

Investigation of Effectiveness of Chitosan Hydrogel to Stop Bleeding and Air Leakage from Lung Fistula: An In Vivo Study

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ABSTRACT

Chitosan is the most important derivative of chitin, the second most abundant biopolymer just after cellulose, which has received great attention because of its unique characteristics. Without doubt, its biomedical usages have gained more importance among the vast variety of chitosan applications owing to its good biocompatibility and biodegradability. In recent years, particular interest has been devoted to chitosan hydrogels as a promising alternative in competition with conventional sutures or bioadhesives. In the current work, we have investigated the effectiveness of chitosan hydrogel to stop bleeding and air leaking of lung fistula. Polycationic chitosan was obtained with solubilization of chitosan powder in aqueous acidic media. Different parameters such as acid type and concentration, and degree of deacetylation (DD%) of chitosan, were altered to modify hydrogel properties including viscosity, pH, cohesive strength, and tissue bioadhesiveness. In vivo experiments have been conducted on sheep models which provide a convenient way to evaluate the efficacy of prepared samples. The lung was punctured in distinctive geometries and hydrogel then injected on. Bioadhesive strength as well as irritant effects were discussed. Samples with higher degree of deacetylation, including Chs-16 (DD% = 99, MW=230,000) and Chs-19 (DD% = 98, MW=300,000) that were dissolved in lactic media showed best sealing effect. Further studies are now conducted to optimize the sealing properties of chitosan based hydrogels.

Key Words:

chitosan;
hydrogel;
injectable;
lung;
in vivo.

INTRODUCTION

Chitin, the second most abundant natural polymer next to cellulose, is the nitrogen-containing member of the great family of polysaccharides. It extracts from fungal cell wall and exoskeleton of arthropods such as insects, crabs, shrimps, and lobsters [1]. The chemical structure of chitin has been shown in Figure 1a. The poor solubility of chitin has limited

its applicability hence, one must chemically modify amide groups of the main backbone to produce poly [(1→4)-2-amino-2-deoxy-D-glucopyranose] which has been known as chitosan (Figure 1b). Chitosan is soluble in aqueous acidic media due to presence of amine functionalities. Moreover, chitosan is the biodegradable, biocompatible, and

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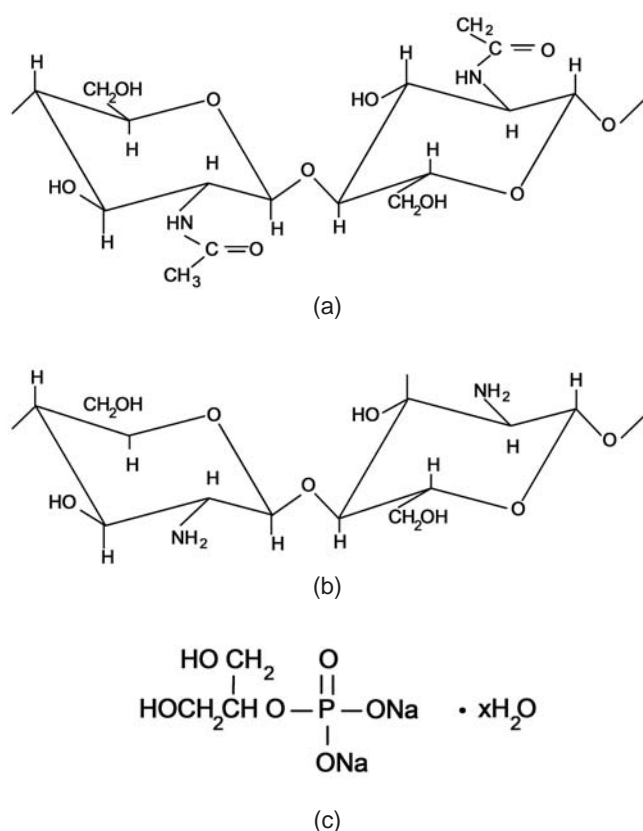


Figure 1. The chemical structures of (a) chitin, (b) chitosan and (c) β -GP .

bacteriostatic polymer [2], which seems to play a significant role in biomedical applications. For instance, air leakage and blood oozing are common problems in lung and thoracic operations [3,4] as well as in tuberculous patients [5]. Up to now, several materials such as fibrin glue, light-cured gelatin hydrogel glue [3], chitosan micropowder [6], and UV-cured chitosan hydrogel [7] have been explored.

In this work, we have been studying a bioadhesive hydrogel based on chitosan to seal lung punctures. Chitosan was solubilized by aqueous solutions of two different organic acids: acetic and lactic acids. Viscosity, pH, and degree of protonation (α) of obtained cationic polyelectrolyte have been related to parameters, e.g., degree of deacetylation (DD%), polymer content, and type and concentration of used acid. To adjust pH solution, β -glycerol phosphate (β -GP) (Figure 1c) which is an organo phosphate salt was added to the system that obviously improves elasticity and cohesive forces in final hydrogel. Gelatin, a partially denatured derivative of the collagen [8] was also used

to develop, eventually the tissue-hydrogel interaction and bioadhesion strength. Finally, animal experiment was carried out as a qualitative study to determine the efficacy of optimized hydrogel samples to stop bleeding and air leakage from lung fistula.

EXPERIMENTAL

Materials

Chitosan with different degree of deacetylation was used. Table 1 shows the supplier, the molecular weight and DD% of different grades. The viscosity average molecular weight was estimated of intrinsic viscosity determined in the solvent 0.3 M acetic acid/0.2 M sodium acetate using the Mark-Houwink parameters $a = 0.76$ and $K_{\eta} = 0.076$ at 25°C when the intrinsic viscosity is expressed in mLg^{-1} [9]. The DD% was concluded from elemental analysis. Gelatin powder (food grade) was kindly provided by Iran Polymer and Petrochemical Institute (Tehran, Iran). Other reagent including acetic and lactic acids and β -GP were chemical grade and acquired from Merck.

Preparation of Chitosan based Hydrogels

At first, 2-3 w/v% solutions were prepared by dissolving of chitosan powder in 1 v/v% aqueous acidic media including either acetic or lactic acids via a magnet stirrer. The pH of these pregels was measured by a Metrohm 744E pH-meter which comprises a combined glass electrode. Sterilizing was then carried out in an autoclave (121°C, 10 min) [10]. Several alkali materials

Table 1. The supplier, the molecular weight and DD% of used Chitosan samples.

Code	Molecular weight (g/mol)	Received from	Degree of deacetylation (%)
Chs-F-M	400,000	Fluka	81
Chs-S-C3646	300,000	Sigma	85
Chs-PGC10	280,000	IPPI [†]	86.67
Chs-CSN-7-3	530,000	IPPI	96.67
Chs-16	230,000	IPPI	99
Chs-19	300,000	IPPI	98
Chs-21	200,000	IPPI	96

[†] Iran Polymer and Petrochemical Institute

were used to neutralize the chitosan solutions. Upon adding strong alkali to the polycationic chitosan solution, e.g., sodium hydroxide, pH would increase to ~6 and further addition results to formation of hydrated gel-like precipitate as perviously reported [11].

Interestingly, β -GP has an effective role to increase pH up to physiological range (6.8-7.4) without immediate precipitation or phase separation [11]. Finally, β -GP solutions were prepared with concentration ranging from 5 to 10% w/v and sterilized by 0.22 μ m filtration. Pregels were cooled down to ~4°C and continuously stirred during adding drop by drop of the β -GP solution. After 15 to 30 min, stirring was stopped and a clear homogenous hydrogel obtained. No immediate precipitation or phase separation was observed even at high phosphate salt contents, as has been previously reported by Chenite et al. [12]. The pH measurement was performed when temperature equilibrium achieved. Gelatin as a biodegradable and biocompatible biopolymer that has sufficient intermolecular interactions with chitosan [13,14] was added to the sterilized chitosan solution to improve bioadhesiveness to the lung tissue.

The gelatin component of the clear homogenous (1:1) chitosan/gelatin hydrogels was completely compatible with protonated chitosan solution [15]. All hydrogels were kept under cold condition till using in animal experiments.

In Vivo Assay

Animal experiments were conducted in accordance with ISO 10993 specifications at in vivo lab of Polymeric Biomaterial Department of Iran Polymer and Petrochemical Institute. Twelve mix bred female sheeps of 2-3 years old weighing 45-55 kg were used as animal models. All these animals were adopted at least 15 days before operation. Antibiotic therapy protocol for each animal was started one day before surgery and continued four days after the operation. General anesthesia was induced with intravenous injection of sodium thiopental (6 mg/kg). After endotracheal intubation and connection to positive pressure ventilator system, halothane (0.5% + 3%) was administered as a general anesthetic agent. During the procedure, the sheep was monitored with arterial line and pulse oximeter. A left thoracotomy through sixth intercostals space was performed. After chest opening, lung exposed and the ade-

quate experiment space has been made. Different incisions were then created on the lung surface so that severity of trauma could be considered (Figure 2). Air pressure was adjusted by means of a mechanical ventilator.

Hydrogel samples were pulled into sterile syringe and then injected on the mentioned fistula. Each hydrogel sample was applied on the three fistulas. After 180 s, ventilation was gradually started and the bioadhesiveness of the samples for preventing air leakage and bleeding was qualitatively investigated. After 30 min observation of the incised sites, chest was closed and the sheep was transferred to postoperative care unit.

RESULTS AND DISCUSSION

Potentiometry of Chitosan Solutions

For dissolution of chitosan in aqueous acidic media, one could consider two equilibrium reactions which surely affect each other. Table 2 indicates equilibrium concentrations of system components before and after adding the chitosan where α' and α'' are degrees of dissociation of acid in the absence and presence of chitosan, respectively. α is degree of protonation of chitosan and C_A and C_p are initial concentrations of acid and chitosan, respectively [9].

Upon adding chitosan as a weak base, the equilibriums would shift and subsequently the degree of dissociation of acid, e.g., acetic acid will change in accordance with Le Chatelier's principle.

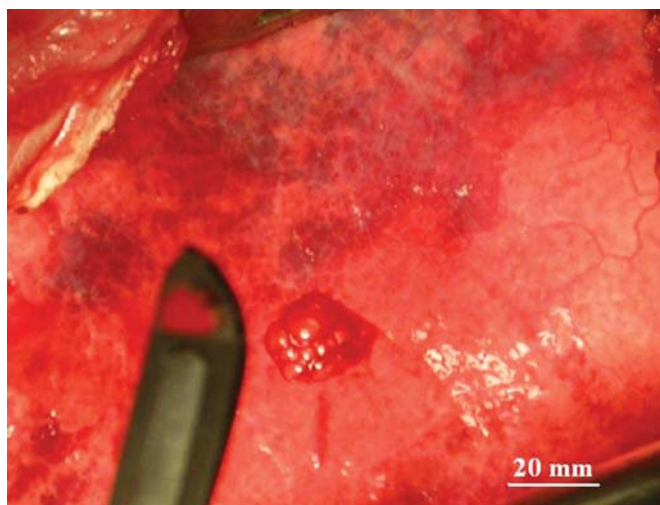
For dissociation of a weak acid in presence of chitosan one can write:

$$K_a = [H_3O^+] \left(\frac{\alpha''}{1-\alpha''} \right) \quad (1)$$

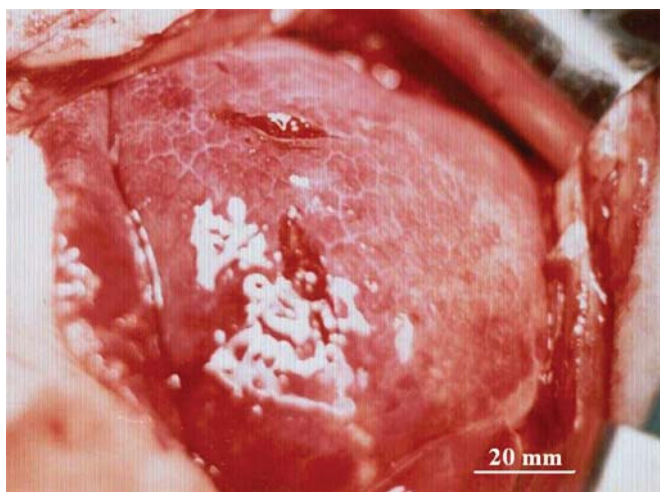
or,

$$\alpha'' = K_a / (K_a + [H_3O^+]) \quad (2)$$

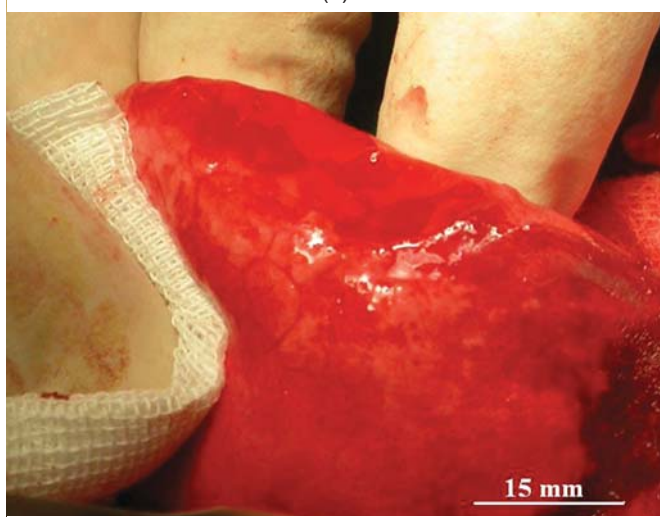
Where K_a is the dissociation constant of weak acid, and $[H_3O^+]$ is the equilibrium concentration of hydrogen ion. The values of pK_a for acetic and lactic acids are 4.75 and 3.86, respectively [16]. The degree of protonation of chitosan is consequently calculated in regards to electroneutrality of the system [9].



(a)



(b)



(c)

Figure 2. Creating of different incision on the sheep lung including: (a) stab incision, (b) longitudinal incision, and (c) massive incision .

$$C_A \alpha'' = C_p \alpha + [H_3O^+] \quad (3)$$

With a simple rearrangement we have [9]

$$\alpha = (C_A \alpha'' - [H_3O^+]) / C_p \quad (4)$$

From pH measurement of acidic chitosan solutions and with respect to equation (2) and (4), degree of protonation, α , was determined. Table 3 shows the value of degree of protonation for different polymer concentrations in the two acidic media.

Depending on type of acid and DD% of chitosan, complete solubilization was established after varying periods of time. As observed, chitosan in acetic acid would reach to equilibrium more rapidly at the same DD% in comparison with chitosan in lactic acid. The higher DD% resulted to the shorter period of time for establishing equilibrium in the same acidic media.

As seen in Table 3, polymer concentration has a crucial effect on the degree of protonation of chitosan at the same acid type and concentration. It was expected because at higher C_p , the number of amine groups in the solution will significantly increase, as though alkalinity of chitosan has been intensified. Moreover, influence of acid type on the degree of protonation is quite clear regarding its dissociation constant. Thus, the solutions that were obtained by lactic acid have greater degree of protonation than the solutions by acetic acid as shown in Table 3. It can be interpreted from the smaller value of pK_a of the lactic acid comparing with the acetic acid. Thus, its greater tendency to dissociate in presence of chitosan (remember the difference between α' and α'') that eventually results in more accessible hydrogen ions for protonation of the chitosan chains. Due to the α'' value greater than 0.5 for all chitosan solutions, it could be concluded that complete solubilization has

Table 2. The equilibrium concentrations of system components including weak acid, e.g., acetic acid, in absence and presence of chitosan and also the chitosan in aqueous acidic media [9].

AcOH+ H ₂ O \rightleftharpoons AcO ⁻ + H ₃ O ⁺			
In presence of chitosan	$C_A (1 - \alpha')$	$C_A \alpha'$	$C_A \alpha'$
In presence of chitosan	$C_A (1 - \alpha'')$	$C_A \alpha''$	
Chit-NH ₂ + H ₃ O ⁺ \rightleftharpoons Chit-NH ₃ ⁺ + H ₂ O			
In presence of chitosan	$C_p (1 - \alpha)$	$C_p \alpha$	

been reached [9]. Also, it was observed that samples with higher DD% take the shorter time to reach equilibrium state.

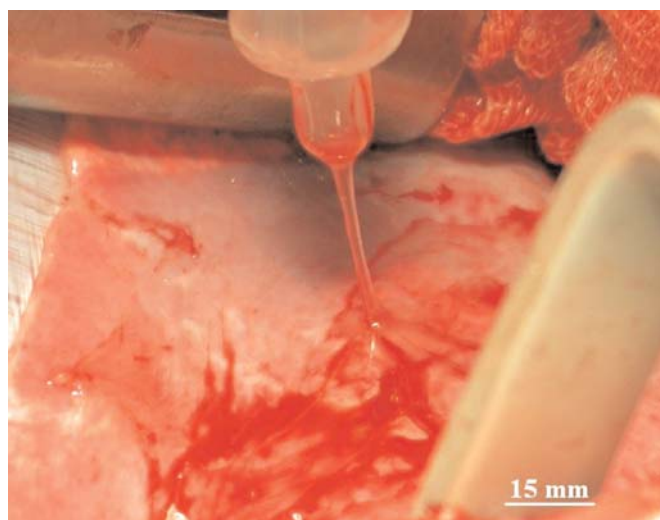
In Vivo Findings

All animals had normal behaviour and performance. Redo surgery was done 2-3 months after experimental surgery for evaluation of local complications including infection, severe lung adhesion and any abnormal tissue reaction. Observations indicated none of the above mentioned signs. Animal experiments revealed that samples containing β -GP had good injectability and could easily apply on the intended puncture and caused

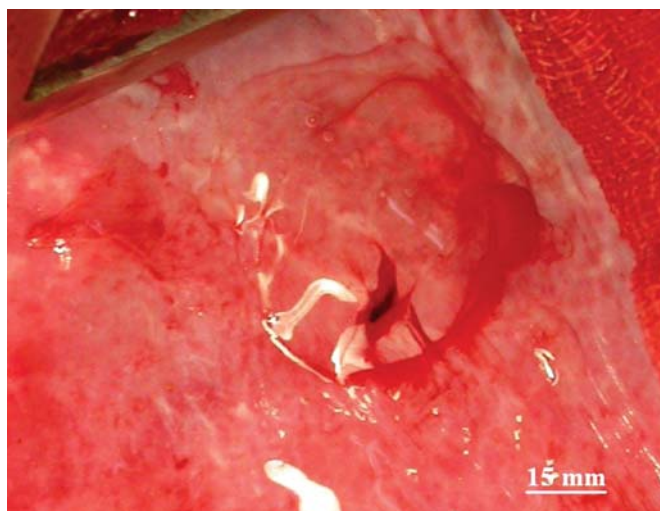
no irritant effect because of its neutrality. Although hydrogels remained on the fistula surface and did not flow, they were not efficient enough to seal lung fistula at high pressure. In this situation a bubble was formed (Figure 3).

However, samples which were prepared by the high DD% chitosan and lactic acid showed better sealing effect. To compensate weak cohesive forces which could be deduced from air pressure of lung via ventilator, we increased the β -GP content, but bioadhesion was strictly reduced though elastic modulus [12] and cohesive forces undoubtedly were increased after applying of those samples.

As an alternative to intensify bioadhesion, gelatin was used in the hydrogel system. The prepared samples preserved their injectability and biocompatibility. Figure 4 clearly shows the efficacy of gelatin rather than β -GP contained hydrogel even at higher air pressure. Bleeding was also stopped after applying of these samples [5]. Again, samples which were prepared by chitosan codes of Chs-16 and Chs-19 (Table 3) had more efficacy to seal air leakage. Also hydrogels which were solubilized in lactic media rather than acetic media showed better bioadhesion to lung surface in vicinity of fistula. Even after washing the lung surface, all samples



(a)

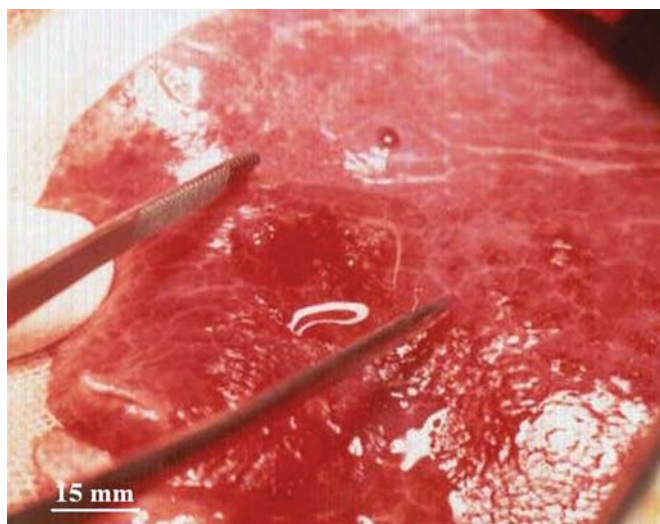


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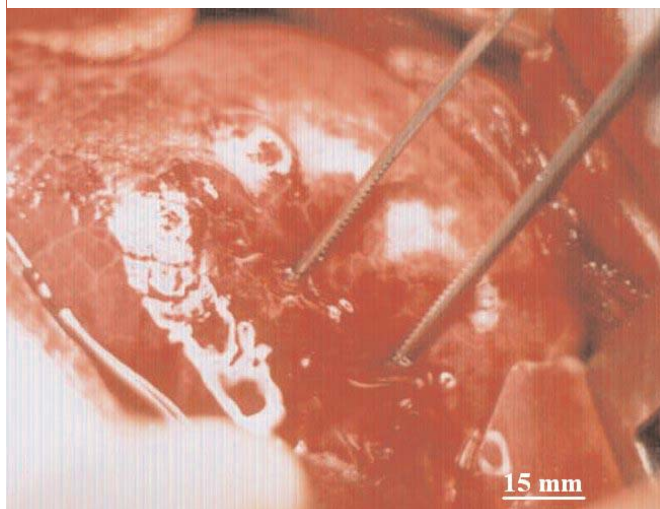
Figure 3. The injection of β -GP-contained chitosan hydrogel on the lung fistula: (a) by a syringe and (b) bubble formation after ventilation .

Table 3. Degree of protonation (α) of chitosan as a function of the polymer content (C_p), the degree of deacetylation, the acid type and concentration (C_A).

Chitosan code	C_A (w/v %)	Acid type	C_A (v/v %)	α (%)
Chs-F-M	2.23	Acetic	1	51
	2.23	Lactic	1	84
Chs-S-C3646	2.23	Acetic	1	47
Chs-PGC10	2.23	Acetic	1	47
Chs-CSN-7-3	2.23	Acetic	1	78
Chs-16	2	Acetic	1	50
	2.23	Acetic	1	59
Chs-19	2	Lactic	1	100
	2.23	Acetic	1	60
Chs-21	2	Lactic	1	100
	2.23	Acetic	1	60



(a)



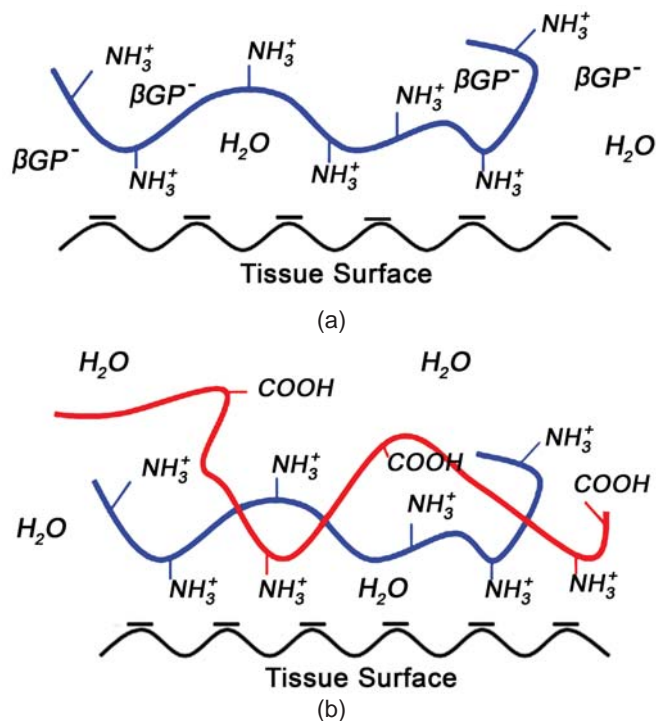
(b)

Figure 4. The injection of gelatin- contained chitosan hydrogel: (a) the length of incision and (b) intensity of air pressure of the lung.

remain on the surface and any air leakage and bleeding were not observed.

To demonstrate above results, we must understand in detail the interactions within the hydrogels. There are several possible molecular interactions while applying the β -GP contained hydrogels on tissue including:

- (i) Repulsive forces between like-charged chitosan chains.
- (ii) Attractive hydrophobic and hydrogen bonding between chitosan chain [17].
- (iii) Electrostatic attraction between cationic amine functionalities of chitosan and anionic phosphate moieties of β -GP.



negatively charge tissue (\curvearrowright) negative moiety of β -GP (βGP^-)
 protonated amine group of chitosan chain ($-$) gelatin chain ($-$)

Figure 5. Schematic representation of interactions in the chitosan hydrogel consisting of (a) β -GP and (b) gelatin.

(iv) Hydrophobic or water- structuring of the glycerol moiety of β -GP.

(v) Owing to negative surface charge of most living tissues [18], bioadhesive forces would make up from attraction forces between the cationic amine groups of chitosan and the negative sites of the tissue.

Figure 5a shows a schematic representation of different interactions within hydrogel and between hydrogel and tissue. We concluded that the higher DD% means the greater active sites to interact with lung tissue as was observed in samples with highest DD% (Ch-16) (Table 3). On the other hand, weakening of bioadhesion upon adding more β -GP would be explained as follows:

when concentraion of β -GP increses, actually a situation will be encounter in which probablity of the occurance of interaction (iii) strongly will be intensified rather than the interaction (v) that eventually causes the decreasing of positive charge of hydrogel and weakening of bioadhesive forces. Then, there is an optimum point that cohesive and adhesive forces must be bal-

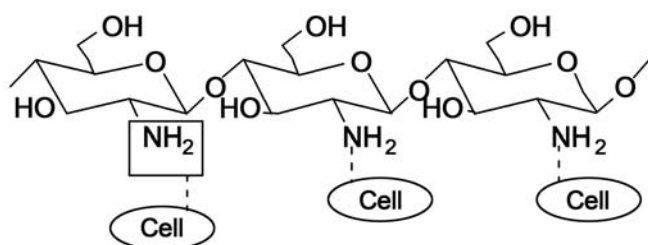


Figure 6. Electrostatic interaction between erythrocyte and chitosan (with permission) [6].

anced. As mentioned before, β -GP contained hydrogels are not strong enough to seal the lung punctures.

When gelatin was added to the system, an improvement in bioadhesion has been reached. As a matter of fact, a polyelectrolyte complex was obtained [19,20] as shown in the Figure 5b. Without doubt, increasing of active sites to interact with tissue (negative sites) due to introducing of amine groups of gelatin has an important effect on strengthening of the bioadhesion forces. Furthermore, effective sealing of lung puncture at high air pressures could be related to attractive forces between carboxylic functionality of gelatin and amine groups of chitosan.

As DD% increases, the both above mentioned interactions will reinforced in a parallel manner that lead to better sealing of hydrogels prepared by chitosan codes of Chs-16 and Chs-19 rather than Chs-21 and Chs-CSN-7-3 (Table 3).

The effect of acid type can be interpreted in the same manner because chitosan solutions which were prepared by lactic acid have the greater degree of protonation than the solutions prepared by acetic acid (Table 3). Therefore, more cationic amine groups have been gained on the chitosan backbone that would modify the balance of different prementioned interactions and finally results in more active sites for bonding to lung surface.

Mechanism for coagulation effect of chitosan is discussed by the same authors elsewhere [6]. In brief, blood also carries negative charge and in vicinity of any positive charged material will form clot (Figure 6). Consequently, with applying chitosan based hydrogels on the bleeding lung fistula, either lung surface or blood would sense oppositely charged substrates. It is a matter of fortunate because all

deductions about mechanism of bioadhesion of chitosan hydrogel and lung might apply to interpret the coagulation effect of the chitosan hydrogel.

CONCLUSION

Different hydrogels were prepared by solubilization of different amount of chitosan in either acetic or lactic aqueous acidic media. Dissolution in the lactic media yields greater degree of protonation rather than acetic media. With respect to $\alpha > 0.5$ for all solutions, it could be interpreted that complete solubilization was reached.

Animal experiment revealed that gelatin-contained chitosan samples are more effective than β -GP's to prevent air leakage and bleeding from lung punctures. However, in the both case, samples with higher degree of deacetylation, including Chs-16 (DD% = 99, MW=230,000) and Chs-19 (DD% = 98, MW=300,000) which were dissolved in lactic media showed best sealing effect. Further studies are now conducted to optimize the sealing properties of chitosan based hydrogels.

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