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Chemical cross-linking abrogates adjuvant potential of natural polymers

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Running title: Loss of adjuvant potential of chitosan and alginic acid polymers

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Abstract

Natural polymers like chitosan and alginic acid are extensively used in biomedicine for different applications. In linear form, these polymers supported growth of human kidney fibrosarcoma cells as confirmed by *in vitro* study and shown no visible toxic effects in mice when injected intradermally. However, polymers in linear form mixed with the cartilage protein, collagen type II (CII), induced significant anti-CII IgG response and arthritis in mice. Histological analysis of the arthritic paws showed massive infiltration of immune cells with extensive damage to joint tissues, similar to the classical arthritis induced with CII emulsified with the Freund's adjuvant. This adjuvant property of the linear polymers is not desirable for their use in tissue engineering. Interestingly, though chemical cross-linking of these polymers supported growth of human kidney fibrosarcoma cells, it abrogated their adjuvant property. Thus, cross-linking of polymers could be used as a strategy in biomedicine to avoid undesirable immune reactions induced or directed against polymers.

Key words: Chitosan, alginic acid, cross-linking, adjuvant, arthritis

1. Introduction

Natural polymers have important applications in the biomedical field such as in tissue engineering as scaffolds for growth of mammalian cells, for drug delivery, polymer coating for immuno-suppression over implants etc. Such biomaterial scaffolds should be degradable, biocompatible and immunologically inert and, polymers with good cell interactions lead to better growth of cells over the scaffolds.¹⁻³ Chitosan (poly-1, 4-D-glucosamine) is a well-known degradable natural polymer that can be formulated in different physical forms such as gels, particulate and films. Due to these properties, chitosan could be used in drug/antigen delivery,

tissue engineering for growing cartilage, bone, liver, nerve etc.⁴ Structurally, it mimics glycosaminoglycan (GAG), which is one of the most important constituents of extracellular matrix that act as substratum for cell growth.⁵ It is also providing environment rigidity to the cells. Presence of protonatable amino groups in chitosan allows the polymer to interact with several cellular proteins, for example in slightly acidic medium amino groups of chitosan (pKa~6.5) can be protonated that could interact with glycoproteins such as mucin of mucus.⁶ In this context, alginic acid composed of β -1, 4 linked D-mannuronic acid and an L-guluronic acid residue is another example of this type of natural polymer. Alginates from different sources have different proportions of these two constituent residues.¹ Alginic acid is water soluble, degradable and biocompatible polymer. In gel format, water molecules bound to the polymeric chains confers viscosity property to the polymer, while loosely bound and free water molecules are movable inside the gel and surrounding milieu making alginic acid as an attractive supporting system for biomedical applications such as cell immobilization.⁷⁻⁹

Cross-linking between the polymeric chains alters the physical and mechanical properties of polymers, and affects the release rate of drug/protein from the polymers. For example, protein polymers in cross-linked form show high adhesion and proliferation properties compared to uncross-linked films.¹⁰ Similarly, drug release profile and muco-adhesive properties of chitosan microspheres were affected after cross-linking them.^{11,12} Interestingly, cross-linking also improves stability, homogeneity and higher production yield of polymeric nanoparticles.¹³ In this context, we evaluated the immunological properties of chitosan and alginic acid polymers in linear and cross-linked forms as substratum for cell growth and as adjuvants mixed with collagen type II (CII) to induce arthritis in mice.

2. Materials and methods

2.1 Mice, antigens and chemicals

B10.RIII/Rhd male mice were used in the experiments. All the animals were housed in a climate-controlled environment having dark and light cycles for 12h. Wood shavings and paper were used as bedding and enrichment materials respectively in the individually ventilated polystyrene cages. Standard food and water were given *ad libitum*. All the animal experiments were performed and approved under Swedish ethical permit number, N166-10. Animals were anesthetized using isoflurane-air (3:1) mixture during experimental procedures. Bovine collagen II was used as an antigen for immunization to induce arthritis. CII was prepared by pepsin digestion and purified as described earlier.¹⁴ Alginic acid and chitosan (low viscous) were purchased from Sigma Aldrich, Germany.

2.2 Polymers characterization

Chitosan (1%, w/v) was cross-linked by 0.05 % glutaraldehyde, while alginic acid (1%, w/v) was cross-linked by calcium chloride (0.05 mM) solution. After cross-linking, polymers were purified by overnight dialysis. Cross-linking of polymers was confirmed by Fourier transform infrared spectroscopy (FTIR). The samples used in this study were dried first and prepared in the form of powders. They were thoroughly mixed with KBr pellet (0.1 g, IR Grade, Merck, Germany) followed by recording of the spectrum using 8300 spectrophotometer (Shimadzu, Japan). Viscosity of polymers was determined using Ostwald viscometer (Ace Glass Inc., Vineland, NJ).

Polymers capacity to support cell growth was analyzed using a colorimetric assay and HT1080 human kidney fibrosarcoma cell line. This assay is based on the principal of oxidizing capability

86 of live cells converting blue color of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
87 bromide (MTT) into blue-violet color.¹⁵

88 **2.4 Adjuvant properties of polymers**

89 Adjuvant capacity of the polymers was analyzed using 7-8 weeks old B10.RIII mice. Mice were
90 divided equally into 7 different groups; complete Freund's adjuvant-collagen II (CFA-CII),
91 linear chitosan-CII, linear alginic acid-CII, cross-linked chitosan-CII, cross-linked alginic acid-
92 CII, linear chitosan and linear alginic acid. CII emulsified in Freund's adjuvant (Difco, Detroit,
93 MI) (CFA-CII group) and linear chitosan or alginic acid alone immunized mice served as the
94 positive and negative controls respectively. For priming, 200 µl of antigen-polymer (100µg CII:
95 100 µg polymer) was used and the animals were boosted on day 21 with 100 µl dose (50 µg CII:
96 50 µg polymer). All the mice were bled at days 21 (early phase) and 50 (chronic phase) and the
97 sera were collected for subsequent antibody analysis. Anti-CII IgG antibody levels were
98 quantified using ELISA. Affinity purified anti-CII antibodies from pooled sera of CII immunized
99 mice or pooled sera were used as the standard. Biotinylated rat anti-mouse IgG kappa (prepared
100 in-house, clone 187.1) or mouse anti-mouse IgG2c (BD), or peroxidase-conjugated goat anti-
101 mouse antibodies specific for IgG1, IgG2b or IgG3 (Southern Biotech, Birmingham, AL, USA)
102 were used as detecting antibodies. The biotin binding was detected by extravidin peroxidase
103 (Sigma-Aldrich) and peroxidase substrate solution (Roche, Germany). Anti-CII IgG levels were
104 measured using the sera collected on both phases of the disease (days 21 and 50), whereas IgG
105 subclass analysis was done using chronic phase sera (d50) only.

106 **2.5 Arthritis evaluation**

The clinical evaluation of inflammation was done, using a standard protocol¹⁶ in which inflammation is defined as swelling and redness. In arthritis scoring each inflamed toe or knuckle gives one point, while the inflamed wrist or ankle gives five points, resulting in a score of 0-15 (5 toes + 5 knuckles + 1 wrist/ankle) for each single paw and 0-60 points for each mouse. The arthritis incidence is the percentage ratio of number of sick mice and total number of mice used in each group. For histological analysis, the arthritic paws were cut from three mice, fixed, decalcified, embedded and sectioned by microtome. Joint sections (3-6 sections per paw) were stained with hematoxylin-eosin to visualize morphology and infiltration of immune cells.

2.6. Statistical analysis

Mann–Whitney U ranking test in Statview 5.0.1 software (SAS Institute, NC) was used for statistical calculations. Significance was considered when $p < 0.05$, for a 95% confidence interval. Polymer characterization experiments were performed in triplicates and animal experiments were done twice with several mice in each group per experiment (total: 110 mice).

3. Results and discussion

Natural polymers like chitosan and alginic acid are widely used in various biomedical applications such as tissue engineering, drug/antigen delivery and transplantation studies.^{1, 17} These polymers exhibit properties such as aqueous solubility, degradability, easy in formulation and modifications. Ideally, polymers should be biocompatible, non-toxic and should not induce any adverse immunological responses. Therefore in this aspect, we assessed the adjuvant properties of chitosan and alginic acid. At first, linear polymers were mixed with CII and used for immunization of naïve, inbred mice twice (days 0 and 21) following a standard protocol for

129 arthritis induction in mice. Interestingly, both these polymers possessed adjuvant capacity. CII-
130 polymer mixture induced arthritis (Figure 1A). CII-chitosan induced arthritis in 15% of the mice
131 with a mean maximum arthritis score of 20 ± 7.5 , while CII-alginic acid induced arthritis in 18 %
132 of mice with a mean maximum score of 13 ± 3 . The positive control, CFA-CII immunized mice
133 induced 80% arthritis with a mean arthritis score of 40 ± 7 , while negative controls chitosan or
134 alginic acid alone without CII did not induce any disease (Figure 1B, C). Anti-CII antibodies
135 with all the major IgG subclasses in polymer groups were observed similar to complete Freund's
136 adjuvant emulsified CII immunized mice. Linear chitosan-CII and alginic acid-CII immunization
137 induced anti-CII antibody response, though significantly lesser than the later (CFA-CII) group
138 but higher than the negative controls, chitosan or alginic acid alone groups without CII (Figure
139 1D). Similar trend was observed in IgG subtypes. IgG1, IgG2b, IgG2c, IgG3 subclass antibodies
140 levels between the groups (i.e. higher in the positive control (CFA-CII) group compared to linear
141 chitosan-CII and alginic acid-CII groups). Linear chitosan-CII group had slightly higher IgG
142 subclass levels than linear alginic acid-CII group but the difference between the groups was not
143 significant. Histology of the arthritic joints clearly showed degradation of cartilage and massive
144 infiltration of immune cells in the linear chitosan-CII and linear alginic acid-CII groups similar
145 to the positive control CFA-CII group mouse joints (Figure 2).

146 However, the adjuvant properties of these natural polymers are undesirable in tissue engineering
147 applications. Hence, we cross-linked the polymers using glutaraldehyde for chitosan and calcium
148 chloride for alginic acid (Figure 3A, B). Cross-linking of the polymers was confirmed by FTIR
149 (Figure 3C) and viscosity analysis. After cross-linking, a new peak of oxime ($-C=N$) bond was
150 found at 1657 cm^{-1} in chitosan, while in alginic acid peak of different functional groups shifted
151 toward higher wave numbers. Frequency of $-OH$ group shifted from 3418 to 3424 cm^{-1} and

=C=O from 1611 to 1629 cm^{-1} . Indirect evidence for polymer cross-linking was obtained by measuring the increase in viscosity of polymers and we observed a significant difference in viscosity before and after cross-linking of the polymers. After cross-linking, viscosity of 1%, w/v chitosan (0.42 Pa.s) and 1% of alginic acid (0.03 Pa.s) were increased to four (2 Pa. s) to 50 folds (1.52 Pa. s) respectively. Although chemical cross-linking led to increased viscosity, these polymers were still injectable in mice. They did not show any significant difference in supporting the growth of human kidney fibrosarcoma cells either before or after cross-linking as confirmed by MTT proliferation assay (Figure 3D). Interestingly, cross-linking of polymers led to loss of their adjuvant capacity and the mice injected with CII mixed with cross-linked polymers did not show any arthritis symptoms (Figure 1B, C). Anti-collagen antibody responses of mice immunized with cross-linked chitosan-CII or alginic acid-CII were significantly reduced compared to linear chitosan-CII and alginic acid-CII groups (Figure 1D).

Polymers are being used extensively in biomedicine, which should not elicit any major immune responses or show *in vivo* toxicity. Here, we demonstrated the adjuvant properties and capacity to support the growth of fibrosarcoma cells of two typical polymers chitosan and alginic acid. Irrespective of difference in their chemical structure, when mixed with CII and injected into mice, they induced anti-CII antibody responses leading to arthritis. Most importantly, our results demonstrate that chemical cross-linking abrogates such adjuvant property of these polymers but still supported the growth of kidney fibrosarcoma cells. However, the exact mechanism by which cross-linking of polymers affects its adjuvant property still needs to be explored further.

4. Conclusions

In order to analyze the immunological response of the natural polymers, chitosan and alginic acid, we characterized the adjuvant capacity of these polymers. Both the polymers in linear and

cross-linked forms supported the growth of kidney fibrosarcoma cells. Linear form of polymers mixed with CII and injected into mice induced arthritis with significantly elevated levels of anti-CII antibody responses. Similar to complete Freund's adjuvant, arthritic joints from mice injected with these polymers had massive infiltration of inflammatory cells. Interestingly, after cross-linking, though they supported the growth of kidney fibrosarcoma cells similar to linear polymers, they did not induce arthritis and anti-CII responses were dramatically reduced. Therefore, clearly chemical cross-linking abrogated the adjuvant potential of these polymers and this strategy could be used to prevent undesirable immunological responses toward different types of polymers *in vivo*.

Acknowledgements

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Conflict of interest

All the authors declare that they have no conflict of interests.

Figures legends

Figure 1: Adjuvant capacity of chitosan and alginic acid in free and cross-linked forms.

Digital photographs of representative mouse hind paws showing arthritis inflammation (A), control mouse without joint inflammation (a), CFA-CII (positive control) (b), linear chitosan-CII

(c) or linear alginic acid-CII (d) immunized mice; arthritis incidence (B) and mean arthritis score (C) on different days; anti-CII antibody response at day 21 and 50 (D) and IgG subclass analysis at day 50 (E). Adjuvant properties of the polymers were analyzed by mixing them with bovine CII and injecting in 8-10 weeks old B10.RIII mice. Mice were divided into 7 different groups viz., CFA-CII, positive control (n=10), linear chitosan-CII (n=20), linear alginic acid-CII (n=20), cross-linked chitosan-CII (n=20), cross-linked alginic acid-CII (n=20), negative controls: linear chitosan (n=10) and linear alginic acid (n=10). Mice were immunized with 100 µg of CII and 100 µg of polymer mixture and boosted with 50 µg of CII and 50 µg of polymer on day 21. Represented results are from two experiments and all the animals were used for calculations. Error bars denote \pm SEM. *, $p < 0.05$.

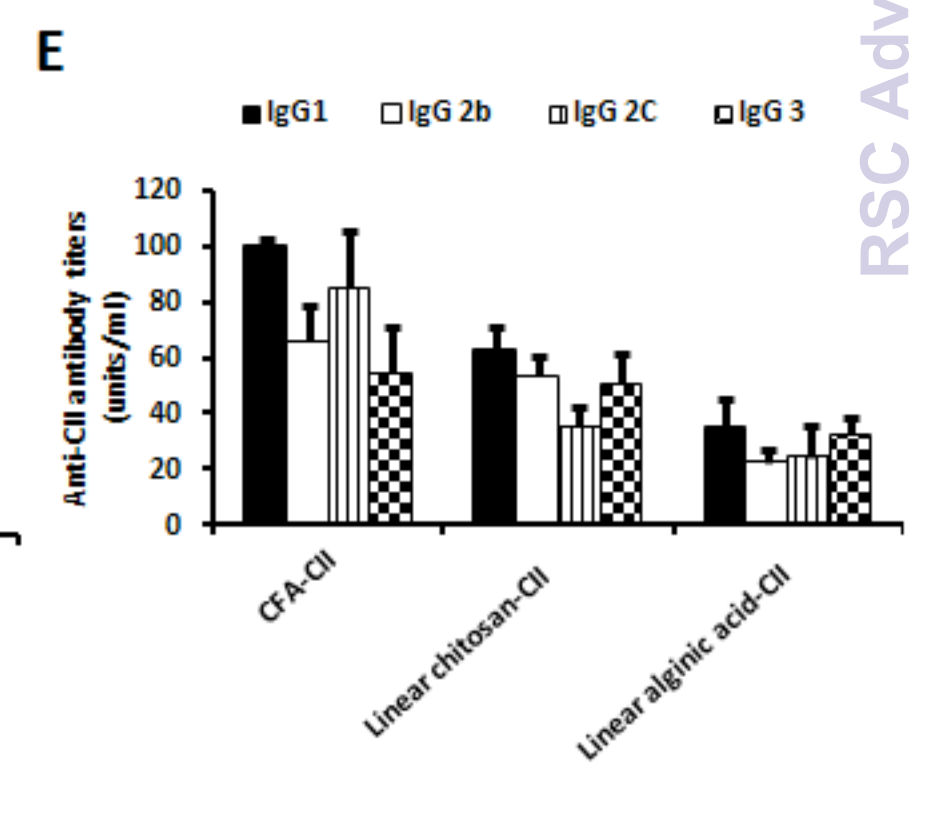
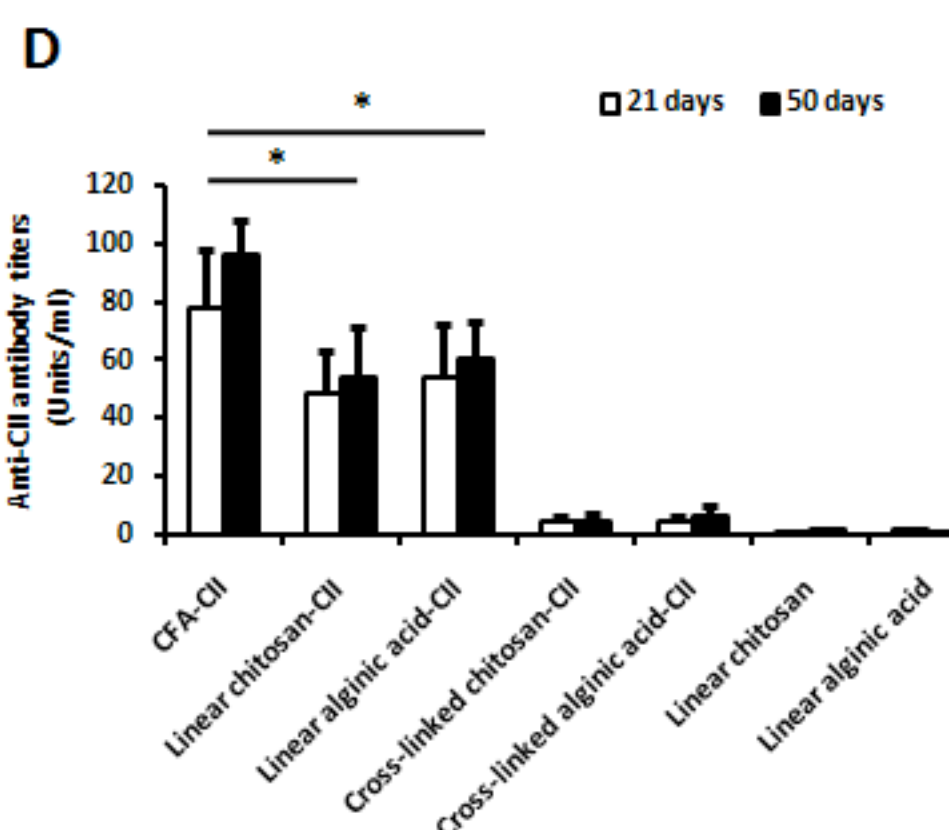
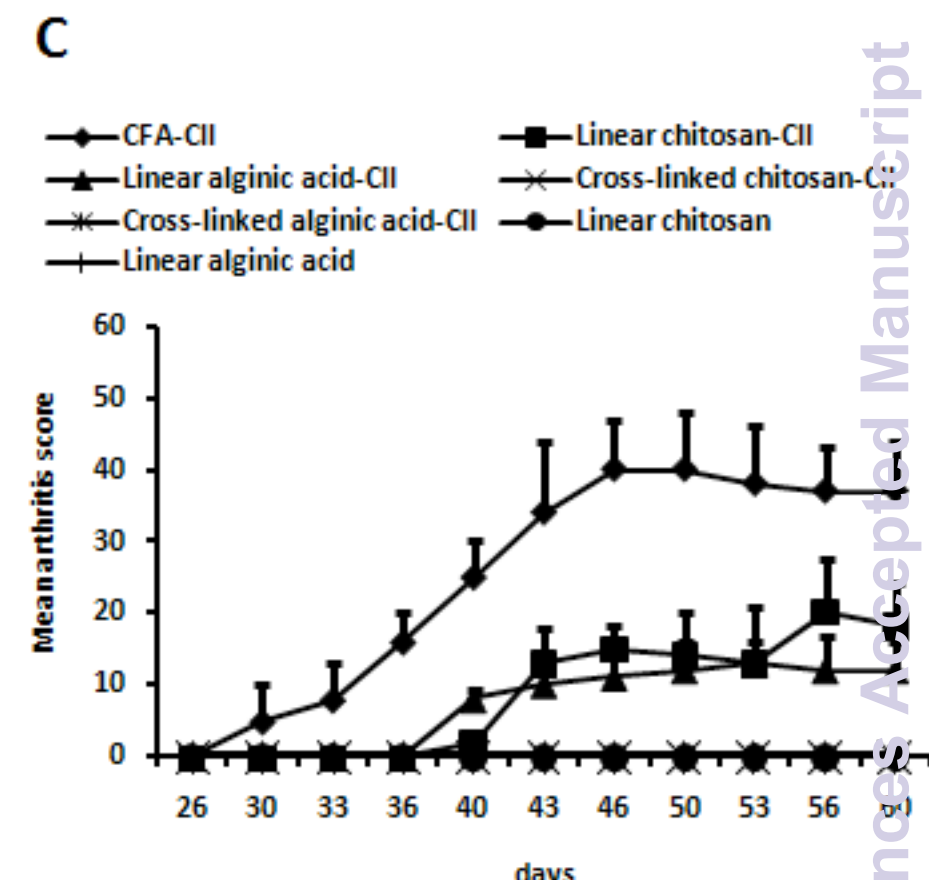
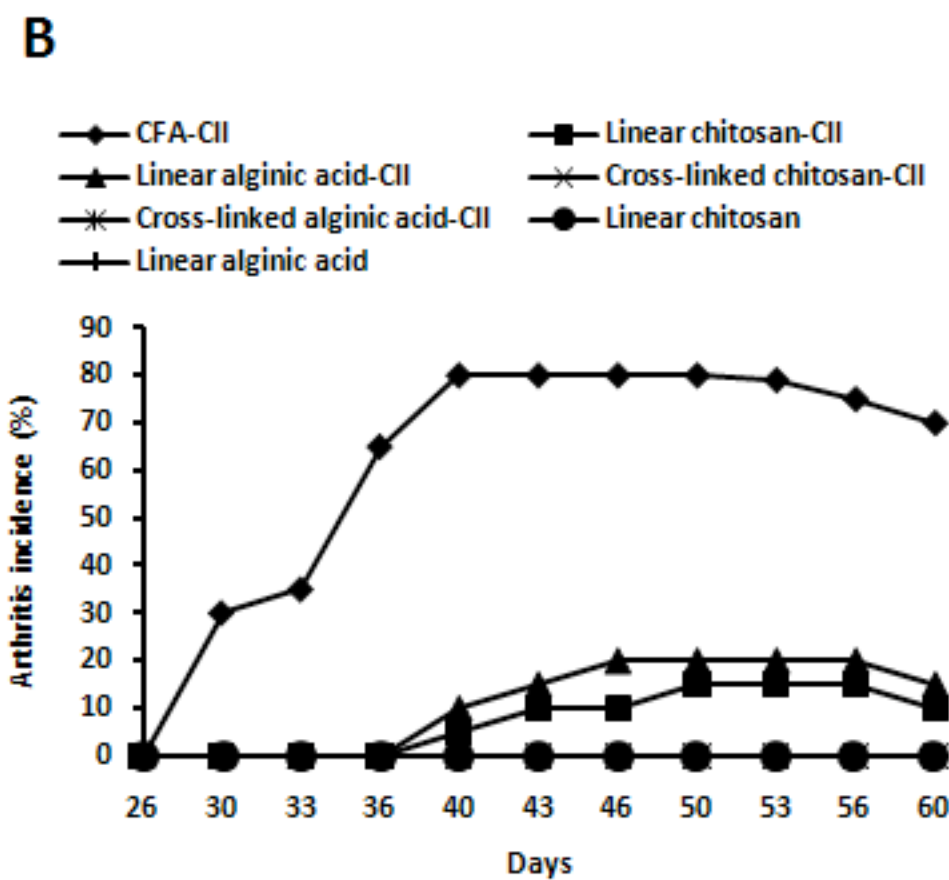
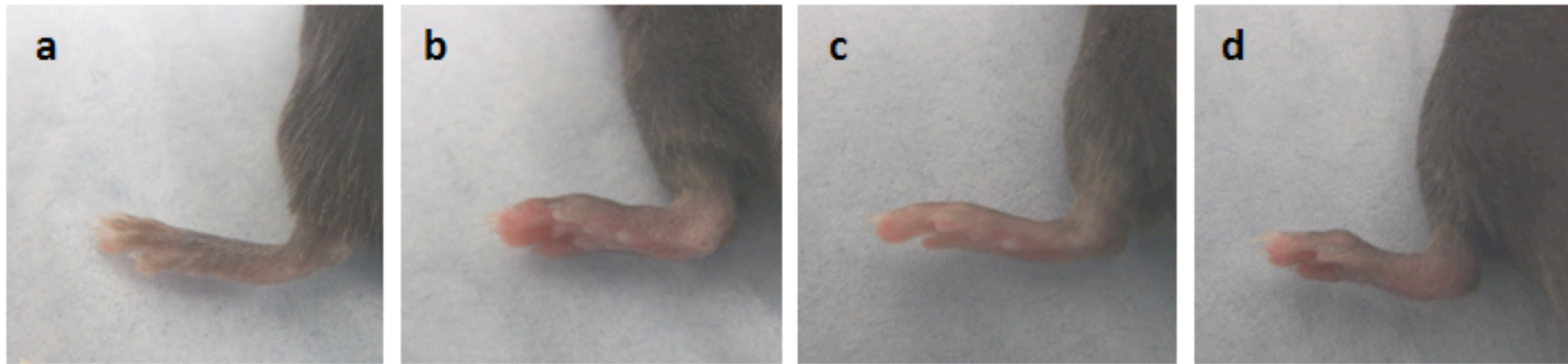
Figure 2: Histological analysis of mice showing arthritis. Hematoxylin and eosin staining of representative mouse joints (n=3 per group), naïve mice (negative control) (a), mice immunized with linear chitosan-CII (b) linear alginic acid-CII (c) and CFA-CII (positive control) (d). All images were taken at 20x magnification. For histological analysis, the arthritic paws were cut and fixed in phosphate buffered paraformaldehyde solution followed by decalcification. They were dehydrated and embedded in paraffin blocks. Joint sections (6 µm) were cut by microtome and stained with hematoxylin-eosin to visualize morphology and infiltration of immune cells.

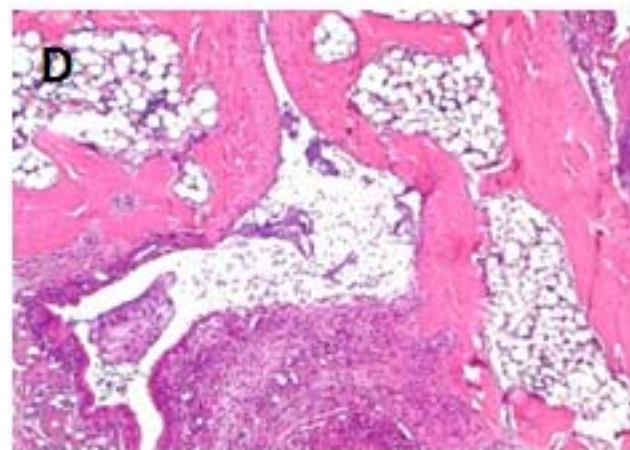
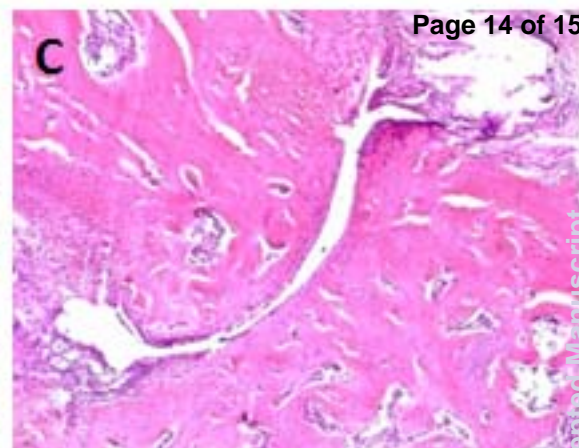
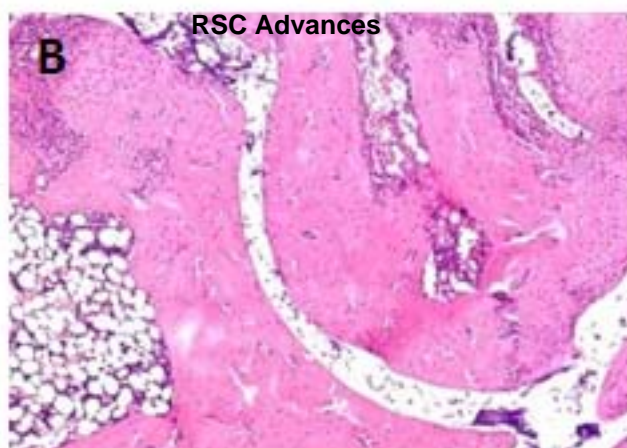
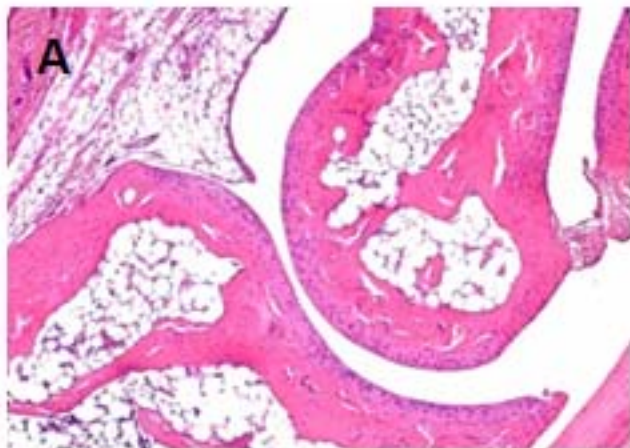
Figure 3: Physiochemical characterization of polymers. Schematic representation of cross-linking polymeric chains of chitosan (A) and alginic acid (B), FTIR of cross-linked forms (C), MTT assay of polymers in linear and cross-linked forms with HT1080 kidney fibrosarcoma cell line (D).

218 **References**

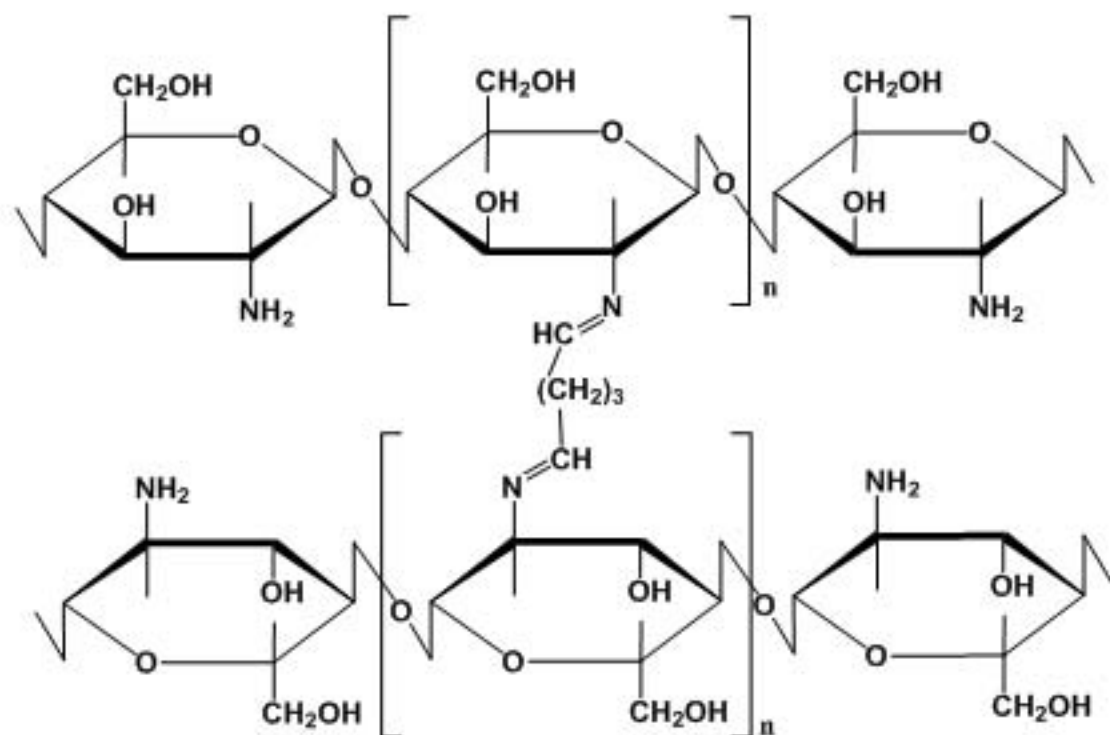
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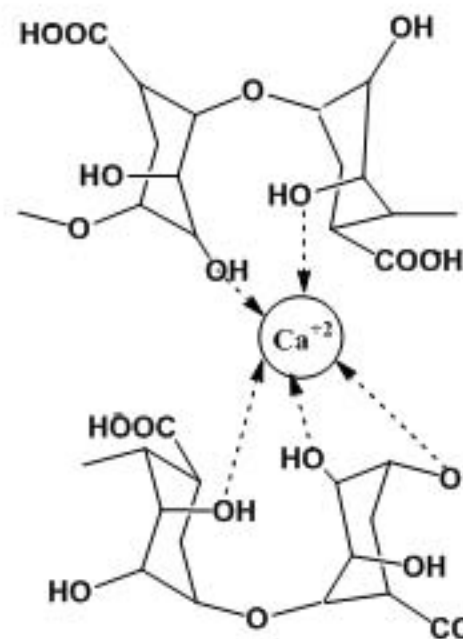




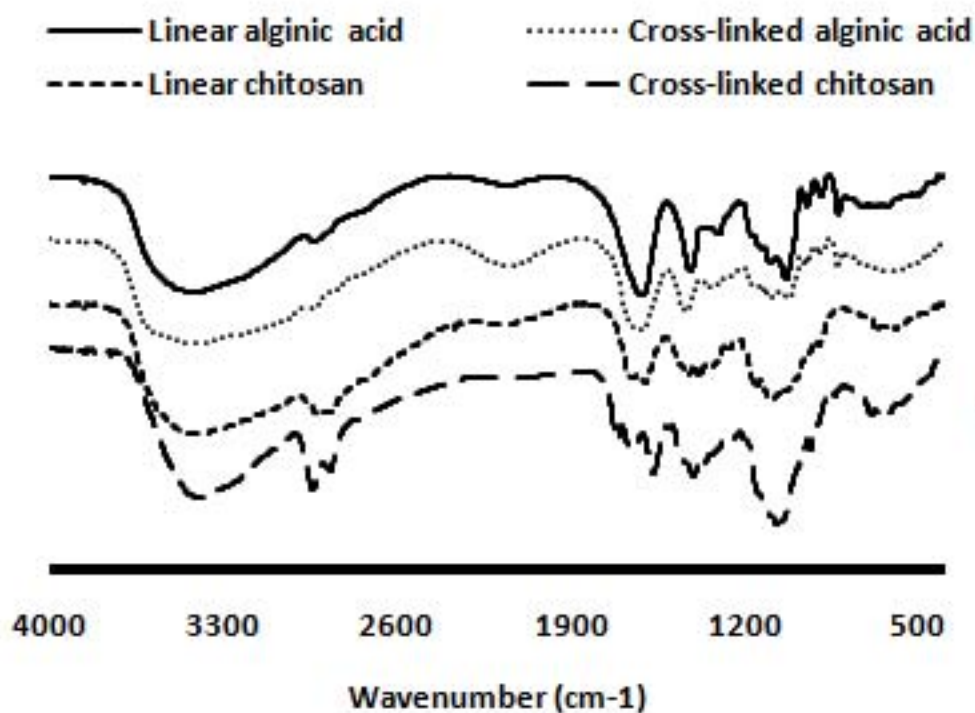
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