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Facile, Shear-Induced, Rapid Formation of Stable Gels of Chitosan through *In Situ* Generation of Colloidal Metal Salts

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A novel method of preparing chitosan gels using *in situ* generated negatively-charged colloidal salts of a variety of metal ions is described. Their potential as scaffolds for tissue-engineering and as recoverable catalysts in aza-Michael addition is demonstrated here. Given their wide range of properties, they have broad scope for applications.

Crosslinking of chitosan, from its solution, is fundamental to the preparation of hydrogels, scaffolds, drug-delivery vehicles etc.^{1,2} Conventionally, chitosan is crosslinked through chemical,^{3–5} physical^{6–13} or coordination complexation^{14–16} routes. Chemical crosslinking often involves the use of toxic reagents, which limits its usage. Physically crosslinked systems, such as those formed by ionotropic interactions between chitosan cation and polyvalent anions like sodium tripolyphosphate, lack precursor versatility and suffer from poor mechanical and environmental stability (pH, temperature and ionic strength), leading to the risk of dissolution, due to their pH sensitive swelling.¹⁷ Similarly, crosslinks based on metal-organic complexes are often toxic¹⁸ and prone to exchange metal ions with the surroundings¹⁴, restricting their applications.

The preparation of chitosan gels with good environmental stability, wide range of mechanical properties and chemical reactivity may have profound and broader applicability. In this work, such gels of chitosan were prepared by a novel approach involving generation of colloidal metal salts, *in situ*, in chitosan solution. Surprisingly, gelation did not occur with all colloidal metal salts, but was restricted to those of Al³⁺, Fe³⁺, Cu²⁺, V²⁺, Cr³⁺, Ni²⁺ or the rare-earths, Ce⁴⁺, Nd³⁺, Pr³⁺, Y³⁺, Eu³⁺, Sm³⁺, Gd³⁺, La³⁺, Tb³⁺ and the like, among those screened. Among these gels, ferric-based gel, in particular, showed very low cytotoxicity and a distinct potential for application as scaffolds in tissue engineering. Another gel, based on cerium, could be

utilized as a recoverable catalyst for aza-Michael addition. The photograph of various chitosan-based gels, prepared by *in situ* precipitation of colloidal metal phosphates, is shown in figure 1. Among the many possible variations of the anions (phosphates, hydroxides, carbonates, sulphates, sulphides, fluorides, chromates, bicarbonates etc.), gelation by *in situ* generation of metal phosphates was chosen as the model reaction for detailed studies.

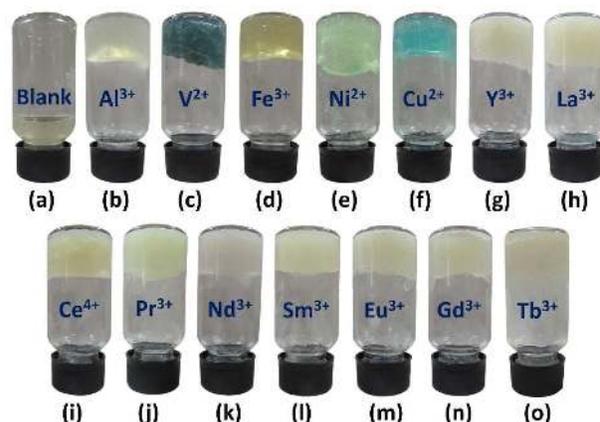


Figure 1. Photographs of (a) chitosan solution and (b-o) gels prepared by precipitation of phosphates of various metals.

The rheological properties of the gels were determined using a parallel plate rheometer. From the steady shear rheological measurements, all the gels were found to be of “Herschel-Bulkley” nature, exhibiting a non-linear pseudo-plastic flow with a yield stress. However, the final properties of the gel were strongly dependent on the choice of metal salts and the molar ratios of the starting materials used (Table S1; Figures S1-S2). The viscoelastic nature of the gels was further confirmed using dynamic shear rheological measurements, wherein the storage modulus was observed to dominate the loss modulus by almost an order of magnitude in all the gels. Among the gels studied, those based on Ce⁴⁺, Fe³⁺ and Y³⁺ exhibited a high shear modulus, for all the compositions. In

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comparison to gels prepared by ionotropic crosslinking using sodium tripolyphosphate (TPP) and sodium pyrophosphate (PPi),¹⁹ the shear modulus was two orders of magnitude higher for the Ce⁴⁺ based gels, whilst having a lower solid-content (Figures S3-S5). This is, probably, the strongest ionotropic gel of chitosan reported, till date. Al³⁺ based gel, on the other hand, showed poor mechanical properties. Gels based on the lanthanides and other transition metal ions gave an intermediate modulus (Figures S6-S7). Some of the rheological properties of Ce⁴⁺, Fe³⁺, Al³⁺, TPP and PPi based gels are presented in table 1.

Table 1. Rheological properties of Ce⁴⁺, Fe³⁺ and Al³⁺ based gels

Gel system	η_0 (kPa.s) ^[a]	G' (kPa) ^[b]	Shear Stability (% strain) ^[c]	Solid Content (%) ^[d]
Ce ⁴⁺	293.6	208.52	13.17	4.99
Fe ³⁺	0.17	1.03	114.17	1.99
Al ³⁺	12.78	0.16	51.33	3.25
TPP ¹⁹	-	2.48	-	~12 ¹⁹
PPi ¹⁹	-	6.87	-	~11-12.5 ¹⁹

[a] Viscosity at zero shear; [b] Storage (G') and loss moduli (G''), obtained at $\omega = 1$ Hz; [c] Stability under oscillatory shear, determined at $\omega = 1$ Hz; [d] Measured using isothermal TGA at 100 °C;

These gels were very stable under low strains, typically 0.1-10 %, while at high strains, the gels lost their stability. Surprisingly, gels based on transition metal ions, gave very high shear stability (Figure S8). Shear stability was the highest for Cr³⁺ based gel (364 % strain), although its shear strength was poor. Interestingly, this strain-induced gel-sol transition was reversible in nature. When the high strain was removed, the sols returned to the initial gel state. This thixotropic behavior was further confirmed using the strain-recovery measurements with alternating low strain ($\epsilon = 0.1$ %) and high strain ($\epsilon = 100$ %) cycles. A full recovery of the gel network, within few seconds of reducing the shear strain, was observed. These recovery cycles could also be repeated many times, without any notable reduction of the average magnitude of G' and G'', confirming the remarkable and rapid thixotropic behavior. Also, when the gel was cut into two halves and again brought into contact with each other, they fused back to give a single continuous solid. This indicated the "self-healing" nature of the gel.²⁰ The self-healing property probably arises from reformation of the electrostatic interactions between the exposed chitosan polycations and colloidal metal phosphate particles, across the interface.²¹ The step stress-strain plots are presented in figure 2 (a), while the photographs showing the self-healing nature of the gel are presented in figure 2 (b). Apart from the mechanical properties, gelation time, in particular, had a very strong dependence on the molar ratio of phosphate to the metal salt (Table S2; Figures S9-S14). Increase in the molar ratio, increased the rate of formation of metal phosphates and thereby, reduced the gelation time. However, beyond a loading percentage, addition of phosphate salt led to poor mechanical properties. Although gelation

happened without any shear, over a very long residence time of 6-8 hours, shear brought the reactants in close proximity to each other in a very short time, allowing instantaneous gelation to occur. The time sweep for the Ce⁴⁺ based gel is presented in figure 2 (c). The gel-time was observed to be 41 s, even at low strains ($\gamma = 10$ %). At higher strains, however, the gel-time was unmeasurable, due to spontaneous gelation. In addition to mechanical stability, these gels were stable at all temperatures and pH.

The mechanism of gelation was probed using multinuclear NMR, zeta potential, ESR, pH metry, WAXS and TGA studies. The formation of the metal phosphates, *in situ*, was confirmed using ³¹P NMR spectroscopy, wherein a new peak between 0 and -20 ppm was observed, in all the gels.²² In the presence of chitosan, this phosphate peak underwent downfield shift, indicating interactions between chitosan and the metal phosphates. This shift was very prominent in the case of Al³⁺ based gel, in which the phosphate peak was deshielded by almost 10 ppm (Figures S15-S21). This complete shift of the phosphate peak, rather than the appearance of a new peak, indicates that the chitosan-colloid interactions dominate the bulk interactions in the colloid. This could, in turn, be due to the precipitation of very fine colloids of metal salts, when generated, *in situ*, in chitosan solution. ¹³C NMR spectroscopy showed that the chemical structure of chitosan was partially modified upon gelation with significant upfield shifts for C1, C4 and C6 carbons of chitosan, suggesting that the phosphate group could be located in the space around C1, C4 and C6, while electrostatically binding with the NH₃⁺ at C2 (Figures S22-S25).

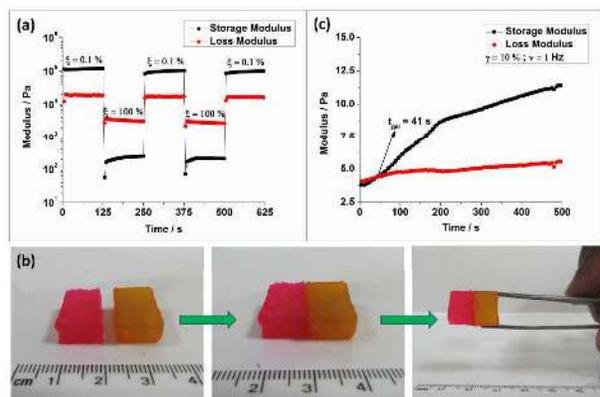


Figure 2. (a) step-stress strain curves for the Ce⁴⁺ based gel at $\epsilon = 0.1, 100$ %; (b) photographs showing the fusion between the two cut halves of Ce⁴⁺ based gel (For contrast, the two halves were individually stained with rhodamine B and fluorescein dyes, respectively); (c) Time sweep ($\gamma = 10$ % and $\omega = 1$ Hz) showing the gelation time for the Ce⁴⁺ based gel.

It is, hence, clear from the NMR studies that the presence of chitosan does not affect the formation of metal phosphates. Hence, gelation occurs only as a result of interactions between chitosan and the surface of the colloidal metal phosphates, generated *in situ*. In order to study the near-surface

characteristics of the metal phosphate colloids, zeta potential measurements were performed. Interestingly, all the metal phosphates, which produced gels, showed a zeta potential greater than -15 mV (Table S3). Electrostatic interactions between these negatively charged, stable colloidal dispersions of metal phosphates and chitosan polycation are, hence, the primary cause of gelation. Noteworthy, the strength of the gels strongly depended on the magnitude of zeta potential. Metal phosphates which showed higher zeta potential values, gave good gel strength, in general. Further evidence of the mechanism involved arises from the interactions between *ex situ* prepared metal phosphate colloidal dispersions and chitosan solution. Upon the mixing of the two, flocculation was observed that could be due to the electrostatic interaction between chitosan and the negatively charged colloids. The ^{13}C and ^{31}P NMR spectra for cerium phosphate and the Ce^{4+} based gel are presented in figures 3 (a) and (b), respectively, while the zeta potential values are presented in figures 3 (c).

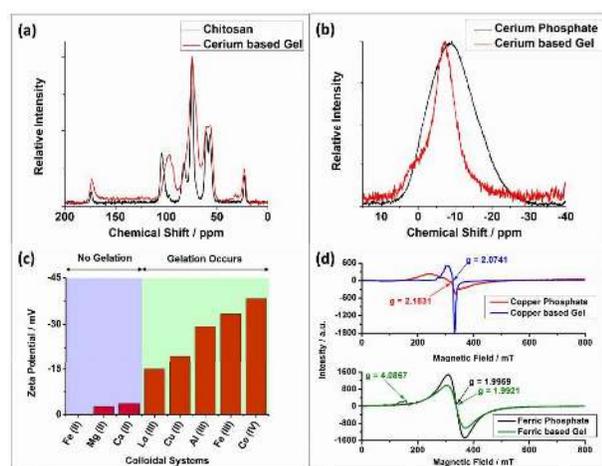


Figure 3. (a) ^{13}C NMR spectra and (b) ^{31}P NMR spectra for chitosan and the Ce^{4+} based gel; (c) Zeta potential (mV) values for few *ex situ* prepared colloidal metal salts; (d) ESR spectra for Cu^{2+} and Fe^{3+} based systems.

The role of metal ions in the gelation process is complex, however. Although ^{27}Al NMR of the Al^{3+} based gel and *ex situ*-prepared aluminum phosphate did not show any difference, indicating that Al^{3+} ions did not play any active role in the gelation process, the same may not be applicable for the other metal ions, especially those of transition metals, whose complexation with the free amino groups in chitosan has been well established (Figures S26-S27).²³ Interestingly, ESR spectroscopy showed that upon gelation, the *g* factor of colloidal Cu^{2+} decreased from 2.18 to 2.07. This change indicates that chitosan acts as a ligand for colloidal Cu^{2+} , forming a complex through its free amine groups. Given the high molar ratio of HPO_4^{2-} anions to Cu^{2+} cations utilized for gelation, it is unlikely that Cu^{2+} ions still exist in solution. The coordination complexation is, thus, likely to arise between chitosan and copper ions on the surface of the colloids. Likewise, in case of Fe^{3+} based systems, a new peak with a *g*

factor of 4.09 appeared, suggesting a similar chitosan-metal complex. However, in the case of other transition metal ions like Mn^{2+} , Zn^{2+} , Cd^{2+} etc., all of which are known to form complexes with chitosan²⁴, gelation did not occur altogether. Thus, these chitosan-metal interactions do not appear to be instrumental in the gelation process and only occur parallel to the gelation process. At this point it is unclear whether these interactions contribute to the strength of the gels. In addition, pH metric studies showed that, upon gelation, the pH remains unmodified, confirming that the gelation does not occur due to precipitation of chitosan (Figure S28-S29). The ESR spectra of Cu^{2+} and Fe^{3+} based systems are presented in figure 3 (d). The extensive crosslinking, brought about by the colloidal particles, prevented any reorganization of the chitosan chains, even after drying or alkali treatment. This led to an amorphous solid, as confirmed by WAXS profiles (Figures S30-S31). The loss in the inter- and intramolecular H-bonding was also evident by TGA measurements, which showed a drastic reduction in the decomposition temperature of the chitosan chains (Figures S32-S35). The WAXS profiles and the thermograms for chitosan, cerium phosphate and Ce^{4+} based gel are presented in figures 4 (a) and 4 (b), respectively.

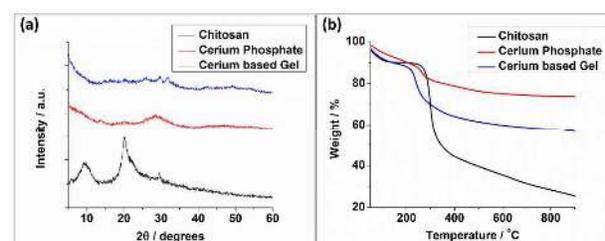


Figure 4. (a) WAXS profiles and (b) TGA thermograms for chitosan, cerium phosphate and Ce^{4+} based gel

The morphology of the *ex situ* generated colloidal metal phosphates was interconnected with quasi-spherical particles of average sizes exceeding 200 nm (Figures S36-S41). However, *in situ* generated colloidal particles were very fine colloidal dispersions. The elemental homogeneity observed with EDS mapping and the absence of any quasi-spherical structures in the freeze-dried gels, further confirm the small-size of the colloidal particles (Figures S42-S46). Surprisingly, freeze-drying of the gels gave a hierarchical structure, composed of many flat layers stacked one over the other. These multilayered structures are likely to be *Liesegang* bands, formed during the gelation process (Figure S47).^{16,25} Although not visible in the gel state, freeze-drying might have introduced shrinkage, leading to a very distinct separation of the boundaries. The formation of these *Liesegang* bands could be explained by the supersaturation theory, according to which precipitates are not immediately formed upon intermixing of two reactants at the interface, but a region of supersaturation is first formed. Cytotoxicity of the Fe^{3+} based gel was evaluated using MTT assay.²⁶ Both the non-neutralized and the neutralized gels gave very low cytotoxicity (88 and 93 % cell viability, respectively; after 72 h, at a concentration of 1 mg/ml) for NIH 3T3 mouse fibroblast cells, very similar to that of chitosan (93 % cell

viability; after 72 h, at a concentration of 1 mg/ml) (Figures S48–S52). Also, the cell viability increased with incubation time, suggesting the proliferation of cells. Under SEM as well, the cells were observed to attach to the scaffold and proliferate significantly, without affecting the morphology. Further, fluorescence microscopy of the DAPI stained scaffolds confirmed nuclear integrity (Figure S53–S54). Given their low cytotoxicity, these gelling systems may also be used as tissue engineering scaffolds, either preformed or *in situ* gelling. The MTT results, fluorescence and SEM micrographs, showing the attachment and proliferation of the live cells, are presented in Fig. 5. In addition, the neutralized gels were observed to be antimicrobial towards the human pathogens- *Staphylococcus aureus* and *Escherichia coli*, while the non-neutralized gels did not suppress the growth (Figures S55–S57, Tables S4–S7). Contrary to the prevalent understanding, free amine groups were found to be central to the antimicrobial activity.²⁷

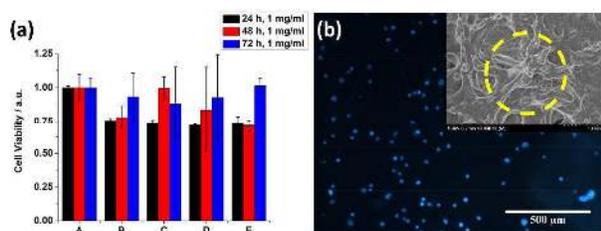


Figure 5. (a) In vitro cytotoxicity of A-TCP, B-chitosan, C-non-neutralized Fe³⁺ based gel, D- neutralized Fe³⁺ based gel, E- ferric phosphate, obtained by MTT assay; (b) Fluorescence micrographs showing the nuclear integrity of attached cells; (inset-SEM micrograph showing the attachment and proliferation of NIH 3T3 mouse fibroblast cells.)

These “metallogeles” could also be utilized as recoverable heterogeneous catalysts for various organic transformations. As an example, aza-Michael addition²⁸ between α , β -unsaturated carbonyl compounds and amines was studied. Reaction between the model reactants, acrylamide and diallylamine, could be efficiently catalyzed using cerium-based gel, at room temperature, to produce 3-(diallylamino)propanamide in high yields (Figures S58–S64). The cerium-based gels could be then extracted by filtration, washed and reused.

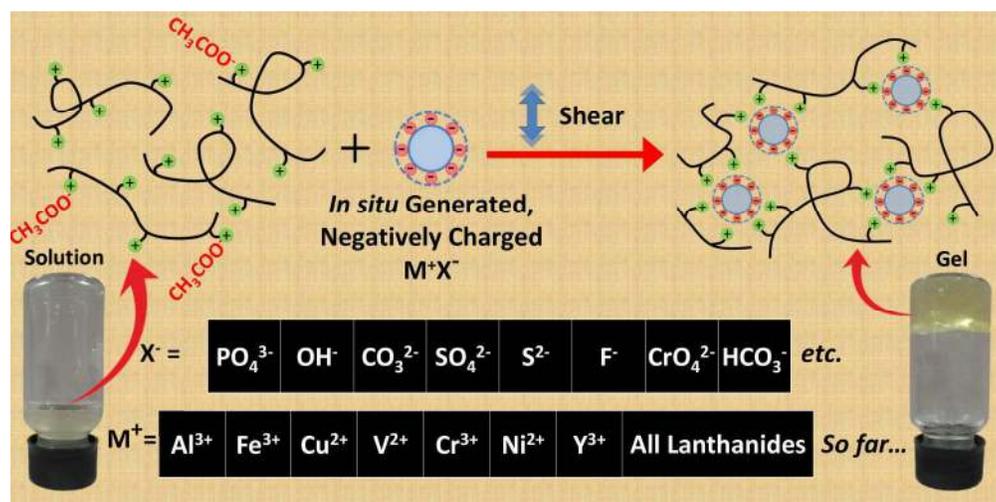
In summary, a wide variety of ionotropic gels of chitosan were successfully prepared by *in situ* generation of colloidal metal salts in chitosan solution. Gelation primarily occurred due to the electrostatic interactions between the chitosan polycation and the negatively charged surface of the colloidal metal salts. These gels showed a wide range of mechanical properties and environmental stability, whilst having a self-healing nature. The antimicrobial nature and low cytotoxicity of some of these gels could be suitable for application as injectable or preformed scaffolds in tissue engineering. In addition, these gels could be used as recoverable catalysts in various organic transformations.

Conflicts of interest

There are no conflicts to declare.

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