JOURNAL OF Applied Polymer SCIENCE

Novel Strategy for the Ionotropic Crosslinking of Chitosan–Alginate Polyelectrolyte Complexes

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ABSTRACT: The formation kinetics of chitosan–alginate polyelectrolyte complex (CAPEC) was investigated in the presence of various ionic crosslinkers. The effects of binary mixtures of some anions with Ca^{2+} cations were also studied as a novel application. CAPEC gelation was enhanced in cases where Ca^{2+} ions coexisted with anionic crosslinkers compared to their conventional individual use, and this resulted in dispersions with higher stability. We demonstrated that the effects of some anions on CAPEC gelation could be reversed in the presence of Ca^{2+} ions, such that the inhibitory anions became enhancers. The results also revealed that Ca^{2+} ions, the most widely used ionic crosslinkers in CAPEC synthesis, exerted inhibitory effect on CAPEC gelation at low concentrations; in addition, the use of Ca^{2+} ions alone resulted in decreased dispersion stability. The effects of the binary crosslinker on the structure and properties of CAPEC were studied by scanning electron microscopy, Fourier transform infrared spectroscopy, particle size analysis, and ζ-potential analyses. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40019.

KEYWORDS: biomaterials; crosslinking; drug-delivery systems; polyelectrolytes; polysaccharides

Received 12 July 2013; accepted 25 September 2013
DOI: 10.1002/app.40019

INTRODUCTION

Chitosan (β-(1→4)-poly-D-glucosamine) is a biodegradable, nontoxic, nonallergenic, and nonirritating (i.e., biocompatible) linear polysaccharide (PS) derived from chitin via deacetylation.[1,2] In addition to these properties and its low production cost, chitosan also possesses some important bioactive properties, such as wound-healing, antiulcerogenic, hypocholesterolemic, anti-inflammatory, antiproliferative, mucoadhesive, and antitumor properties[3,4] and osteogenic, hemostatic, bacteriostatic, fungicidal, and immune-system-stimulating activities.[5–8] Alginates are linear, unbranched natural PSs composed of α-L-guluronic acid and 1→4-linked β-D-mannuronic acid residues. Its residues can be in different sequences and compositions. Sodium alginate is a negatively charged polyelectrolyte (PE) in aqueous solution. Because it is biocompatible and biodegradable, it can be used in the encapsulation and/or delivery of various biological agents, such as proteins, DNA, and functional cells, without damaging their structures and properties.[9–12]

Ionotropic gelation is based on the crosslinking of PEs by counterions to form hydrogels. The ionic crosslinking of PEs can occur under mild conditions, and most ionic crosslinkers are nontoxic. The most widely used ionic crosslinkers for chitosan and alginate are sodium tripolyphosphate (NaTPP) and Ca^{2+} ions, respectively.

Polyelectrolyte complexes (PECs) are formed between two oppositely charged PEs in aqueous solution by electrostatic interactions. There is no need for auxiliary molecules such as catalysts and initiators for the formation of a PEC network. PEC formation can be monitored by conductivity,[13] viscosity,[14] pH,[15] and turbidity[16,17] measurements.

Over the last several decades, there has been a substantial increase in the number of studies dealing with drug- and antigen-delivery systems, but in these systems, properties such as the resistance to degradation in drug-releasing media, drug-loading capacity, and controlled release capabilities still need to be improved. In recent years, researchers have focused on PECs rather than on neat PSs as drug carriers to improve these properties. Of these, chitosan–alginate polyelectrolyte complexes (CAPECs) are the most synthesized and the most commonly used. These are more effective in limiting the release of encapsulated materials compared to either polymer alone. Complexation with chitosan to form CAPEC reduces the porosity of alginate beads and thus reduces the leakage of the encapsulated active materials. Moreover, the dissolution of chitosan at low gastric pH is prevented by the alginate in the CAPEC network because alginate is insoluble at low pHs. On the other hand, the dissolution of alginate at high intestinal pH is prevented by the chitosan moieties.

Ionic crosslinkers are preferred to organic crosslinkers for strengthening CAPEC gel network so that the delivery system can be biocompatible and biodegradable. CAPEC is crosslinked mostly by Ca^{2+} ions and, to a limited extent, by tripolyphosphate (TPP) anions. Alginate binds approximately 100 times more chitosan molecules in the presence of Ca^{2+} ions than in the absence of Ca^{2+} ions.[18] CAPEC microparticles are prepared in various ways in practice: (1) chitosan and alginate solutions at suitable pHs are mixed and processed,[18,19] (2) a solution containing both chitosan and alginate is dropped or sprayed into a Ca^{2+} solution,[20] (3) solid $CaCl_2$ is added to a chitosan+alginate binary solution,[21] (4) a solution of alginate is dropped into a chitosan solution containing Ca^{2+},[22,23] (5) a chitosan solution is dropped into an alginate solution containing TPP,[22] (6) CAPEC beads crosslinked with Ca^{2+} are treated with an SO_4^- solution or glutaraldehyde, or [24] (7) alginate, chitosan, or CAPEC microparticles prepared with or without crosslinker are converted into microcapsules by their coating with chitosan or alginate one or more times or by treating them with Ca^{2+}or polymer solutions to improve their mechanical strength and the delivery characteristics of the active ingredients.[18,21–28] In these methods, the drug or any other active material to be delivered is added to chitosan, alginate, or Ca^{2+} solutions.

The intramolecular and intermolecular interactions in PEC hydrogels to be used in drug delivery and controlled release need to be strong enough to resist gel degradation in drug-releasing media. This can be achieved when gel formation occurs in an enhanced manner. Therefore, in this study, we aimed to develop crosslinkers which promote the gelation of CAPEC more effectively compared to conventional ones. For this purpose, the kinetics of the formation of CAPEC gel dispersions was investigated in the presence of various anions and Ca^{2+}cations. More effective ionic crosslinkers are reported for CAPEC compared to conventional Ca^{2+} and TPP ions. In addition to their individual use, a novel strategy in which the anionic crosslinkers and Ca^{2+}cations were used simultaneously was also applied. This strategy enhanced the formation of the CAPEC gel particles strikingly and also increased the stability of the resulting CAPEC dispersion. The results of this study also demonstrate that Ca^{2+} ions, which are the most widely used crosslinkers in CAPEC microparticle and nanoparticle syntheses, can exert negative effects on CAPEC gel formation and on the stability of the CAPEC dispersion when used alone, as they conventionally applied. Nine main conclusions were drawn from the results.

EXPERIMENTAL

Materials

Medium-viscous chitosan (CMV, Fluka 28191) and medium-viscous alginate (AMV, Sigma A2033) were used as the PEs. The viscosity of a 1% w/v CMV solution in 1% v/v acetic acid at 20°C was 200–400 mPa s. The viscosity of a 2% w/v solution of AMV in water at 25°C was ≈3500 mPa s. All of the other chemicals used were of analytical purity. Sodium polyphosphate (NaPPhos), sodium phosphomolybdate (NaPhosMo), ammonium molybdate (AmMo), NaTPP, and sodium pyrophosphate (NaPyPhos) ionic salts were used as sources of anionic crosslinkers, and $CaCl_2$, which bears the most effective cation in the gelation of alginate and CAPEC, was used as the source of cationic crosslinker. NaPPhos, NaPhosMo, and AmMo were used for the first time in the ionotropic gelation of CAPECs. Deionized water was used throughout.

Instrumentation

A Eutech TN-100 brand turbidimeter was used for turbidity measurements. A homogenizator (Janke and Kunkel, Ultra-Turrax) and a sonicator (Sonics VC 505) were used to prepare the solutions. The solutions were prepared in jacketed vessels and cooled during sonication by the circulation of water at 25°C. Fourier transform infrared (FTIR) spectra were recorded with a MATTSON 1000 spectrometer equipped with an IR pyroelectric DTGS (pyroelectric type IR detectors containing triglycine sulfate) detector. All of the spectra were taken at a spectral resolution of 2 cm^{-1}. The size and ζ potential analyses of the CAPEC particles were performed with a Malvern Zetasizer Nano-ZS instrument (Malvern Instruments, United Kingdom), which measured both the ζ potential and particle size of the dispersed particles and was equipped with a 4-mW He–Ne laser operating at $l = 633$ nm, and with noninvasive backscatter (NIBS2, angle = 173°) optics (Worcestershire, United Kingdom). The autocorrelation function was analyzed with the Malvern dispersion technology software supplied by the manufacturer (NanoApplication.exe) to obtain the particle size. The surface and cross-sectional morphologies of the CAPEC films were assessed by scanning electron microscopy (SEM; JSM-6060LV, JEOL, Japan).

Methods

Preparation of CAPEC Dispersions. The CAPEC dispersions were prepared in the presence or absence of anions and/or Ca^{2+} ions. The chitosan solutions were prepared in 1% v/v acetic acid and allowed to stand overnight before use. The concentrations of chitosan and alginate in the resulting mixtures were 0.05% (w/v). The CAPEC dispersions were prepared by sonication at 60% amplitude. Alginate and salt solutions were added to the chitosan solution during sonication. After a sonication period of 90 s, the turbidity measurements were carried out at certain time intervals. The experimental details of the preparation processes of the CAPEC dispersions are represented in Table I.

In the experiments performed with ionic crosslinkers, the salts were used in equimolar concentrations to enable comparison. In the CAPEC syntheses carried out in the presence of binary crosslinkers, $CaCl_2$ was used in equimolar concentrations of the accompanying anions so that any competition between the cationic chitosan molecules and Ca^{2+} ions and between the alginate polyanions and the crosslinker anions could be minimized. The equimolar Ca^{2+} concentrations of the 1.0×10^{-3} mol/L NaPPhos, NaPhosMo, AmMo, NaTPP, and NaPyPhos were 7.0×10^{-3}, 1.5×10^{-3}, 3.0×10^{-3}, 2.5×10^{-3}, and 2.0×10^{-3} mol/L, respectively.

Preparation of the CAPEC Films. We prepared the CAPEC films by casting the CAPEC dispersions into glass Petri dishes and drying them to a constant weigh at 60°C. The concentrations of CMV (0.25% m/v), AMV (0.25% m/v), NaPPhos (2.0

Table I. Preparation of the CAPEC Dispersion in the Presence and Absence of Crosslinking Anions and Ca^{2+}

Experiment number	0.2% w/v CMV (mL)	0.2% w/v AMV (mL)	4.0×10^{-3} *M* salt (mL)[a]	Ca^{2+} in equivalent concentration (mL)	H_2O (mL)
1	10	10	—	—	20
2	10	10	10	—	10
3	10	10	10	10	—
4	10	10	—	10	10

[a] NaTPP, NaPyPhos, NaPhosMo, NaPPhos, or AmMo.

$\times 10^{-4}$ mol/L), and $CaCl_2$ (1.4×10^{-3} mol/L) were the optimized concentrations for the formation of the CAPEC film.[29]

Characterization Studies. The photon correlation spectroscopy technique was used to determine the particle size distribution and average particle sizes of the CAPEC dispersions. The ζ potentials of the dispersions were measured by the laser Doppler electrophoresis technique. The average particle size and ζ potential values are given as the mean values of three measurements of each sample made at 25°C. The FTIR and SEM analyses were performed with the CAPEC films. Before SEM observations, the samples were coated with a gold layer *in vacuo* for 120 s with a Polaron SC502 sputter coater (Fisons Instruments, United Kingdom).

RESULTS AND DISCUSSION

Formation of the CAPECs

The formation kinetics of the PECs between CMV and AMV were investigated by turbidity measurements. The variation of the turbidity of the dispersion of these CAPECs as a function of time is illustrated in all of the figures to enable comparison.

Comparison of the Effects of Different Crosslinker Systems on the CAPEC Gel Formation

The effects on the formation and stability of the CAPEC dispersions were examined for (1) the effect of the salt alone, (2) the effect of the salt in the presence of equimolar Ca^{2+}, and (3) the effect of equimolar Ca^{2+} alone. The results are illustrated in the same figure for each anion in Figures 1–5 to enable comparison.

Effect of the Anions on the Formation of the CAPEC Gel. The effects of NaPPhos, NaPhosMo, AmMo, NaTPP, and NaPyPhos salts bearing crosslinking anions on the gelation of CAPEC were studied.

NaTPP and NaPyPhos, which are the conventional salts used for the crosslinking of chitosan and CAPEC gels, enhanced the formation of the CAPEC gel almost to the same but small extents. On the other hand, NaPhosMo, AmMo, and NaPPhos, which were observed in our previous study[29] to be remarkably more effective salts in the crosslinking of CMV compared to NaTPP and NaPyPhos, exerted unexpectedly inhibitory effects on the CAPEC gel formation. Another observation was that AmMo led to the formation of CAPEC dispersions with poor stability (Figures 1–5).

Effect of Ca^{2+} on the Formation of the CAPEC Gel. Ca^{2+} is the most commonly used ionic crosslinker for CAPEC. However, there are no reports in the literature on the effect of the Ca^{2+} concentration on CAPEC gel formation. We examined this for

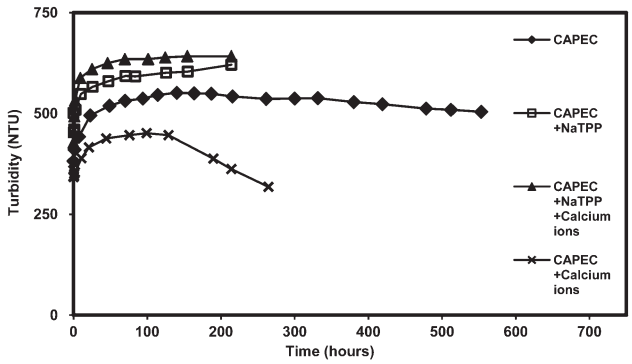

Figure 2. Effects of 1.0×10^{-3} *M* NaTPP, equivalent Ca^{2+}, and their binary mixture on 0.05% (m/v) CMV–0.05% (m/v) AMV PEC formation.

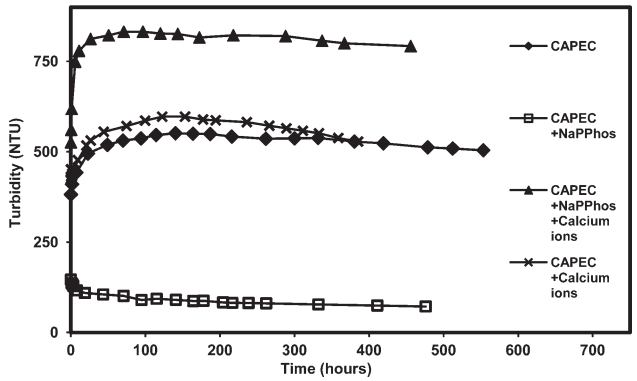

Figure 1. Effects of 1.0×10^{-3} *M* NaPPhos, equivalent Ca^{2+}, and their binary mixture on 0.05% (m/v) CMV–0.05% (m/v) AMV PEC formation.

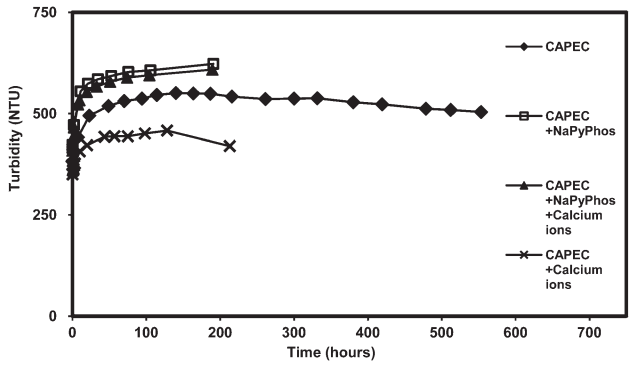

Figure 3. Effects of 1.0×10^{-3} *M* NaPyPhos, equivalent Ca^{2+}, and their binary mixture on 0.05% (m/v) CMV–0.05% (m/v) AMV PEC formation.

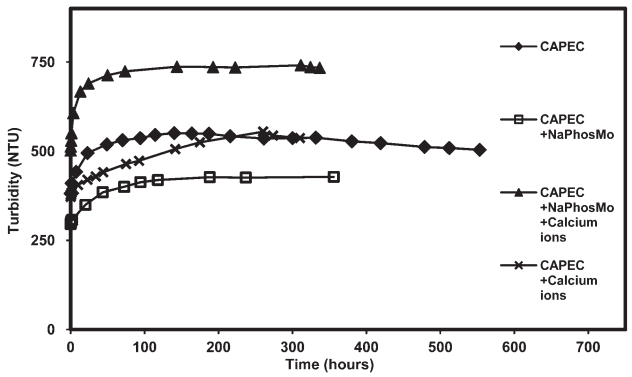

Figure 4. Effects of 1.0×10^{-3} *M* NaPhosMo, equivalent Ca^{2+}, and their binary mixture on 0.05% (m/v) CMV–0.05% (m/v) AMV PEC formation.

the first time in this study, and the results reveal that the mode of the Ca^{2+} effect on the crosslinking of CAPEC varied according to its concentration. Five different graphs were plotted of the turbidity results in the presence of equimolar amounts of Ca^{2+} with respect to 1.0×10^{-3} mol/L NaPPhos, NaPhosMo, AmMo, NaTPP, and NaPyPhos salts. These graphs are given in Figures 1–5. After inspecting these graphs in a comparative way, we concluded that Ca^{2+} had no effect or exerted an inhibitory effect on the formation of CAPEC at concentrations less than 7.0×10^{-3} *M*. Somewhat higher turbidity values were attained in the presence of 7.0×10^{-3} *M* Ca^{2+}. Another observation made from these graphs was that Ca^{2+} ions exerted a negative effect on the stability of the CAPEC dispersion when it was used alone.

Effects of the Anions on the Formation of the CAPEC Gel in the Presence of Ca^{2+} Ions. Figures 1–5 also illustrate the effect of crosslinker anions on the formation and stability of the CAPEC dispersion in the presence of equimolar Ca^{2+}. Graphs plotted in the presence of different anions accompanied by equimolar Ca^{2+} ions are given in separate figures specific for each anion to enable comparison.

Figures 1–5 revealed that the anionic crosslinkers NaTPP and NaPyPhos enhanced the formation of the CAPEC gels, whereas the other three exhibited an inhibitory effect when used alone. AmMo also led to the formation of unstable particles. However, surprisingly, when these three inhibitory salts (AmMo, NaPhosMo, and NaPPhos) were used in the presence of Ca^{2+}, they became remarkably more effective enhancers compared to NaTPP and NaPyPhos. On the other hand, the presence of Ca^{2+} did not markedly increase the effectiveness of NaTPP and NaPyPhos.

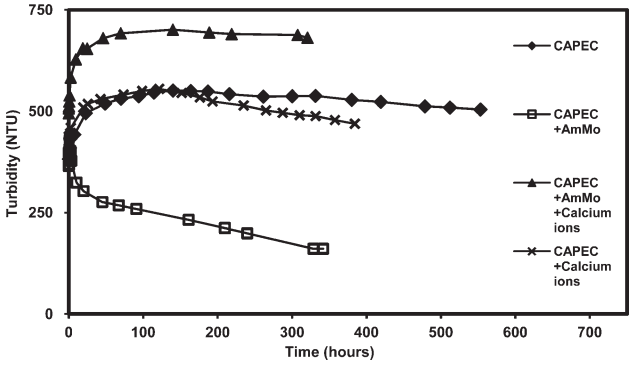

Figure 5. Effects of 1.0×10^{-3} *M* AmMo, equivalent Ca^{2+}, and their binary mixture on 0.05% (m/v) CMV–0.05% (m/v) AMV PEC formation.

It should be noted here that the NaPPhos–Ca^{2+} binary system unexpectedly constituted the most effective crosslinker for the CMV–AMV PEC; this was in contrast to the strong inhibitory effect of NaPPhos on the formation of this CAPEC when used alone. This revealed that the inhibitory effect of an anionic crosslinker on the formation of the CAPEC gel may be effectively eliminated and can even be reversed to an enhancing effect by the simultaneous use of Ca^{2+} ions.

Another conclusion we drew from the comparison of Figures 1–5 was that the simultaneous use of anionic crosslinkers and Ca^{2+}cations enabled the synthesis of high-stability CAPEC particles; that is, any stability problem, as experienced with AmMo, can be eliminated by the use of binary crosslinkers containing Ca^{2+} ions. Figures 1–5 provide evidence that the CAPEC particles with prolonged dispersion stability, which maintained their sizes for longer periods of time, could be synthesized with such binary crosslinkers.

Thus, it can be conclusively said that the simultaneous use of an anionic crosslinker with Ca^{2+} remarkably enhances the formation of CAPEC compared to the use of either anion or Ca^{2+} alone and, in addition, increases the stability of the CAPEC dispersion.

Characterization of the CAPEC Synthesized in the Presence of the Binary Crosslinker

FTIR Analyses. The formation of CAPEC in the presence of the NaPPhos–Ca^{2+} binary crosslinker was confirmed by FTIR analyses. The FTIR spectra of alginate, chitosan, CAPEC synthesized without any crosslinker, and CAPEC synthesized in the presence of the NaPPhos–Ca^{2+} binary crosslinker are given in Figure 6. We observed that spectral alterations occurred in the spectra of

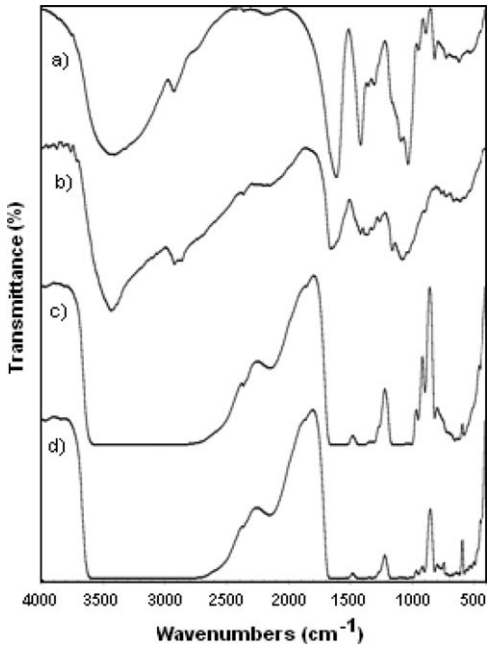

Figure 6. FTIR spectra of the (a) alginate, (b) chitosan, (c) CAPEC synthesized without a crosslinker, and (d) CAPEC synthesized in the presence of the NaPPhos–$CaCl_2$ binary crosslinker.

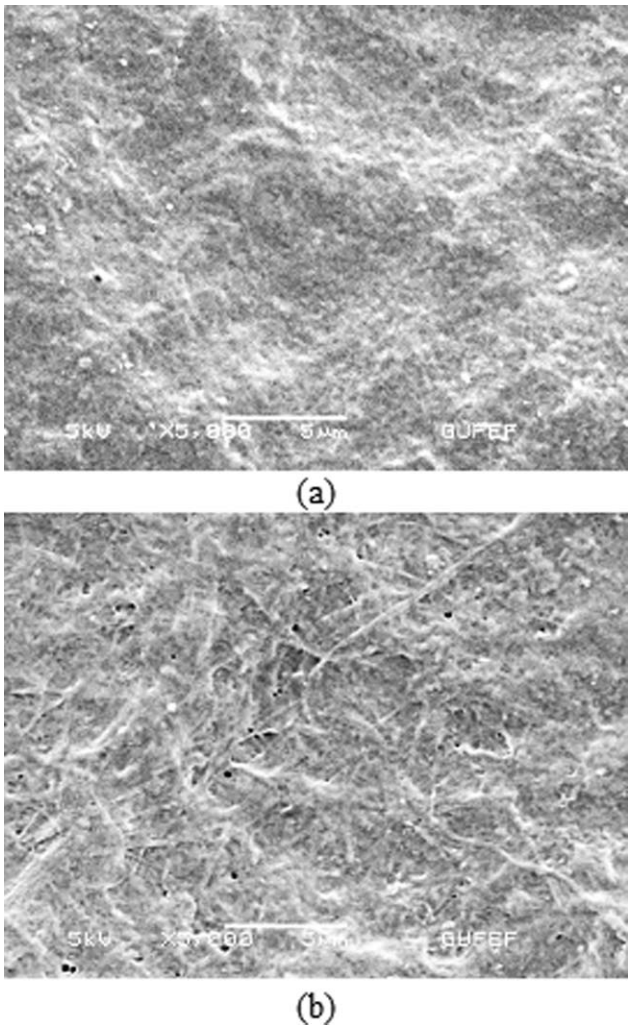

Figure 7. SEM images of the surfaces of the CAPEC films prepared in the (a) absence and (b) presence of the NaPPhos–Ca^{2+} binary crosslinker.

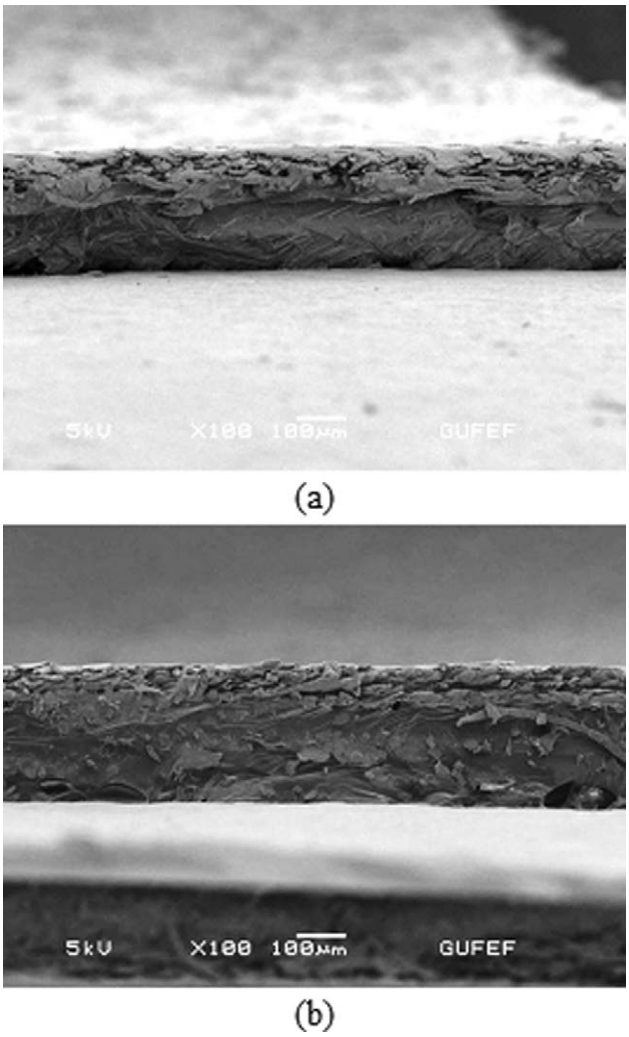

Figure 8. Cross-sectional SEM images of the CAPEC films prepared in the (a) absence and (b) presence of the NaPPhos–Ca^{2+} binary crosslinker.

chitosan and alginate on complexation.[18,30–34] By inspecting these spectra in a comparative way, we concluded that CAPEC was formed both in the absence and in the presence of the binary crosslinker.

ζ Potential and Size Analyses of the CAPEC Particles. The particles of the CAPEC dispersion prepared in the presence of the NaPPhos–Ca^{2+} binary crosslinker were found to have an average ζ potential of −14.7 mV. This implied that the CAPEC particles bore a negative charge high enough to render the dispersion stable. In an additional experiment, the average ζ potential of another CAPEC dispersion, prepared in the presence of NaPPhos and Ca^{2+} at two times lower concentrations compared to their concentrations in Table I, was measured as −14.3 mV. This result indicates that the concentrations of NaPPhos and Ca^{2+} could be lowered to about half their values without a decrease in the stability of the resulting CAPEC dispersion.

The average particle size of the CAPEC dispersion (Table I) was determined as 1917 nm with a relative standard deviation of 24.7%.

SEM Analysis of the CAPEC Films. The surface and cross-sectional morphologies of the CAPEC membranes prepared in the presence and absence of the NaPPhos–Ca^{2+} binary crosslinker were studied by SEM analyses and compared.

Figure 7 illustrates the SEM images of the surfaces of these films. From the comparison of Figure 7(a) and 7(b), we concluded that the film prepared in the presence of the binary crosslinker had a flat surface morphology and a more uniform and compact structure compared to the film prepared without the binary crosslinker.

Cross-sectional SEM images of these CAPEC films are given in Figure 8. Figure 8 reveals that the film prepared in the presence of the binary crosslinker was thicker and had a firmer structure compared to that of the film prepared in its absence. The latter looked more fragile at its cut surface.

CONCLUSIONS

The results of this study show that the functionality of anionic crosslinkers is closely related to their own structures. NaPhosMo, AmMo, and NaPPhos, which are more effective salts in

the crosslinking of chitosan compared to NaTPP and NaPyPhos, exerted an inhibitory effect on the CAPEC gel formation, whereas NaTPP and NaPyPhos exerted enhancing effects.

The drawbacks of the use of Ca^{2+} ions alone, which are the most common ionic crosslinkers for CAPECs, were pointed out with the results of this study. The mode of the effect of Ca^{2+} on the crosslinking of CAPECs depended on the Ca^{2+} concentration. Ca^{2+} at concentrations lower than 7.0×10^{-3} *M* exerted an inhibitory effect. Also, the use of Ca^{2+} ions alone, as applied conventionally, resulted in a decreased colloidal stability; that is, Ca^{2+} addition decreased the stability of the CAPEC sol.

The results revealed that the use of binary mixtures of anionic–cationic crosslinkers promoted the enhancing effects of anionic crosslinkers on the formation of PEC gels. In addition, the inhibiting effect of an anionic crosslinker on the formation of the CAPEC gel was reversed to an enhancing effect by the simultaneous use of Ca^{2+} ions.

Molybdate-ion-bearing anions, namely, NaPhosMo and AmMo, and NaPPhos inhibited the formation of CAPECs. On the other hand, binary crosslinkers consisting of one of these anions and Ca^{2+} enhanced the formation of CAPECs.

One of the main findings of this study was that the simultaneous use of anionic crosslinkers and Ca^{2+}cations increased the stability of the CAPEC dispersion.

NaPPhos–Ca^{2+}, NaPhosMo–Ca^{2+}, and AmMo–Ca^{2+} binary systems were more effective crosslinkers in the preparation of CAPEC gel particles than the binary mixtures of NaTPP and NaPyPhos with Ca^{2+}. The most effective of these was the NaPPhos–Ca^{2+} system, even though NaPPhos alone inhibited the formation of CAPECs. This binary crosslinker is strongly suggested as a superior alternative to both NaTPP and Ca^{2+}, which are the most commonly used ionic crosslinkers in preparation of CAPEC microparticles and nanoparticles.

SEM studies revealed that the film prepared in the presence of the NaPPhos–Ca^{2+} binary crosslinker was thicker and had a firmer and more uniform structure than the film prepared in its absence.

ACKNOWLEDGMENTS

The financial support of the Gazi University Scientific Research Fund is gratefully acknowledged. The authors also thank Prof. Dr. Zekiye Suludere for her very kind and helpful cooperation during the SEM analyses.

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