	76.7 ± 2.9	48.5 ± 3.4	50.6 ± 3.2

Water contact angle of chitosan films and protein adsorption on chitosan films

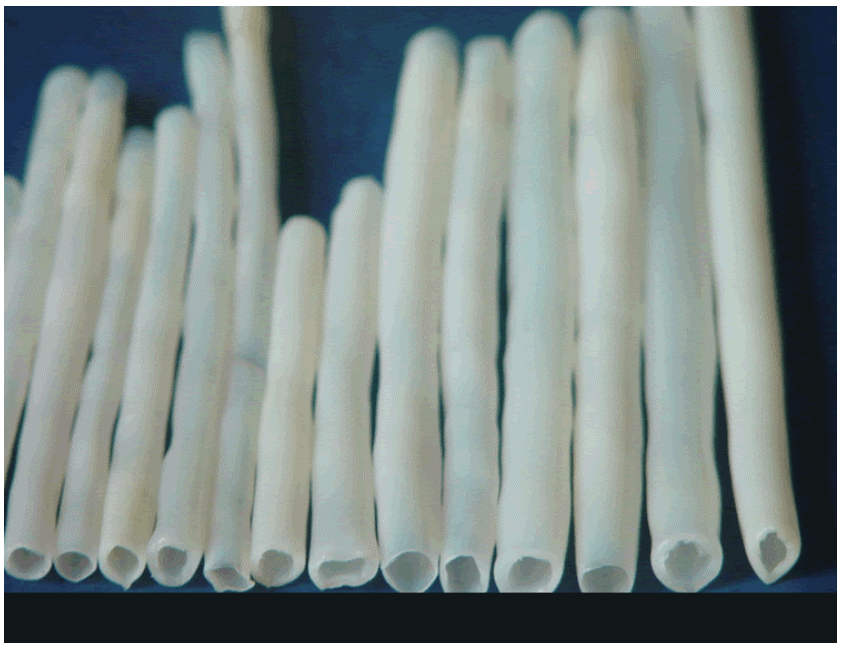
a. n = 6, Mean ± SD.

b. Tissue culture plastics were defined as controls.

Conclusion

- There were more crystalline regions in higher DD chitosan films.
- Swelling index of chitosan films decreased and their elastic modulus and tensile strength increased with the increase in DD.
- DD of chitosan plays an important role in their physicochemical properties and affinity with Schwann cell. The chitosan films with higher DD provided better substrata for Schwann cell spreading and proliferation.
- Chitosan with DD higher than 90% is considered as a promising material for application in peripheral nerve regeneration.

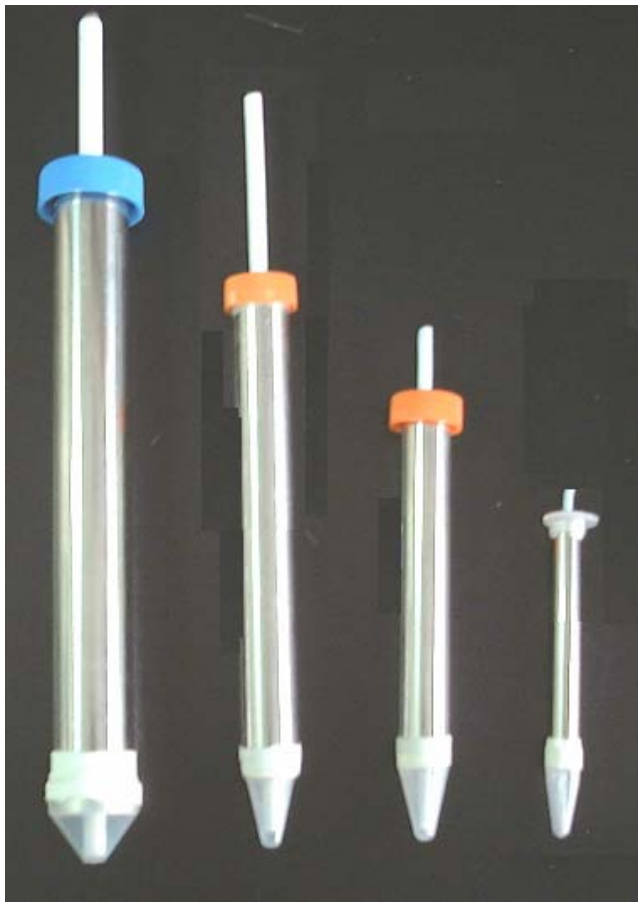
Chitosan nerve conduits prepared by rotary evaporation method



Kept in saturated vapour

Kept in ether

Chitosan nerve conduits prepared by freeze-drying method



Moulds for freeze-drying

Chitosan nerve conduit

Experiment with animal model

Material test and evaluation of biological performance (GB/T16886-2001)

The nerve conduit is a type III medical graft according to a Chinese National regulation, it should be examined in following aspects:

- **cytotoxicity test (in vitro)**
- **genetic test**
- **scratch test**
- **implantation test**
- **degradability test**

...

Preliminary tests have shown that the chitosan nerve conduit is safe in implantation.

Summary

- **Chitosan is a biomaterial with good nerve cell affinity and biodegradability. After proper chemical and physical modification, the material is suitable for nerve conduit making.**

- **Experiment using animal model shows the possibility of nerve repair using chitosan nerve conduit.**

Co-workers

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