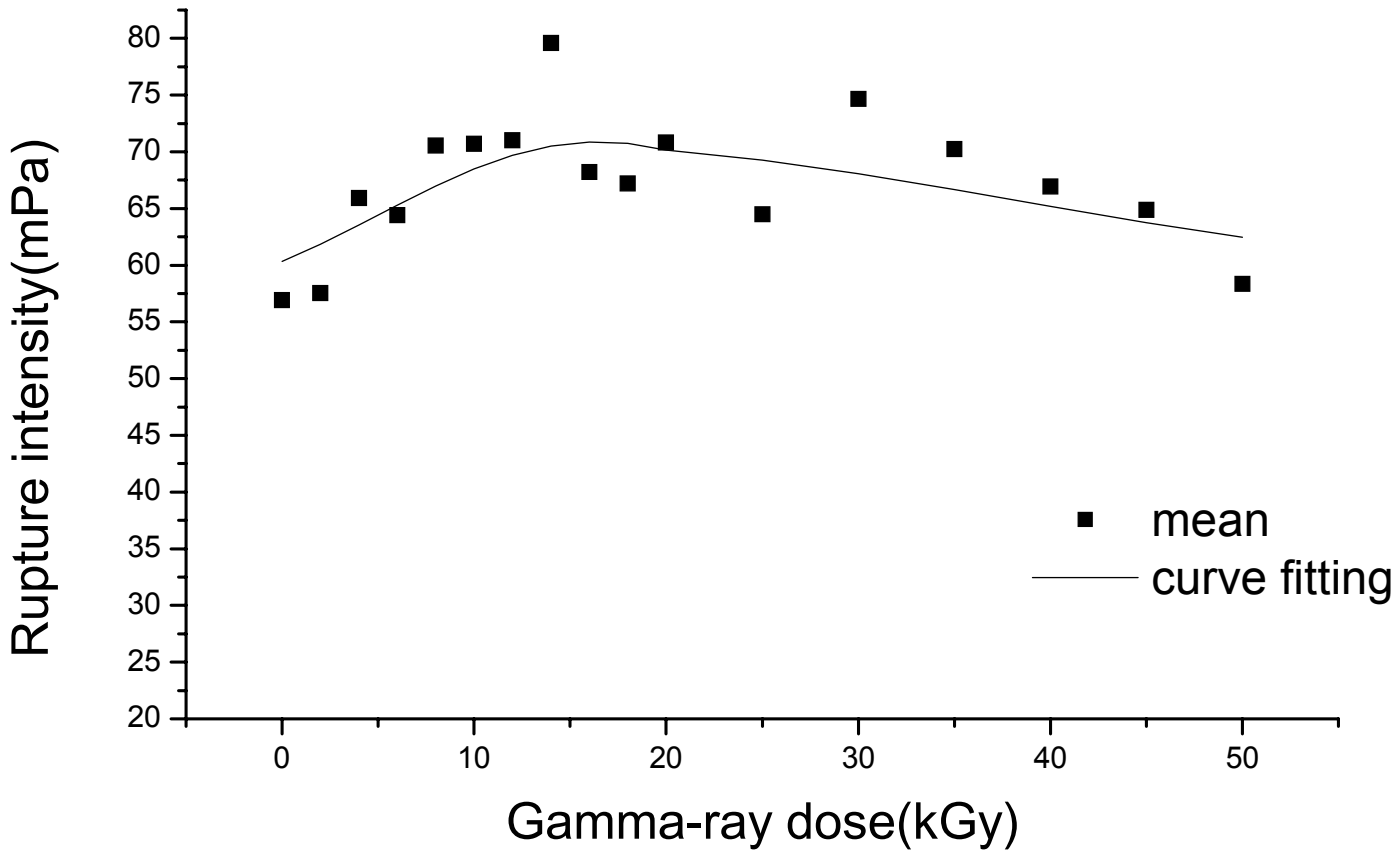
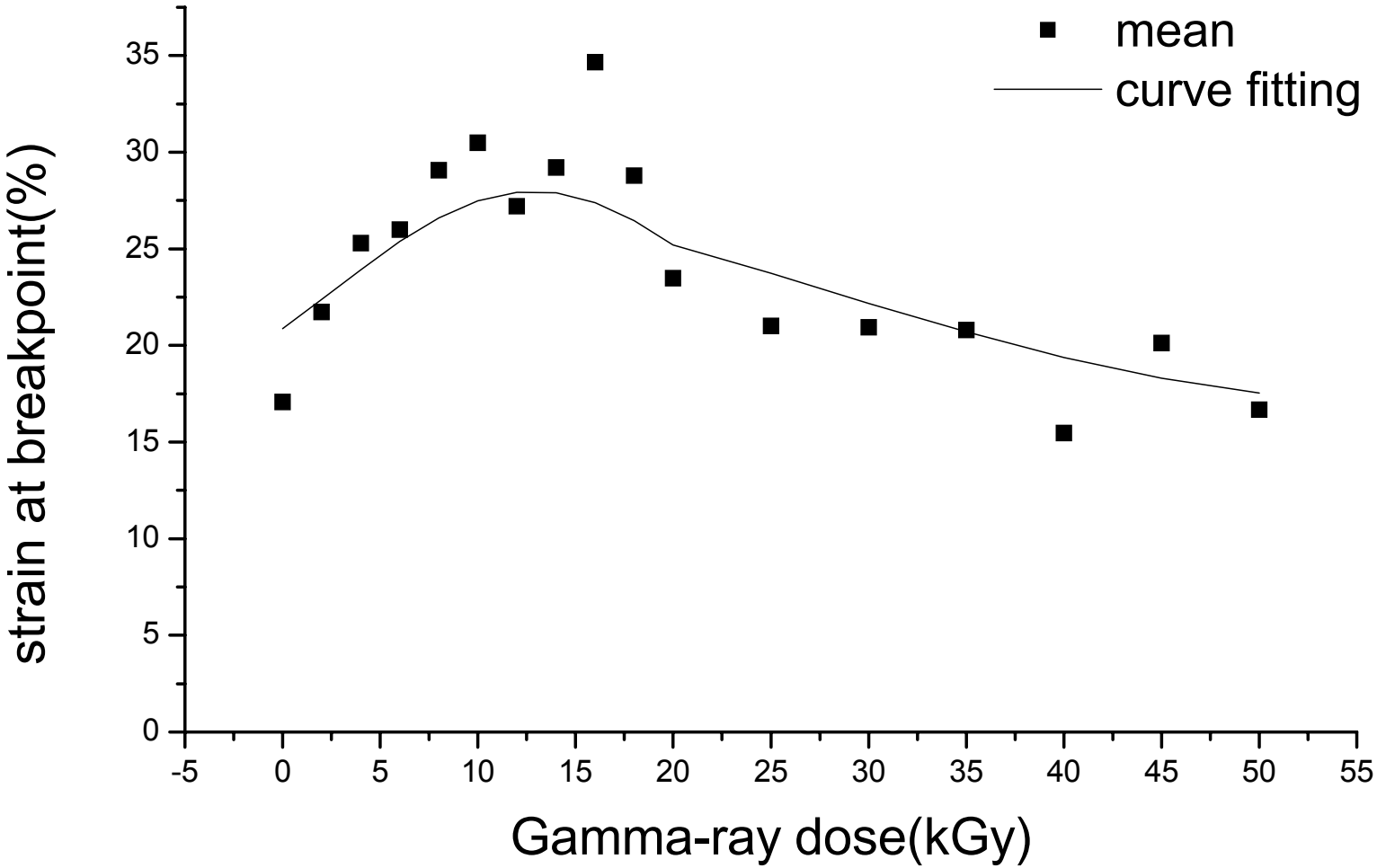
Harvard-MIT Division of Health Sciences and Technology
HST.535: Principles and Practice of Tissue Engineering
Instructors: Xiufang Zhang, Nanming Zhao, Yandao Gong

Chitosan modification (2)

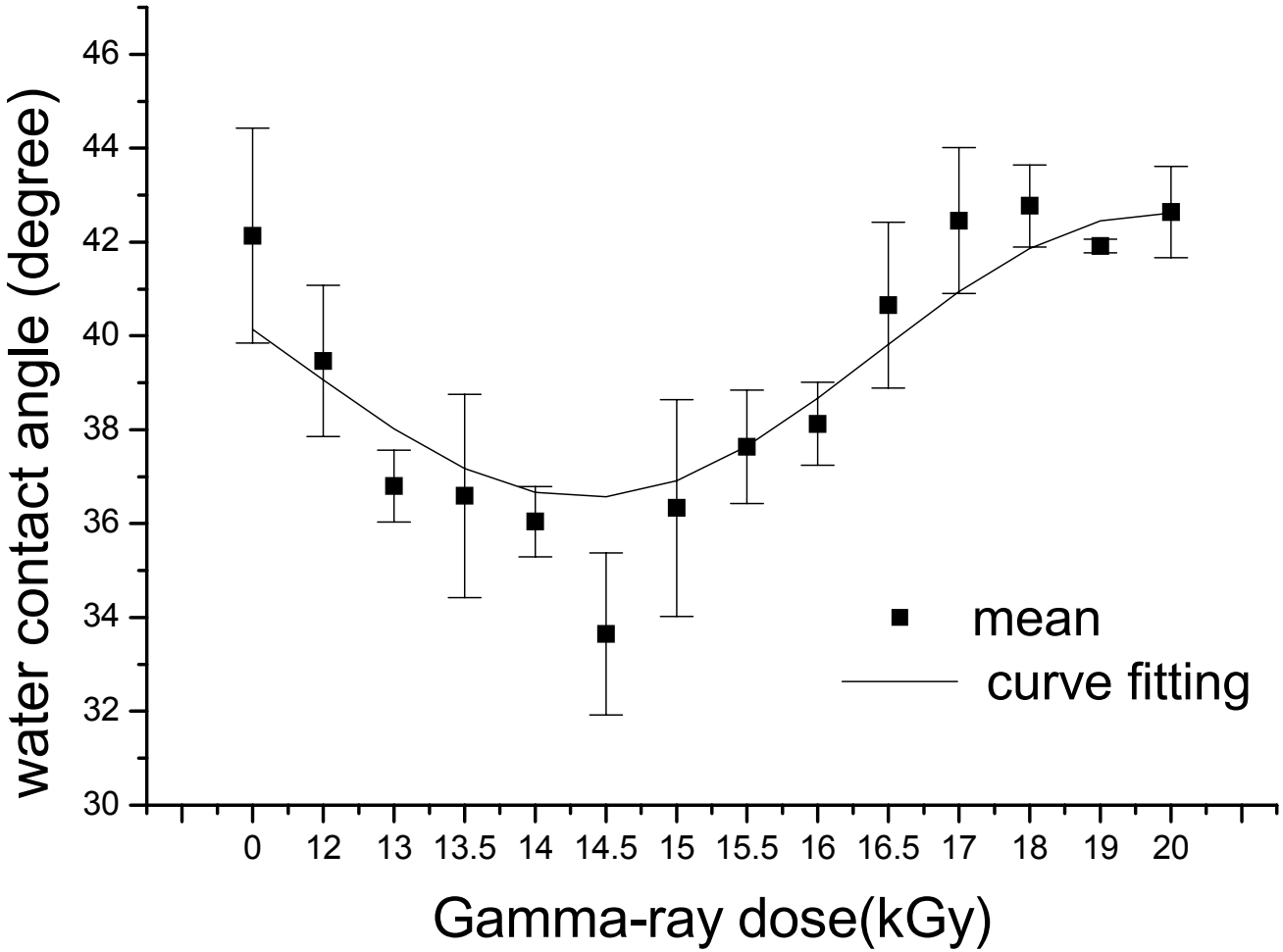
γ-ray irradiation



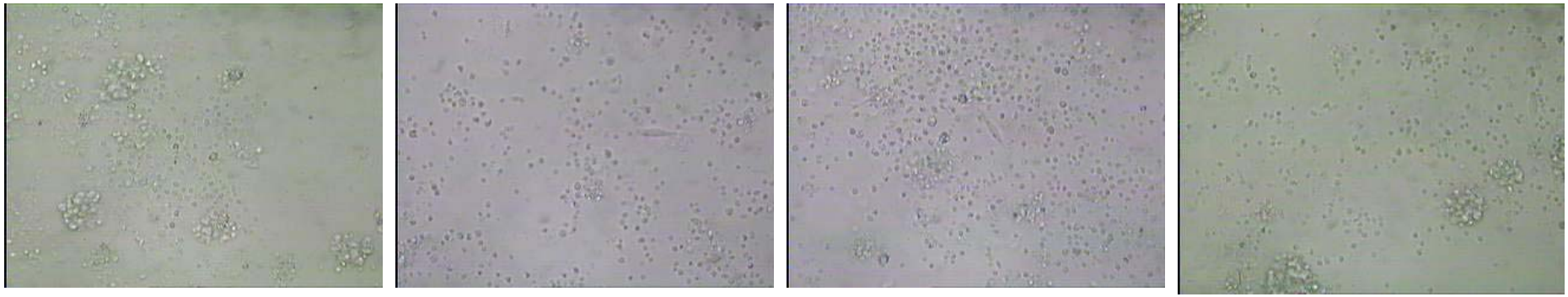
Effect of Gamma-ray irradiation on rupture intensity of chitosan membrane



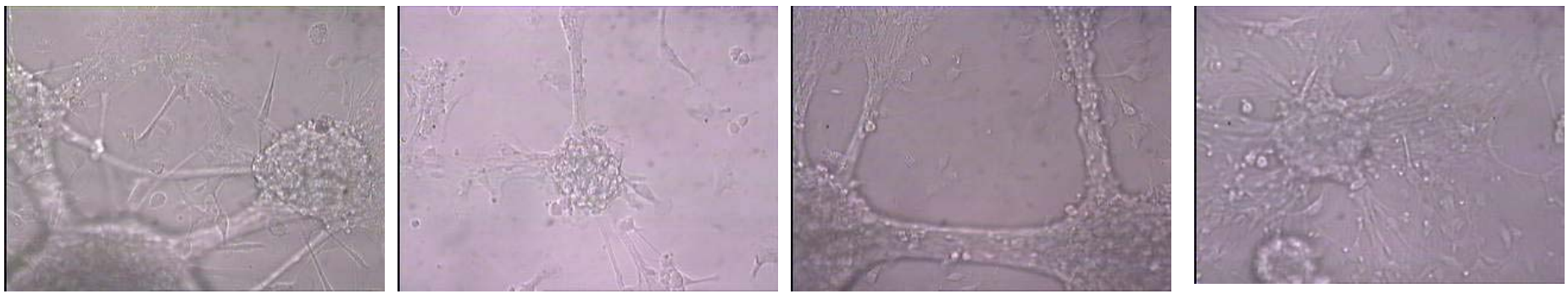
Effect of Gamma-irradiation on strain at breakpoint of chitosan membranes



Change in water contact angle of chitosan membrane by gamma irradiation



a1 b1 c1 d1



a6 b6 c6 d6

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

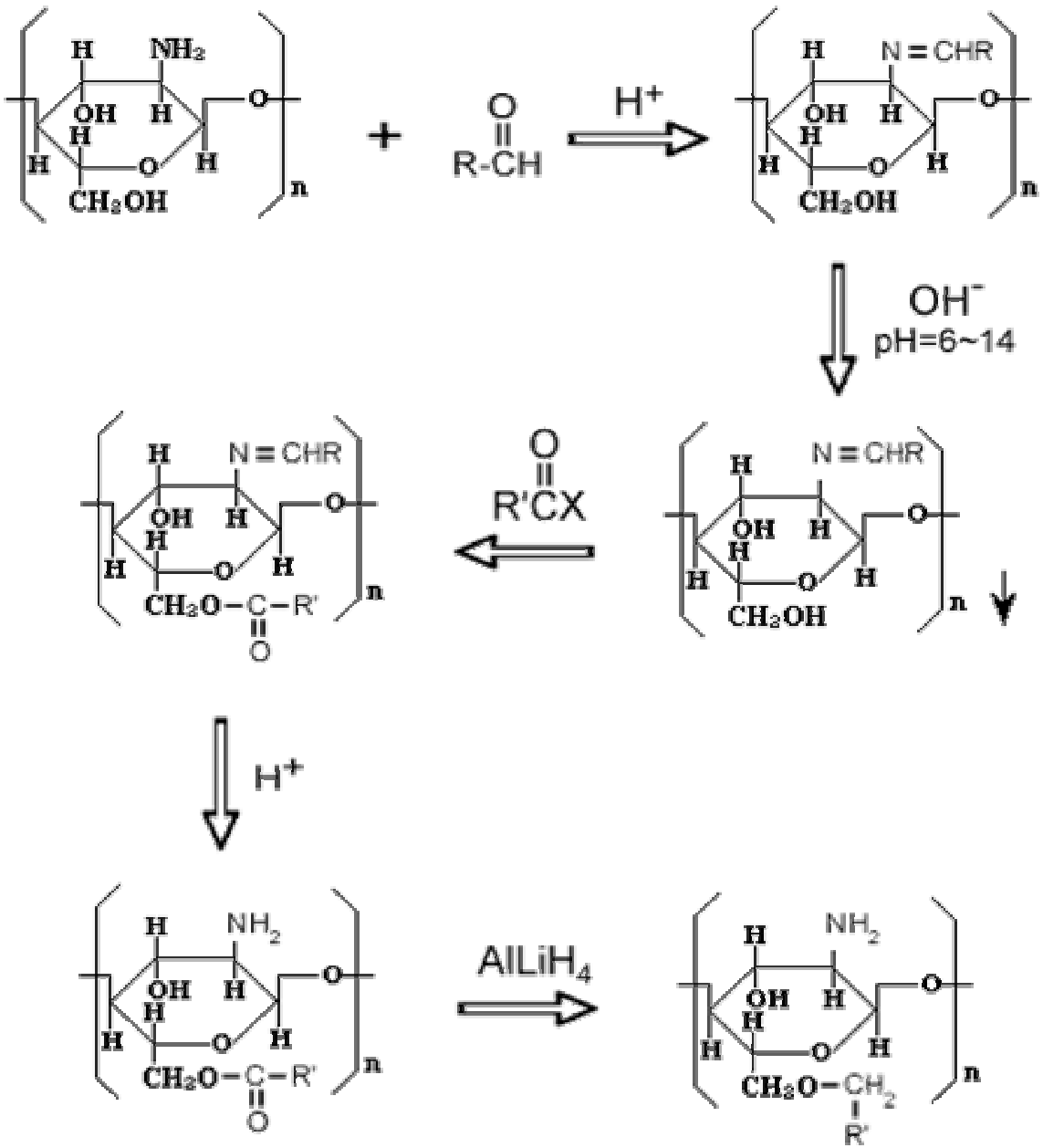
Culture time: a1, b1, c1, d1: 1 day; a6, b6, c6, d6: 6 days.
Irradiation dose: a－0 kGy，b－14 kGy，c－16 kGy，d－18 kGy；
magnification: ×200

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

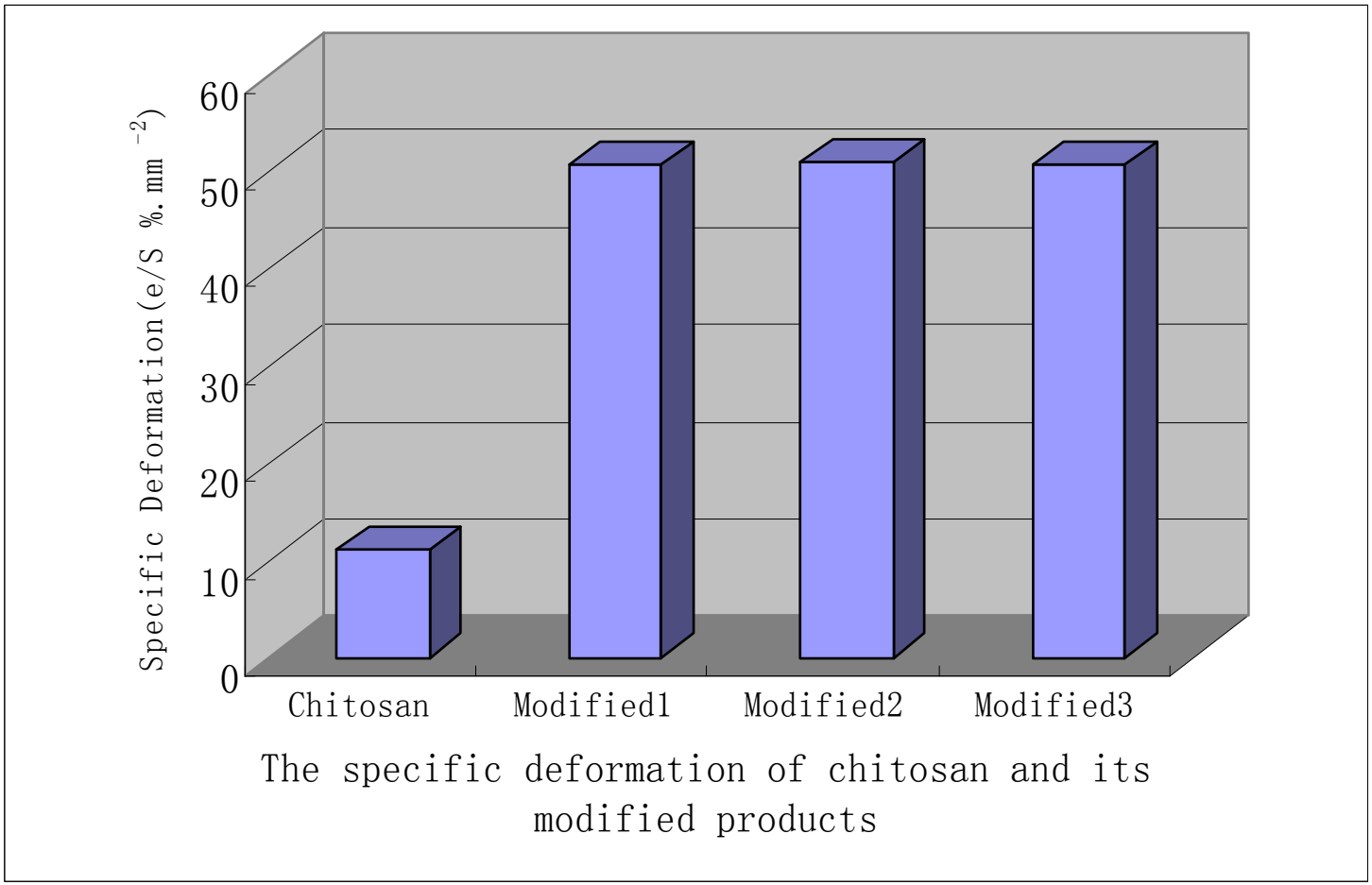


The diagram of chitosan O-alkylation

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.



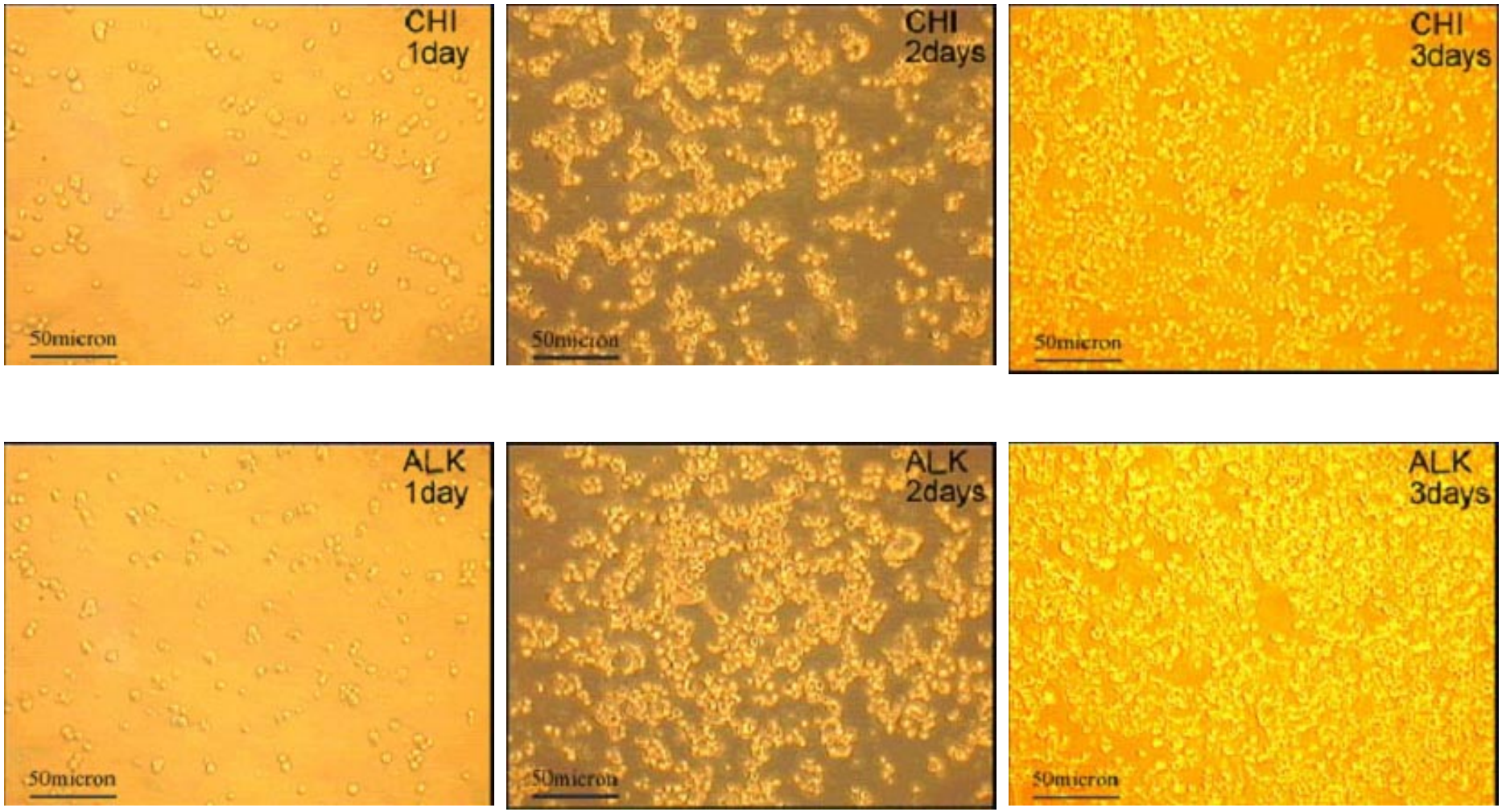
The specific deformation of chitosan and alkylated chitosan

The specific deformation is an index of material toughness.

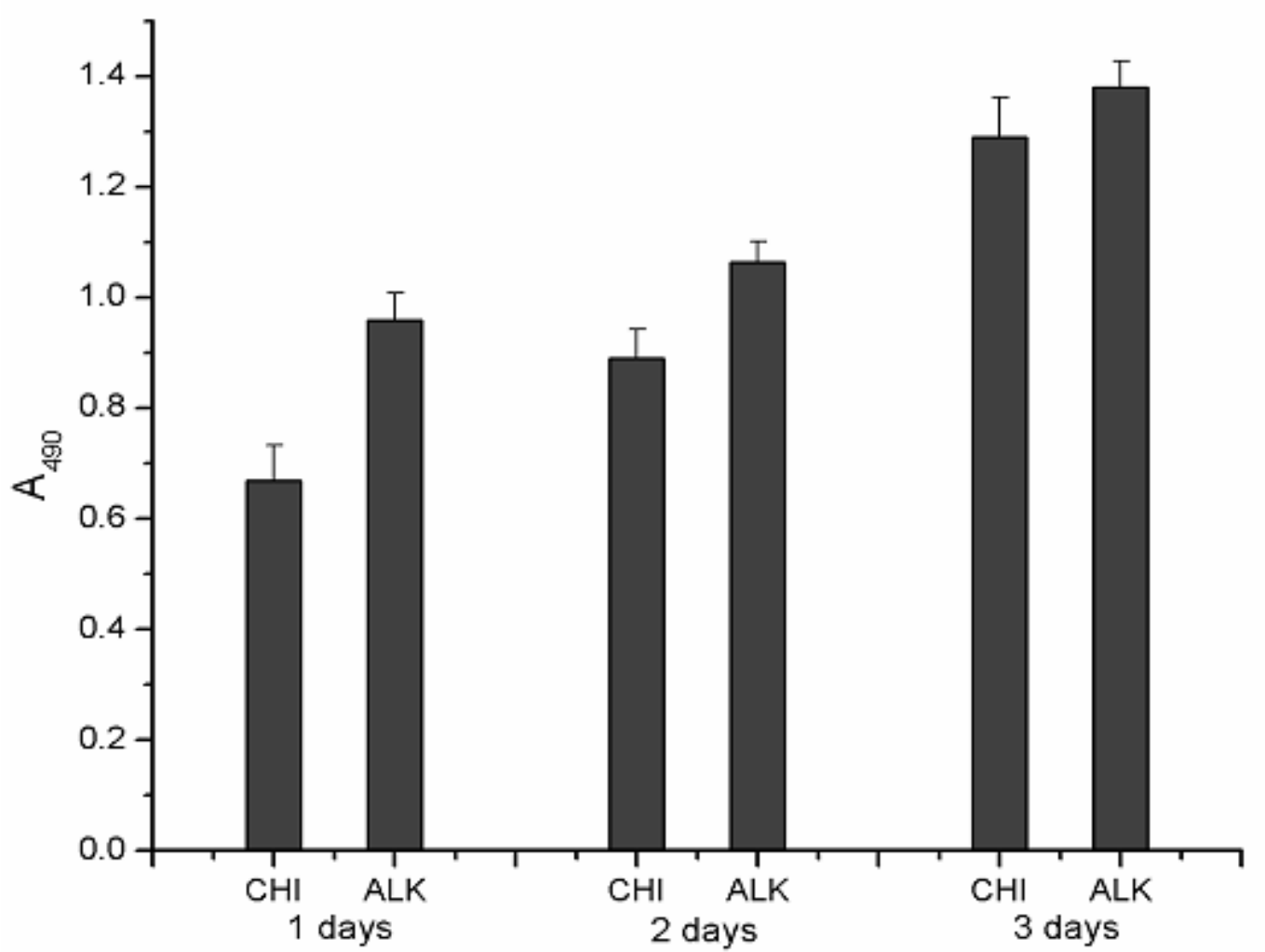
Solubility of alkylated chitosan of different Deacetylation Degree (DD)

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



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



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

means ± s.d. n=8

Chitosan modification (4)

Crosslinking with different reagent

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

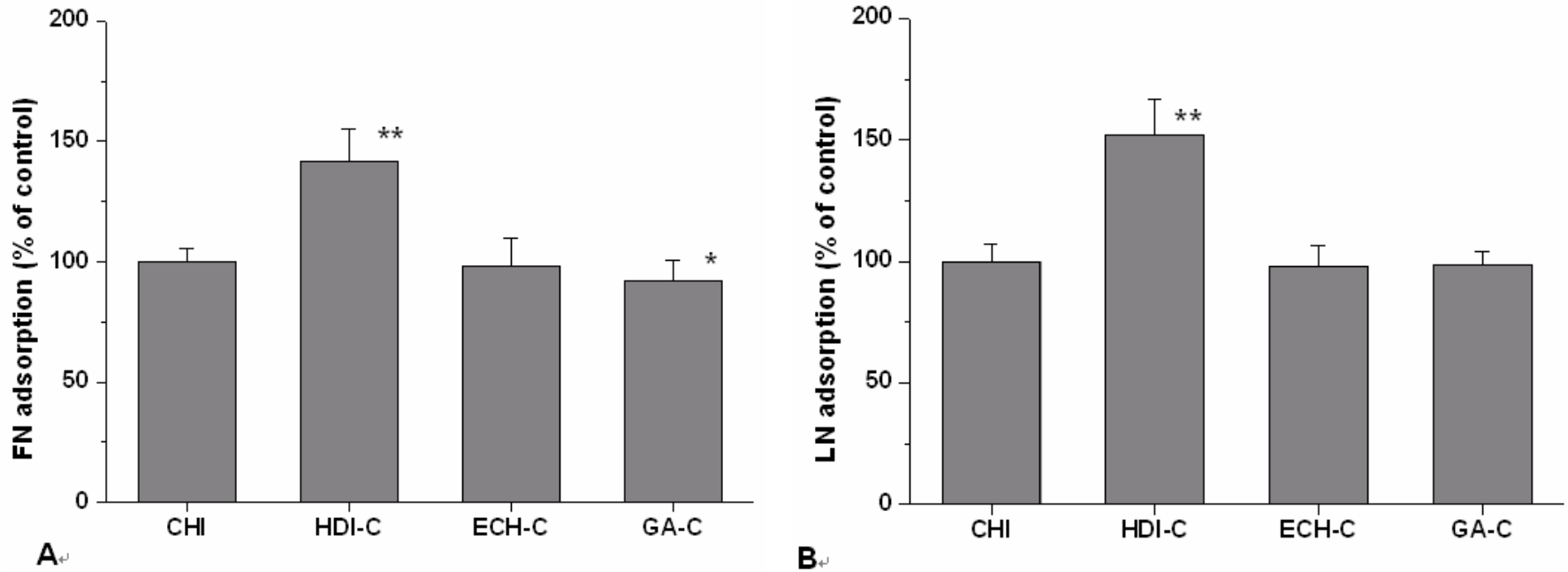
Swelling index, water contact angle and mechanical properties of uncrosslinked and crosslinked chitosan films

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

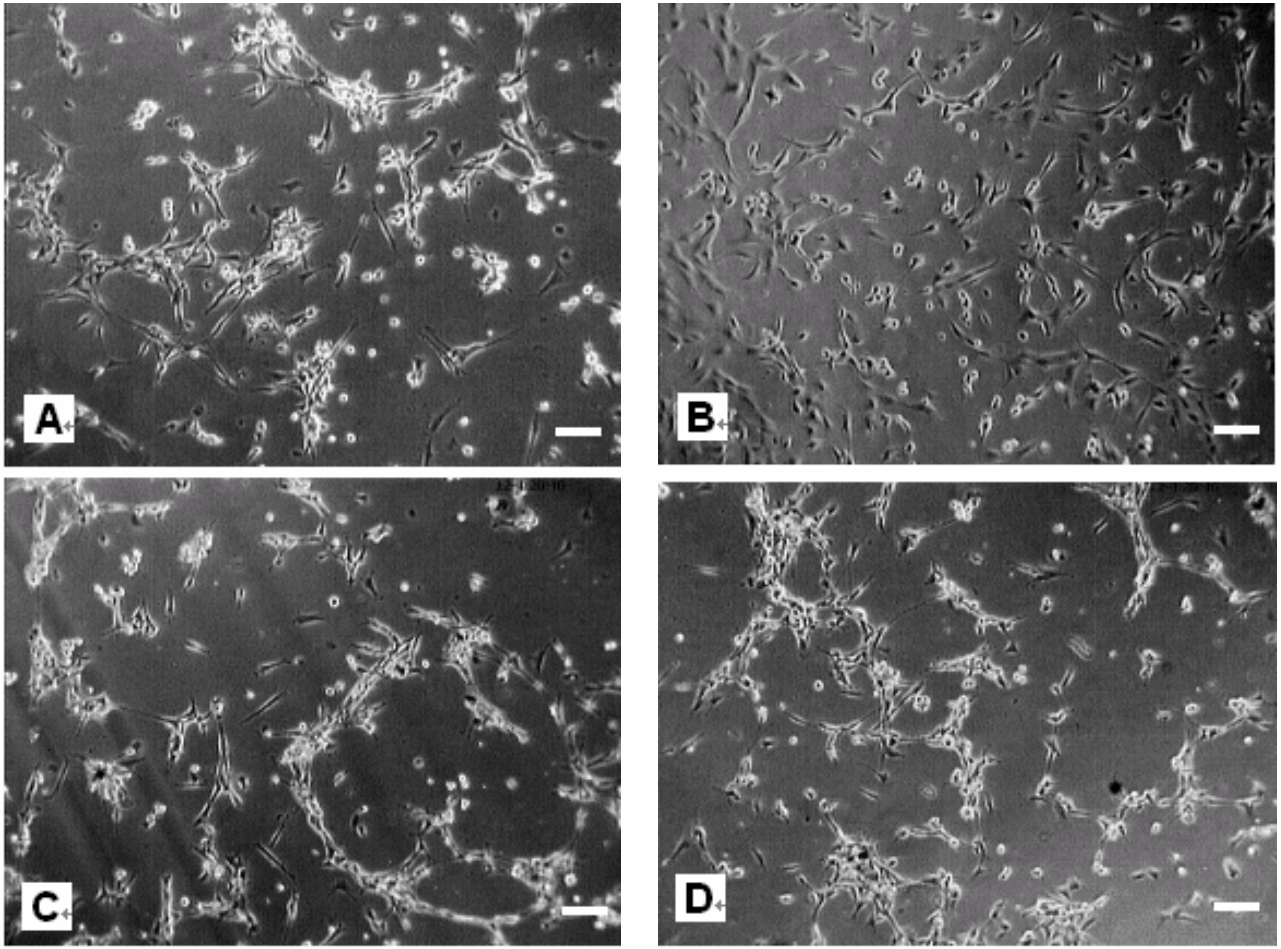
Quantification of fibronectin (A) and laminin (B) adsorption on chitosan films



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 ± SD (n = 6).



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

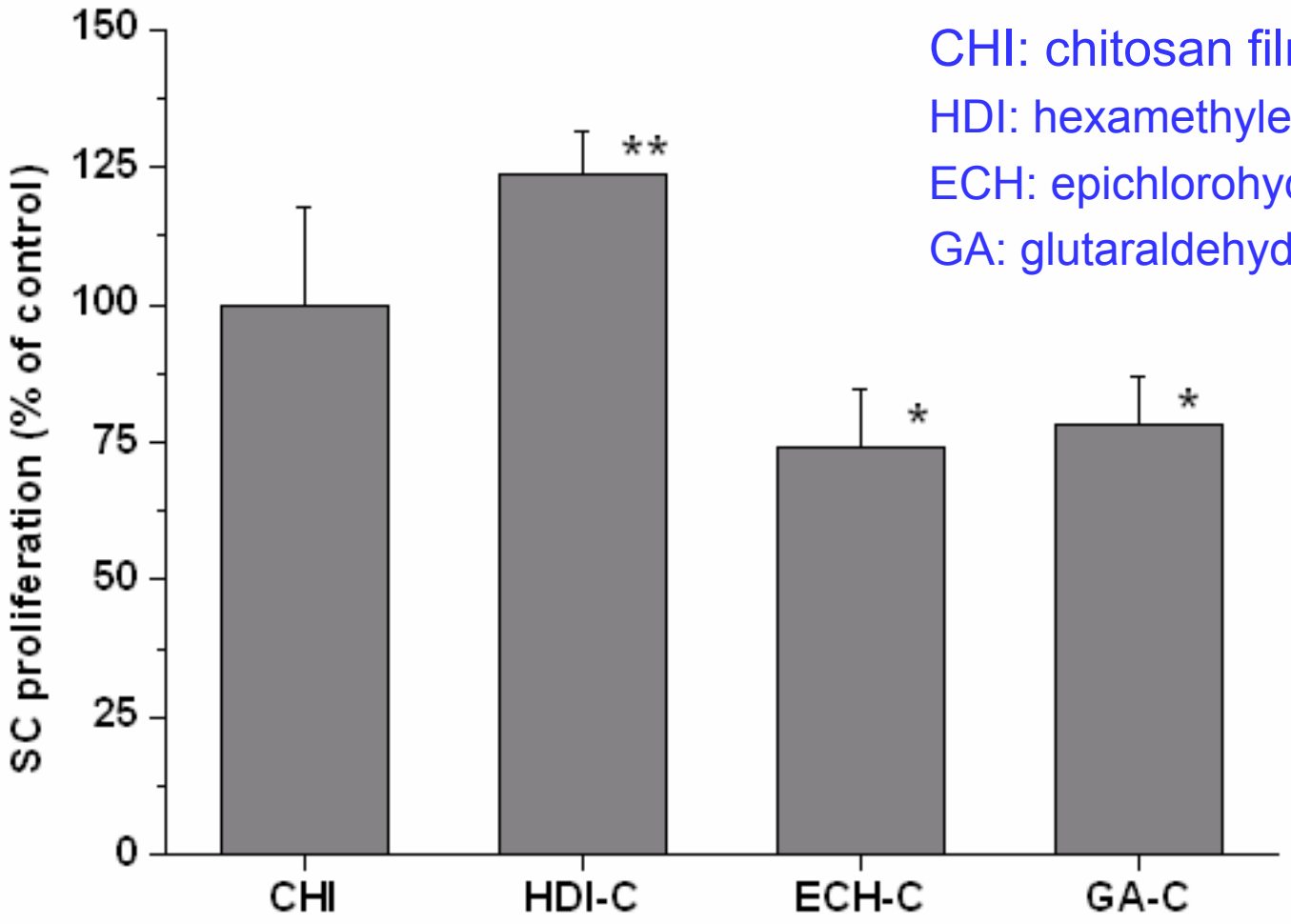
CHI: chitosan film, control.
HDI: hexamethylene diisocyanate;
ECH: epichlorohydrin;
GA: glutaraldehyde

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

Bar = 100 μm

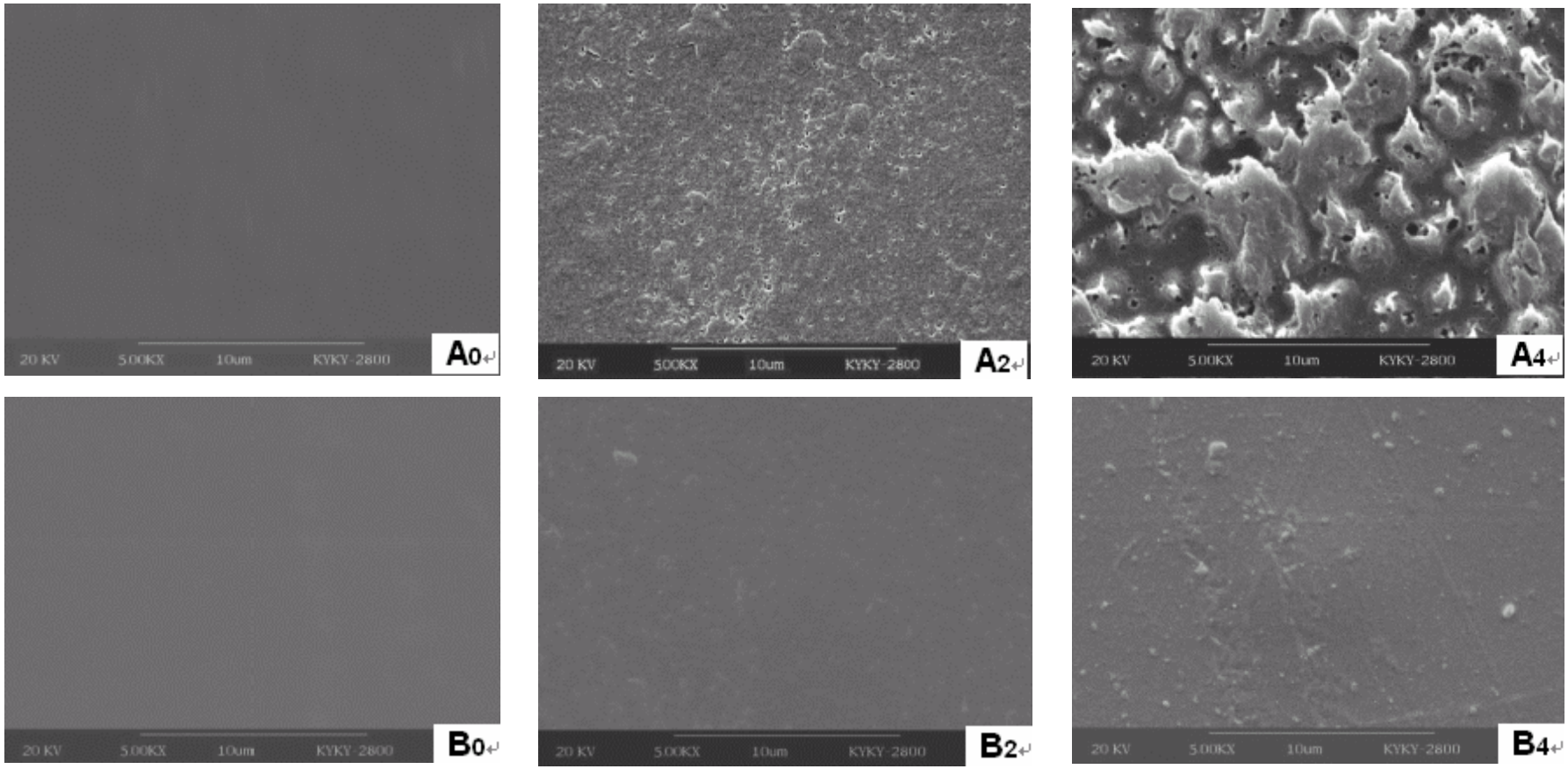
HDI-C films presented a better substratum for the adhesion and spreading of Schwann cells compared with the other films.

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



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

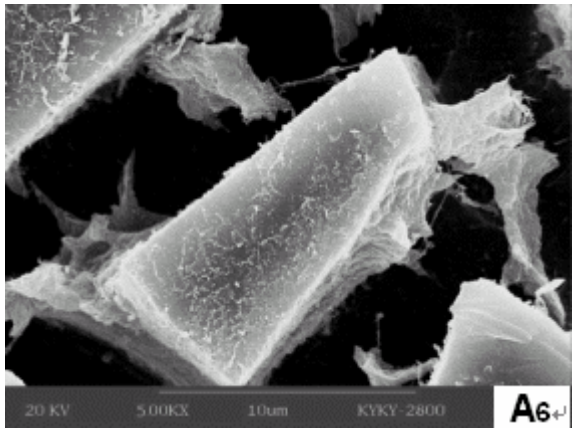
Data are plotted as mean ± SD (n = 6).
Asterisks indicate a significant difference (* for $p<0.05$ and ** for $p<0.01$) compared with uncrosslinked chitosan films.

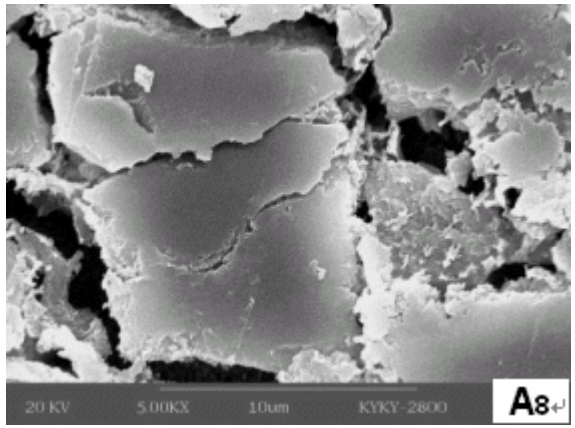


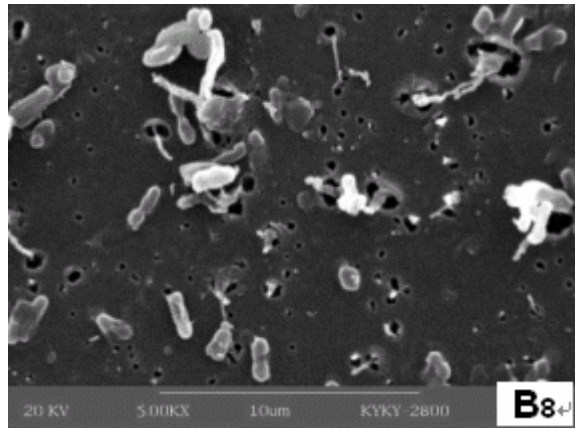
SEM micrographs of the undegraded (A_0, B_0) and degraded chitosan films by lysozyme after 2 weeks (A_2, B_2), 4 weeks (A_4, B_4).

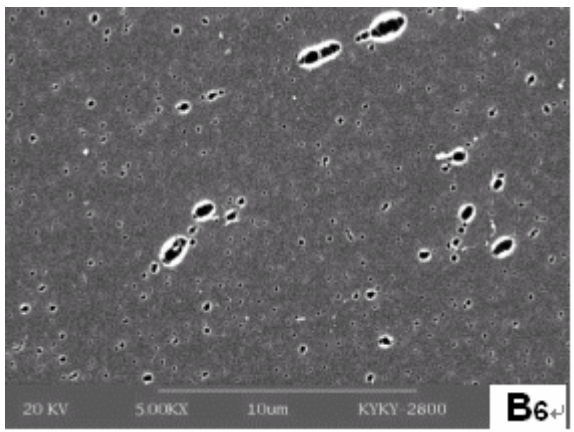
A: CHI; B: HDI-C;

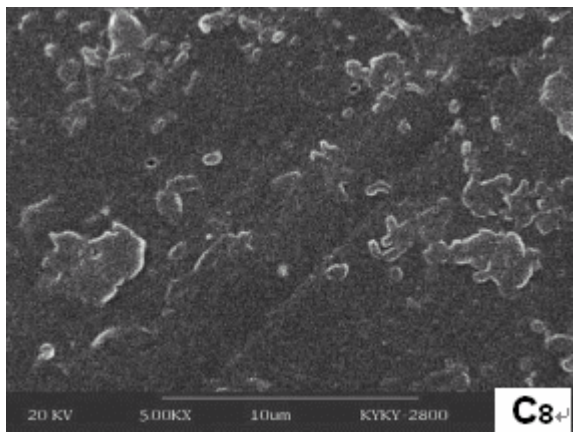
After crosslinking, the degradation rate of chitosan films significantly decreased.

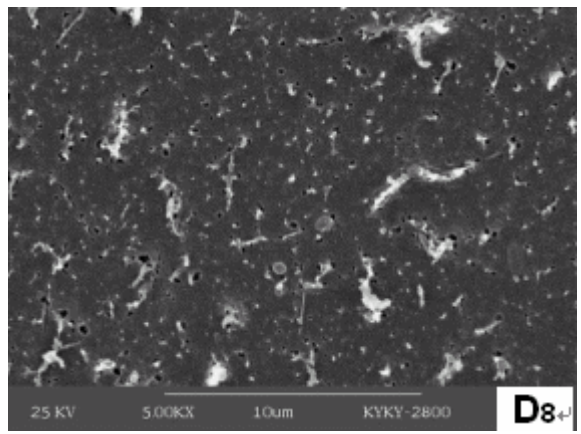












SEM micrographs of the degraded chitosan films by lysozyme after 6 weeks (A_6, B_6) and 8 weeks (A_8, B_8, C_8, D_8).

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

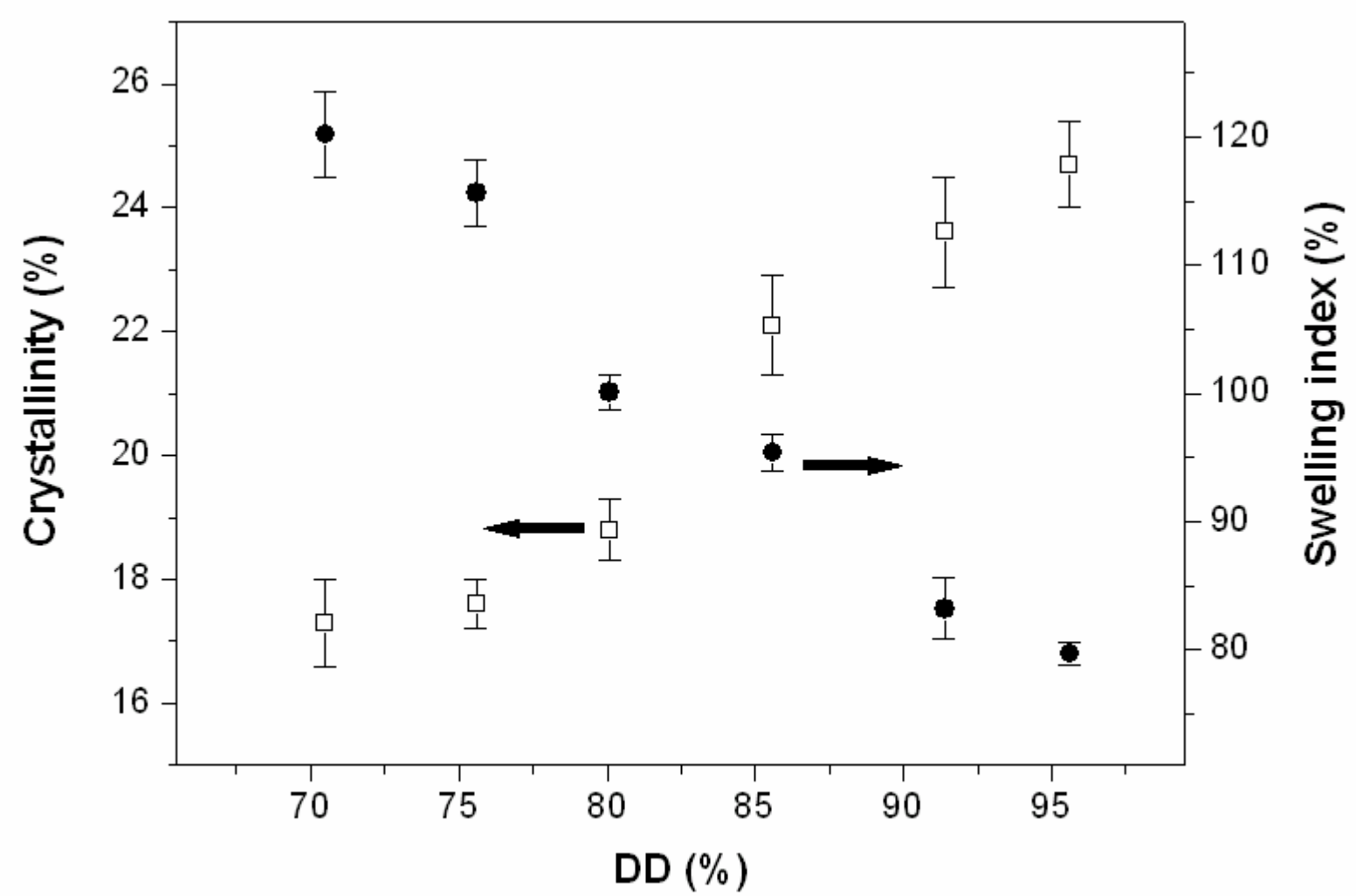
Sample code [a]	DD (%) [b]	M_v	Preparation conditions [c]
CS-70	70.1±1.4	8.6×10^5	Reacetylation of CS-80, 0.05 M acetic anhydride in methanol, 25 ºC, 30 min
CS-76	76.0±1.2	8.6×10^5	Reacetylation of CS-80, 0.05 M acetic anhydride in methanol, 25 ºC, 15 min
CS-80	80.1±0.6	8.6×10^5	Acid hydrolysis, NM-CS, 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.

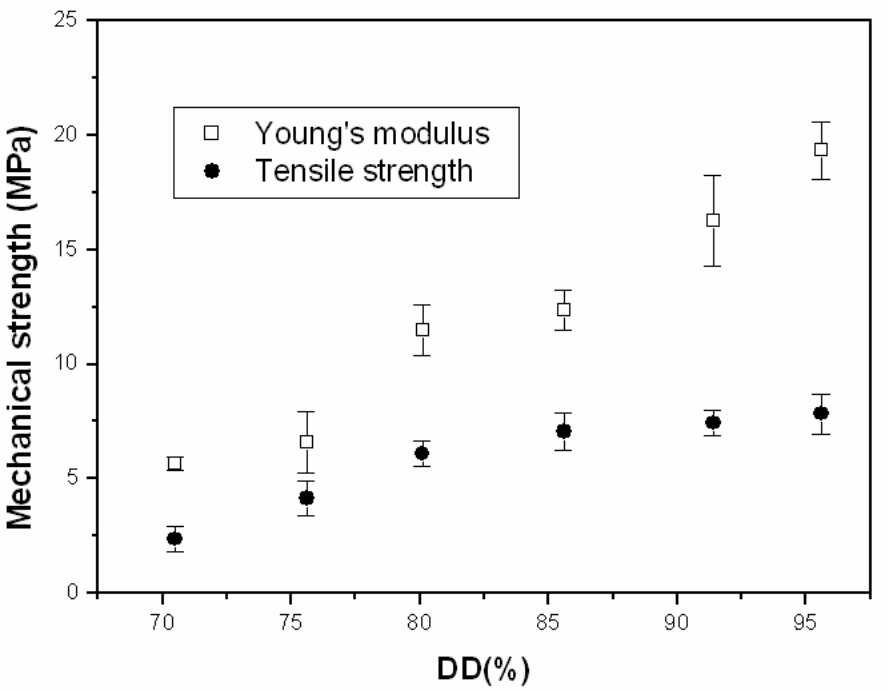
c. The preparation conditions include the kind of reaction, raw material, reaction solution concentration, reaction temperature and reaction time.

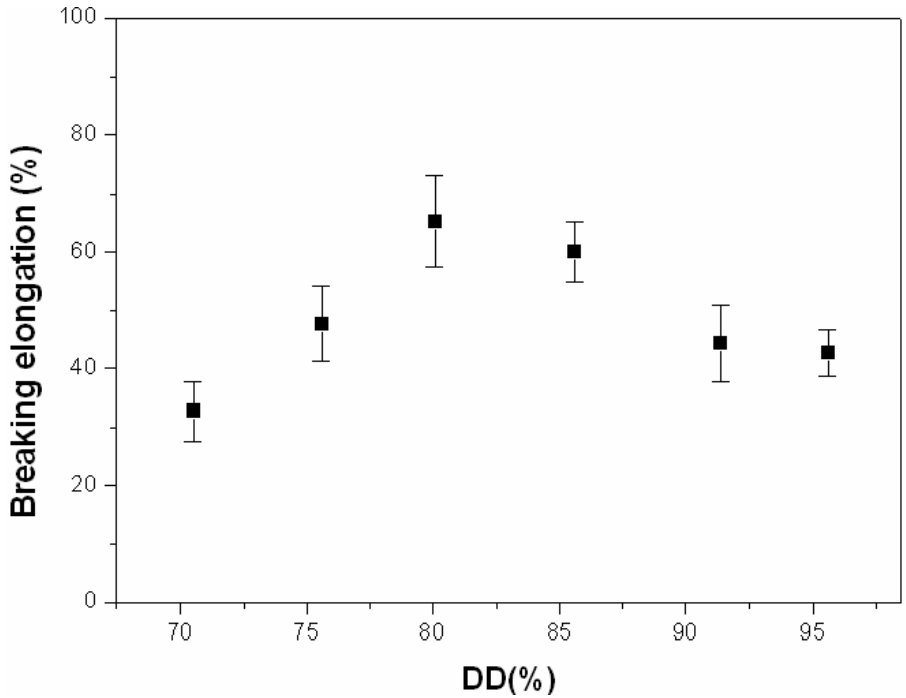
The variance of crystallinity and swelling index of chitosan films with deacetylation degree (DD)



Swelling index of chitosan films decreased and their crystallinity increased with the increase in DD

Effects of deacetylation degree (DD) on mechanical properties of chitosan films in wet state

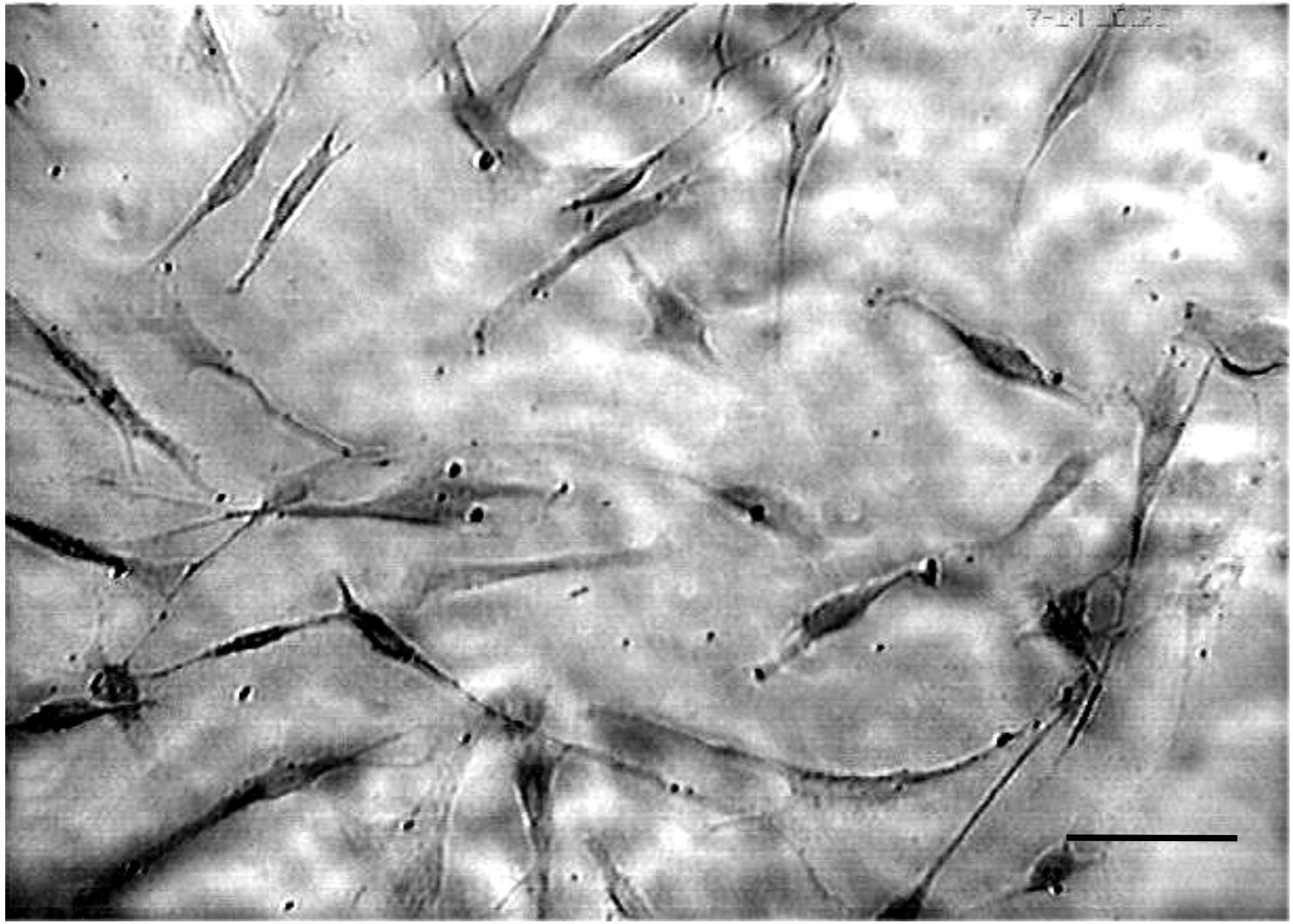




Left: Young's modulus and tensile strength;

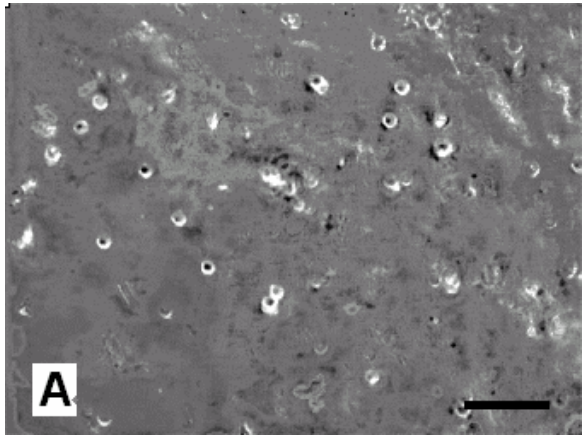
Right: Breaking elongation.

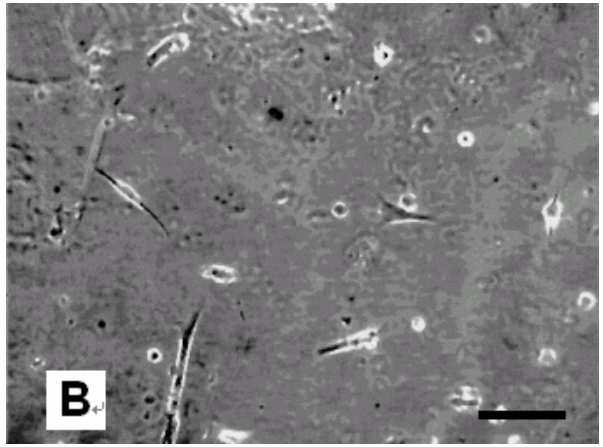
The elastic modulus and tensile strength of chitosan films increased with the increased DD.

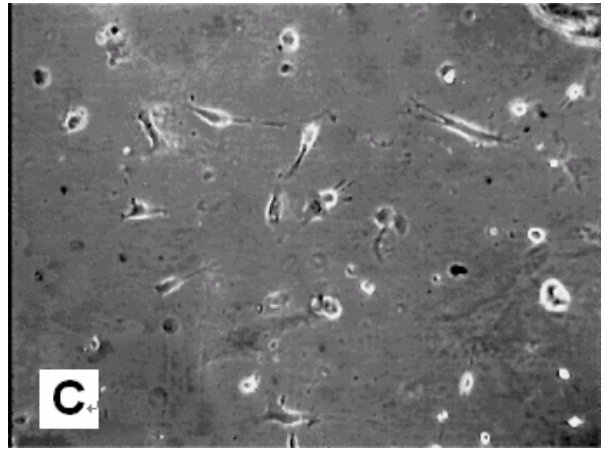


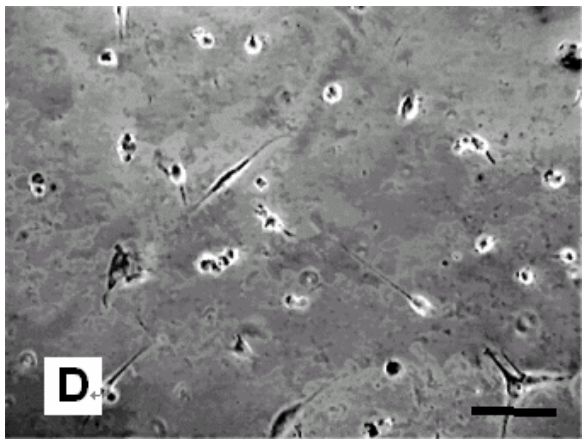
Rat primary Schwann cells stained for S-100

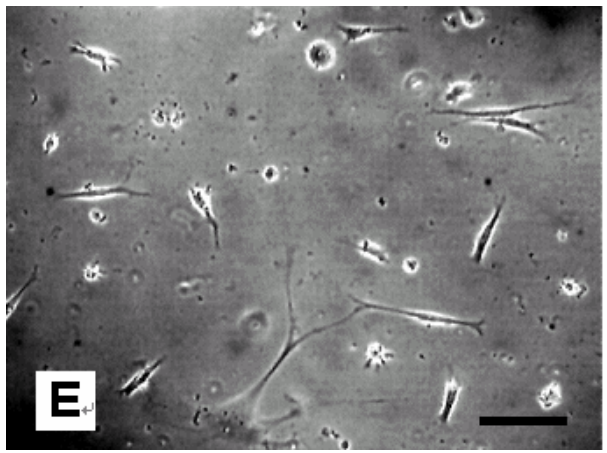
Bar = 100 μ m.

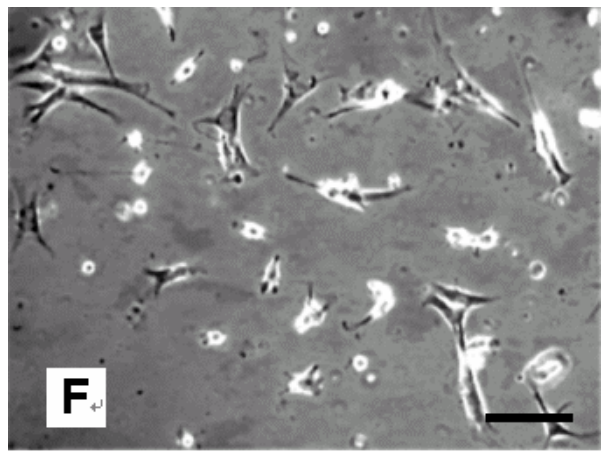


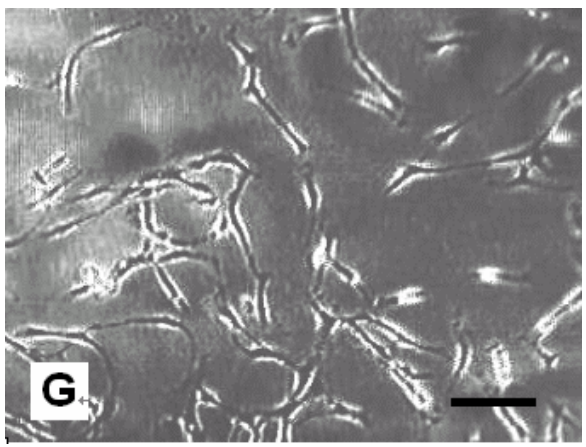








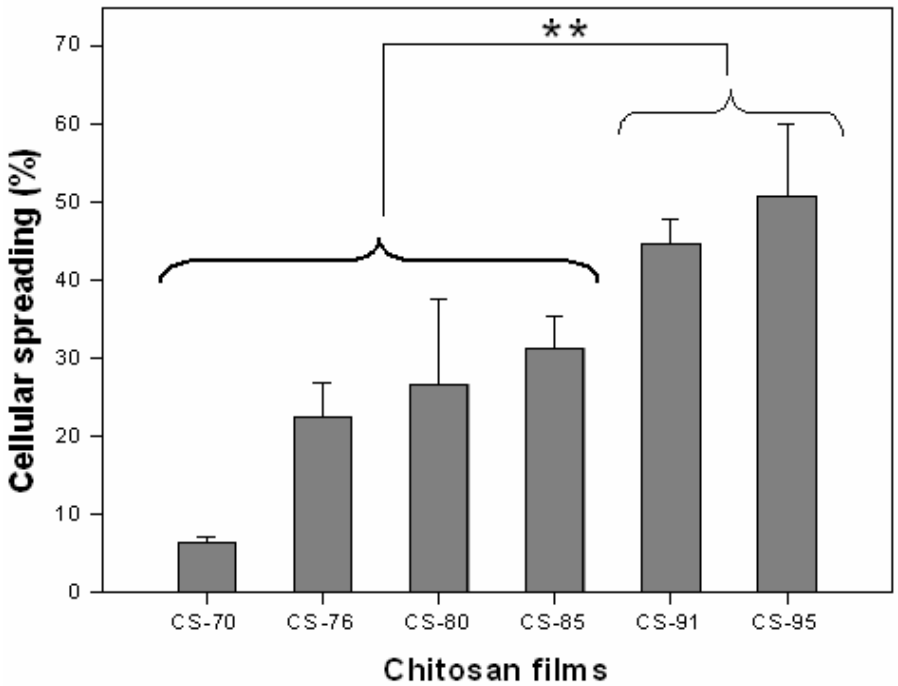


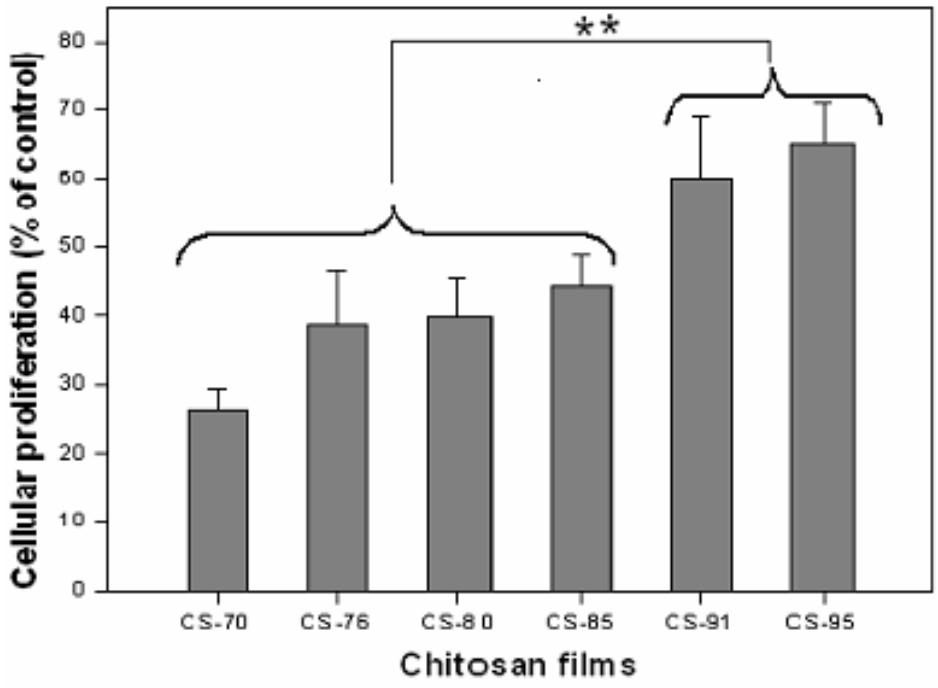


Schwann cells cultured on chitosan films and the control substratum for 24 h

A: CS-70; B: CS-76; C: CS-80; D: CS-85; E: CS-91; F: CS-95; G: tissue culture plastics (control). Bar = 100 mm.

The chitosan films with higher DD are more compatible for Schwann cell growth.





Schwann cells spreading after being cultured for 24 h (Left) and proliferation after being cultured for 7 days (Right) on chitosan films

Asterisk indicates a statistically significant difference ($p<0.01$) compared to other chitosan films of lower degree of deacetylation.

The chitosan films with higher DD provided better substrata for Schwann cell spreading and proliferation.

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

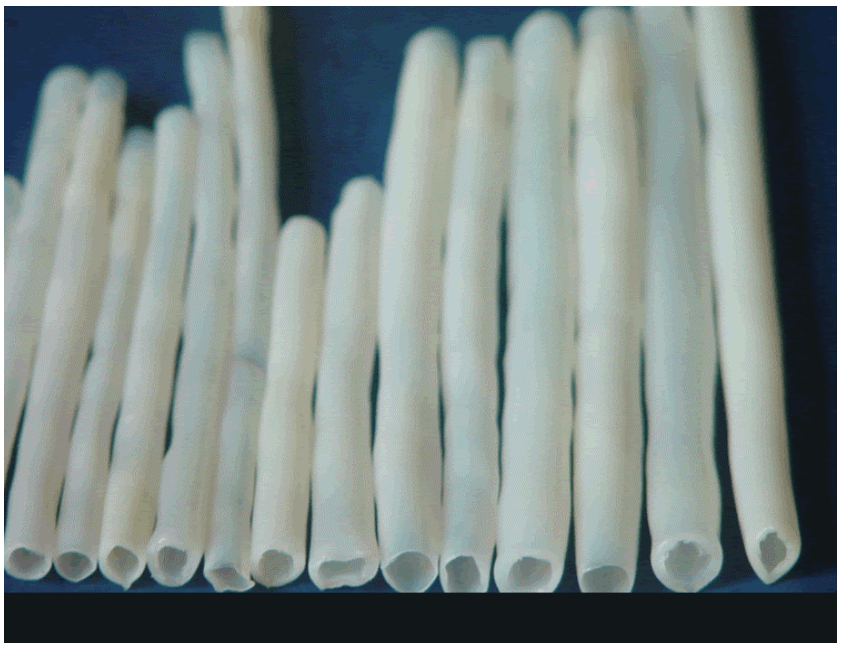
a. n = 6, Mean ± SD.

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.

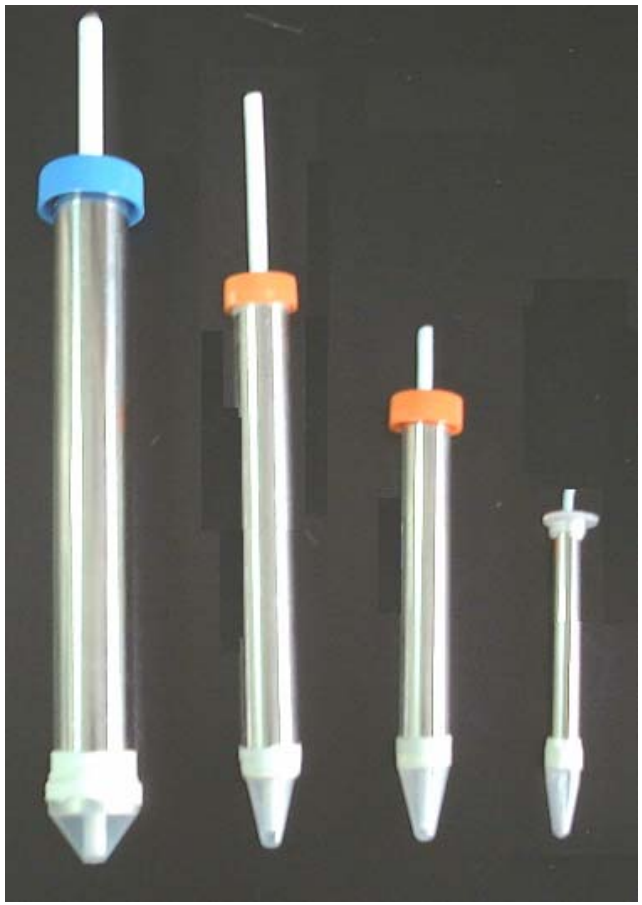
Chitosan nerve conduits prepared by rotary evaporation method



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**

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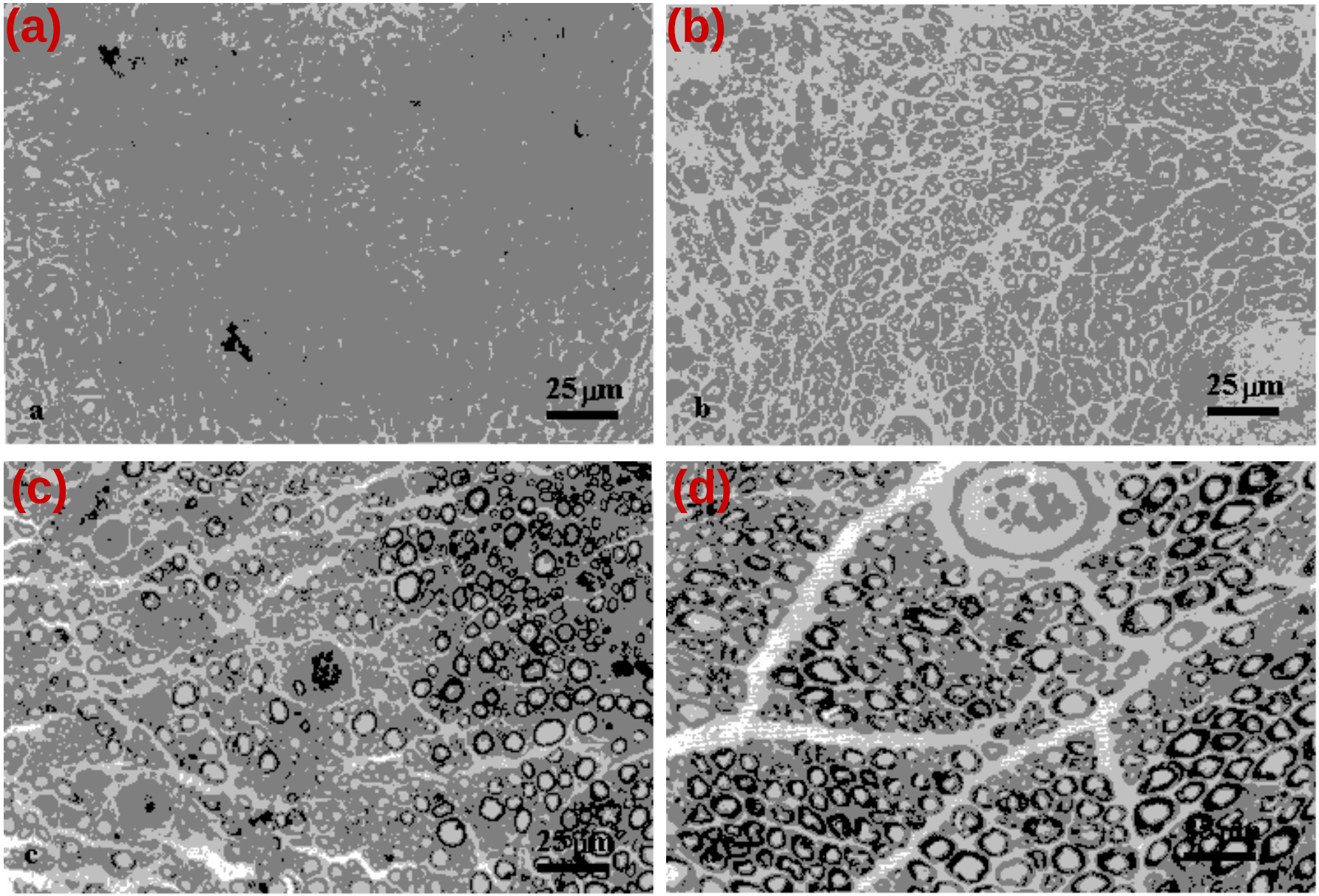
Preliminary tests have shown that the chitosan nerve conduit is safe in implantation.

Implantation in animal (Functional evaluation)

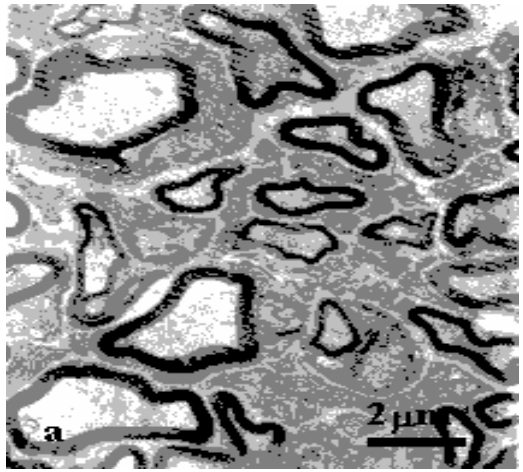
Rat

The chitosan nerve conduit has been used for rat sciatic nerve with gap of 2 cm.

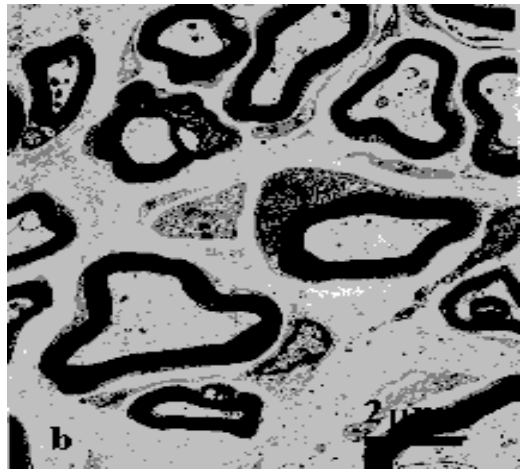


Regenerative and normal nerve

(a) regenerative nerve 2 months after the implantation;
(b) 4 months after; (c) 8 months after; (d) normal nerve



(1)



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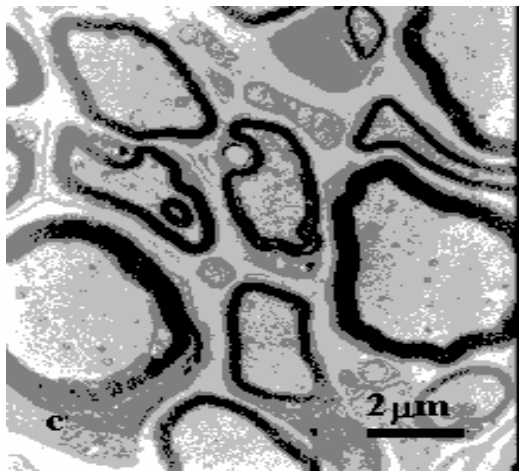

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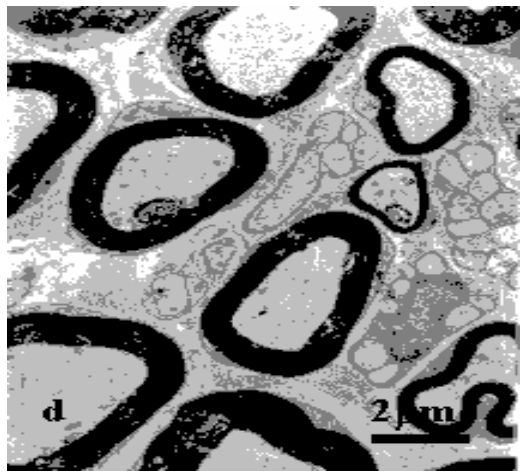
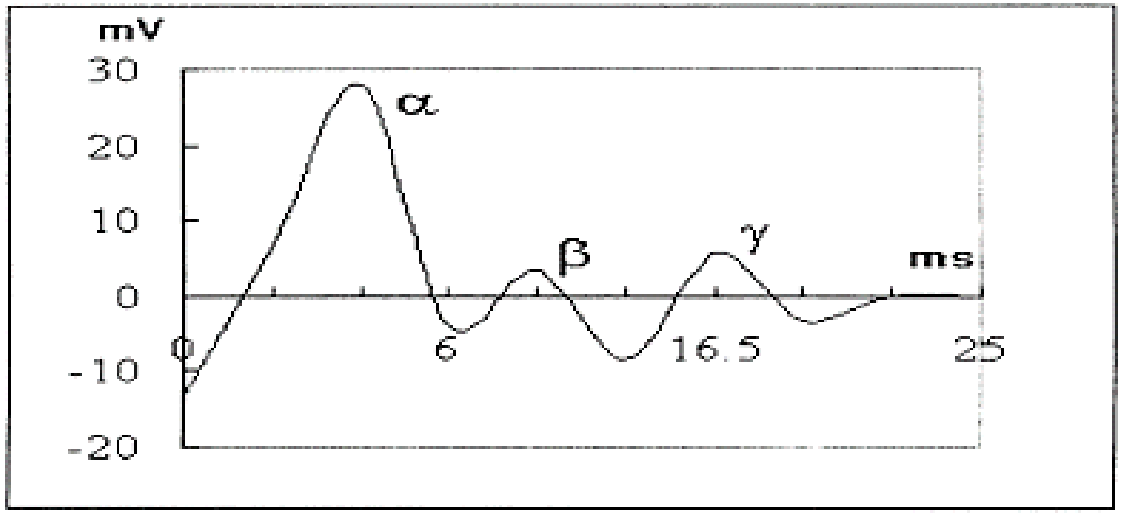


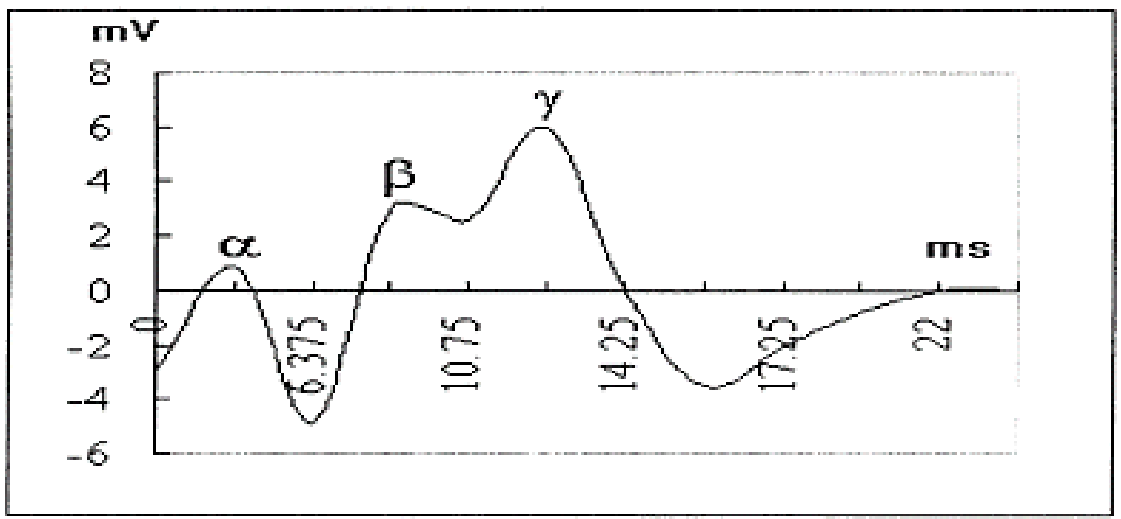
(4)

The changes in thickness of myelin sheath and diameter of nerve fiber are obvious.

TEM photographs of regenerative nerve and normal nerve (1) 2 months after implantation; (2) 4 months after implantation; (3) 8 months after implantation; (4)normal nerve (control).



(a)



(b)

Evoked potentials in normal (a) and regenerated (b) nerves

Goat and Monkey

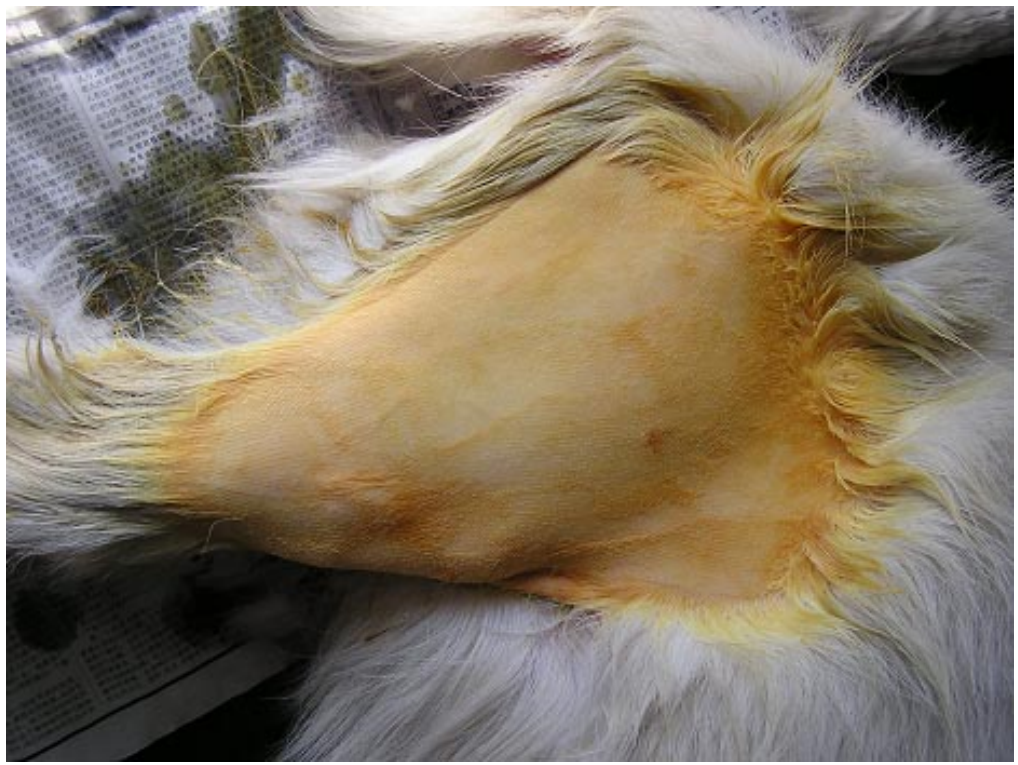
Chitosan conduit implantation

and

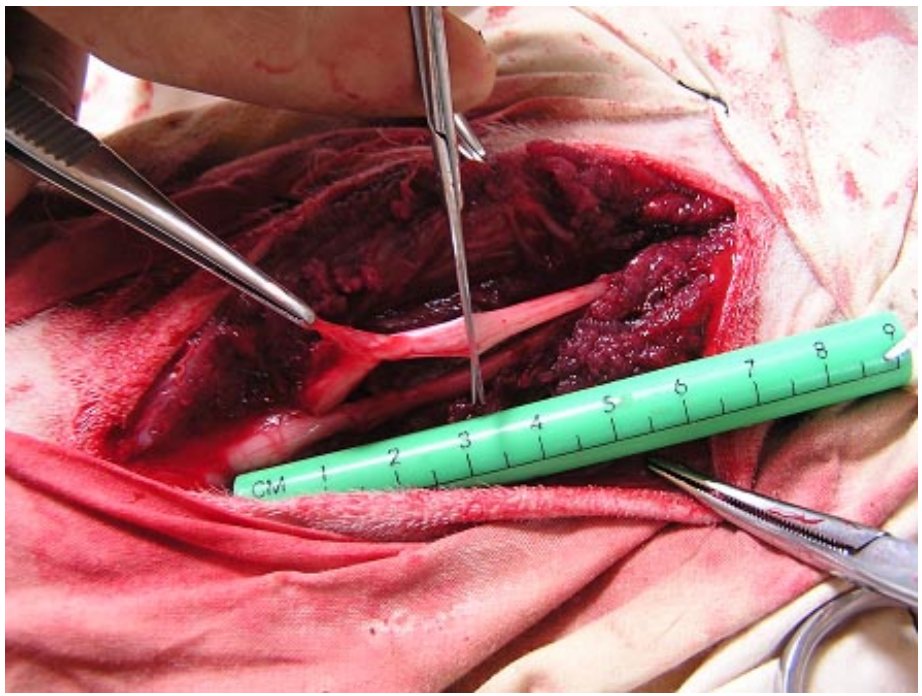
functional recovery

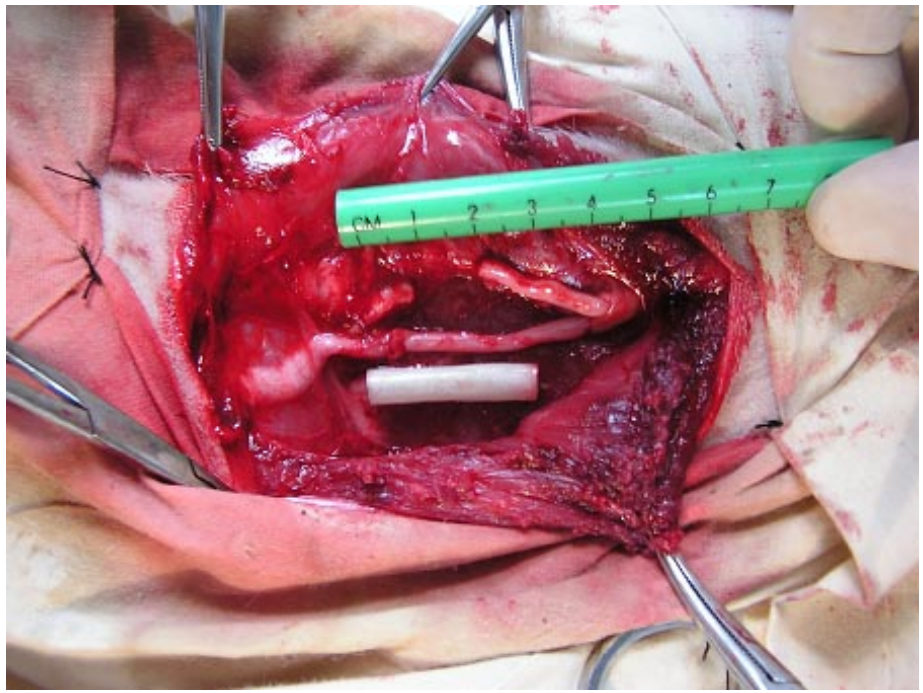
Preliminary result

Operation for goats

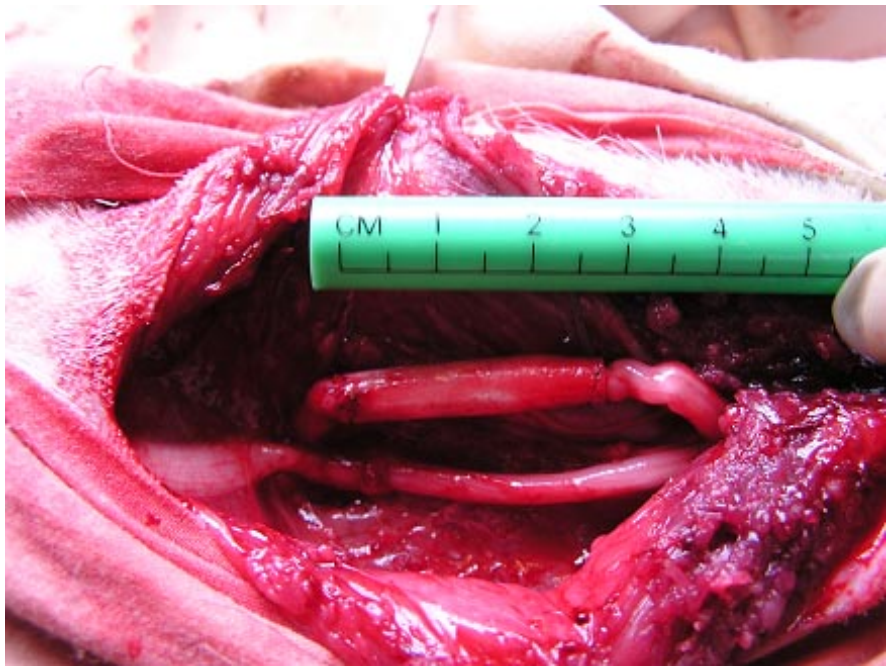


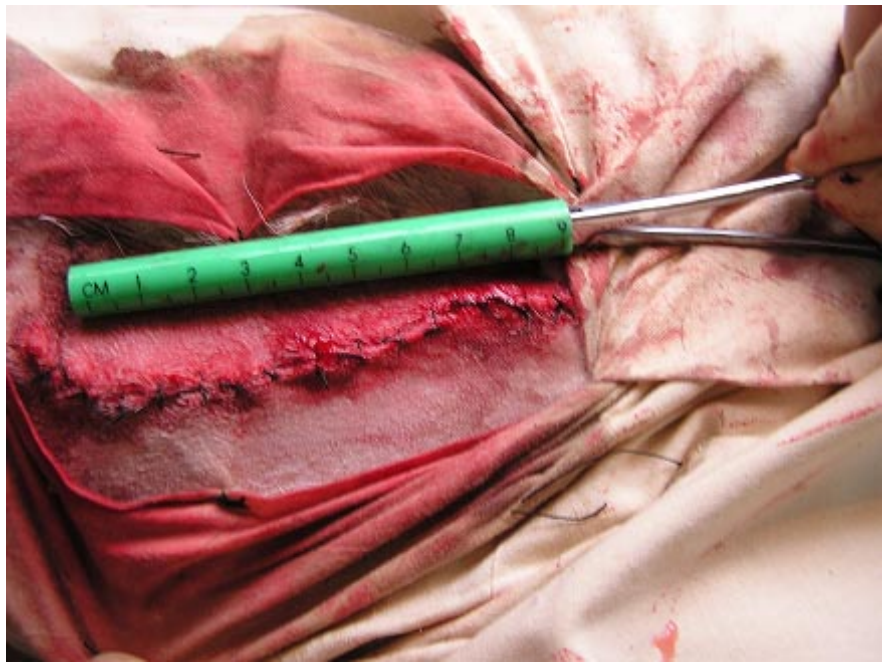
Two adult goats weighing approximately 20 kg were used for the experiment.



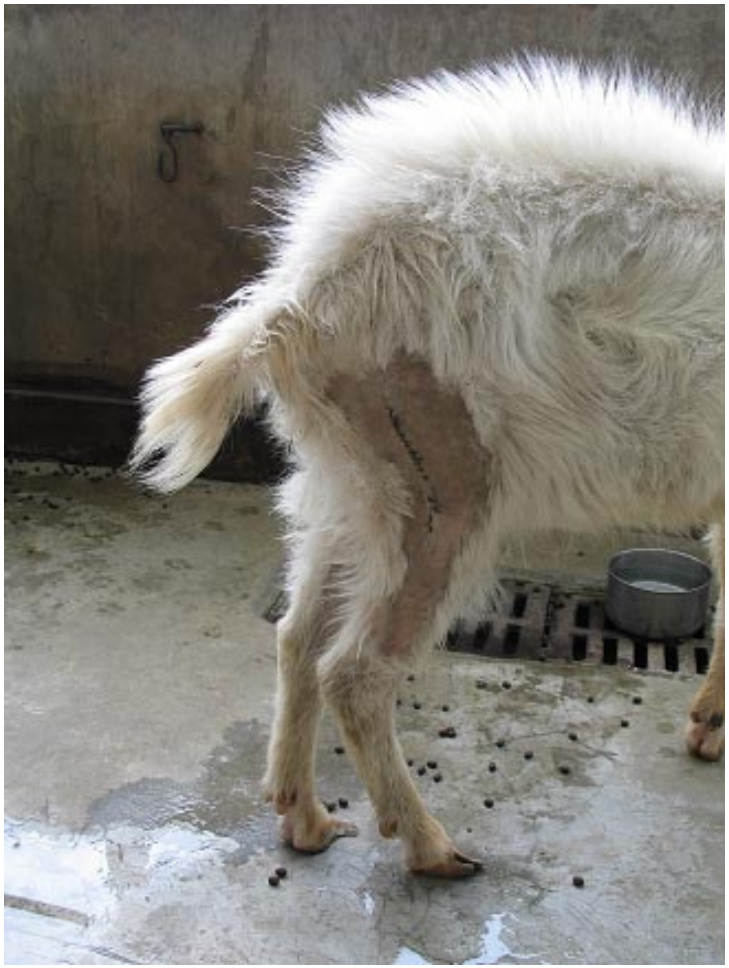


1 cm of tibial nerve or common peroneal nerve was removed, then chitosan nerve conduit was inserted into the gap. The conduit was precoated with laminin.





The graft was sutured compactly with the two nerve stumps, leaving a 2.5 cm gap within the chitosan tube. In all animals the contralateral unoperated side was served as control.

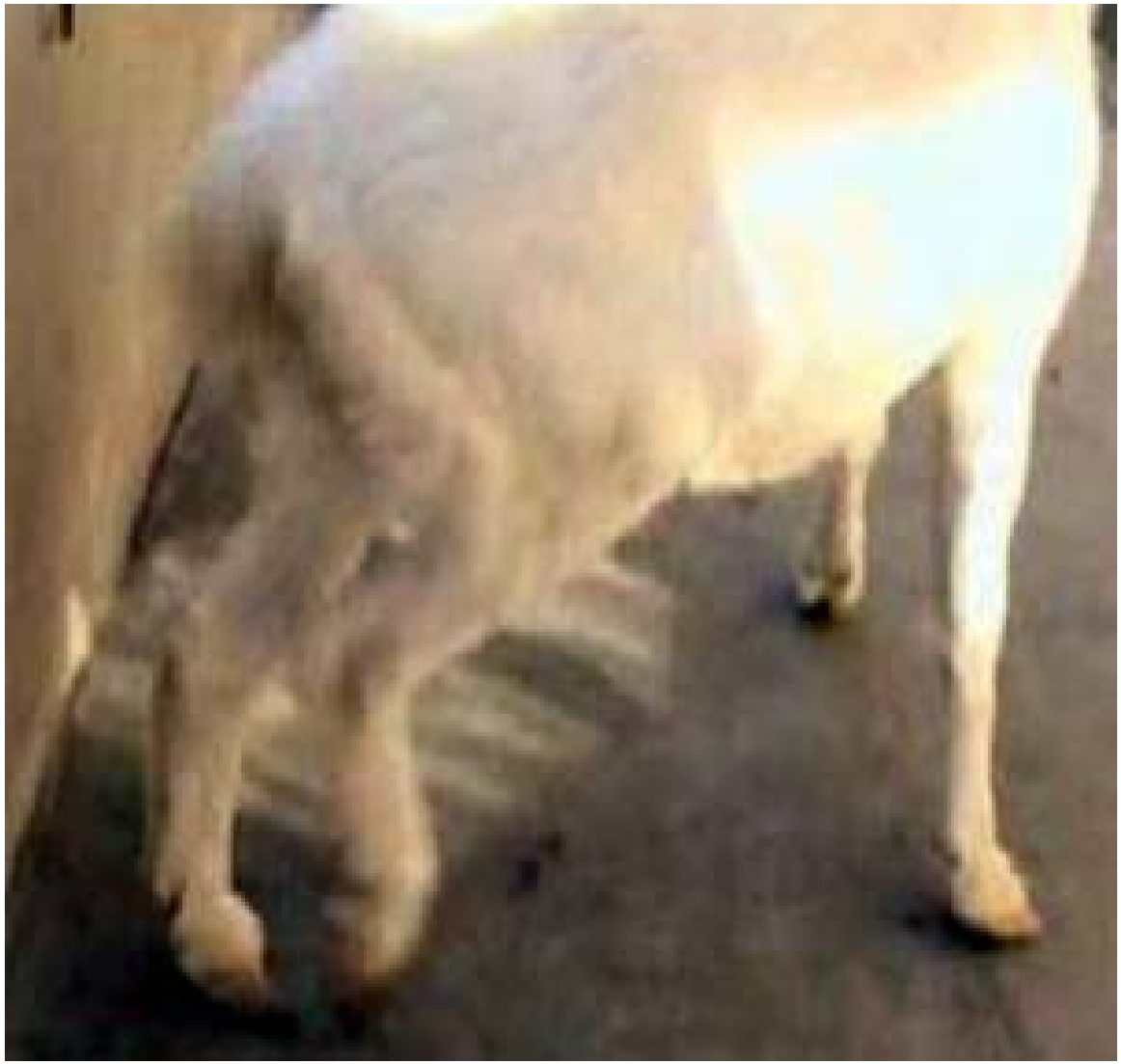


Tibial nerve injury model

Physical sign: inability of active palmar flexion (right-hind hoof)

Common peroneal nerve injury model

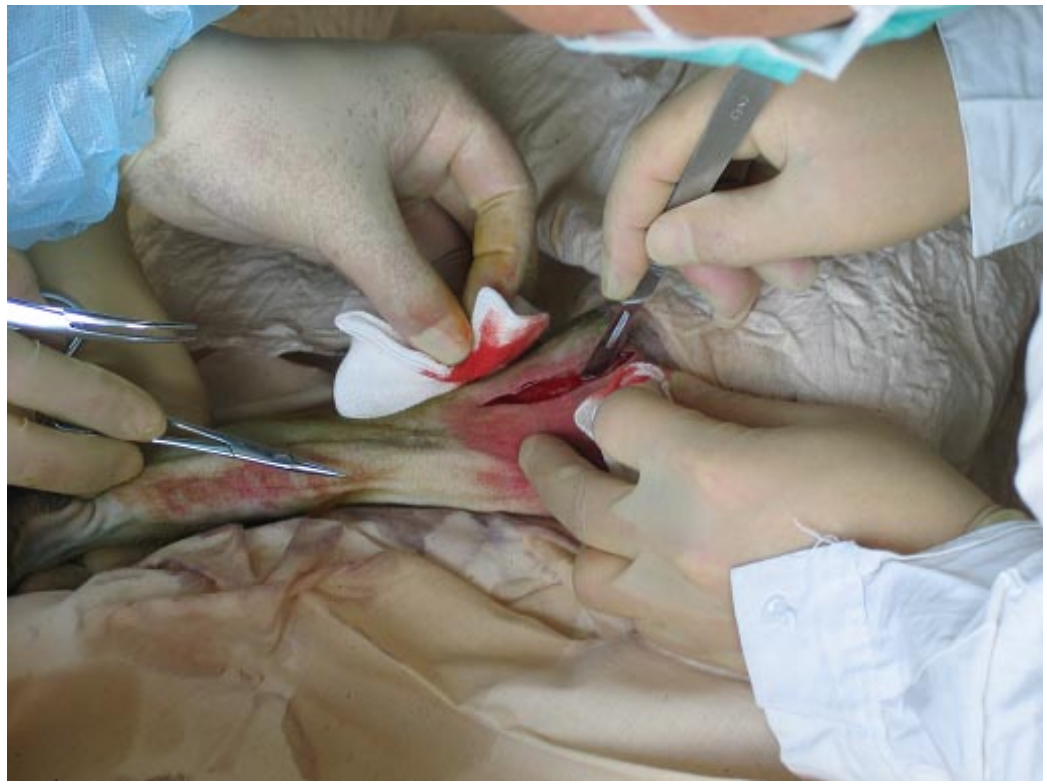
Physical sign: inability of active dorsiflexion (right-hind hoof)



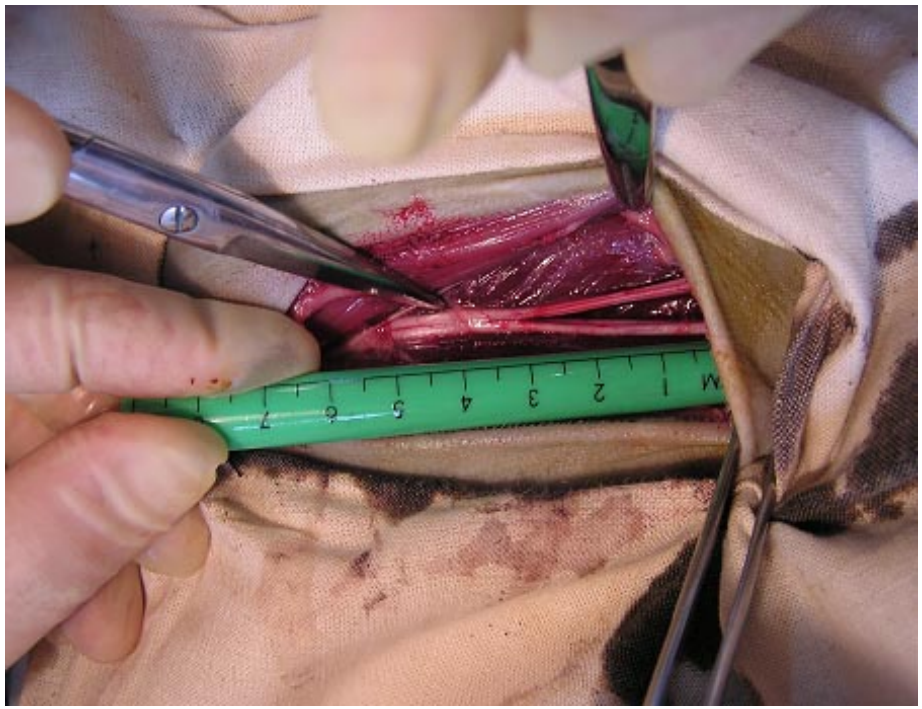
The goat with tibial nerve injury could make active palmar flexion of right-hind hoof (8-month postop)

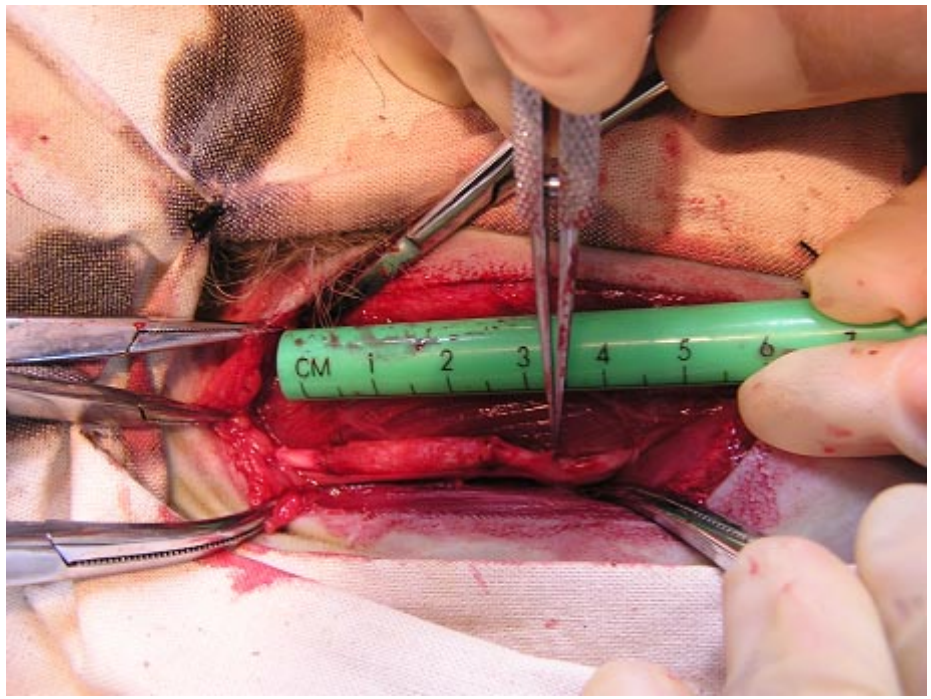
The goat with common peroneal nerve injury could make active dorsiflexion of right-hind hoof (8-month postop)

Operation for monkey



Two adult monkeys *(Macaque)* weighing approximately 3.5 kg were used for the experiments.





Similarly, a gap of 1 cm was repaired with a chitosan tube leaving a 2 cm gap within the chitosan tube precoated with laminin

Tibial nerve Injury model

Physical sign: inability of active palmar flexion (right-hind foot and toes)

Common peroneal nerve injury

Physical sign: inability of active dorsiflexion (left-hind foot)

The monkey with tibial nerve injury could make active palmar flexion of right-hind foot and toes (8-month postop)

The monkey with common peroneal nerve injury could make active dorsiflexion of left-hind foot and toes (8-month postop).

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.**

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Thank You for your attention!