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Review Article

CHEMICAL MODIFICATIONS OF ALGINATE AND ITS DERIVATIVES

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ABSTRACT

Alginate is a polysaccharide obtained from seaweeds that are abundantly available and have shown great potential for diverse industrial applications. However, alginate lacks properties such as stability under aqueous conditions and it is difficult to control the rate of degradation of alginate-based materials, crucial for various medical applications. Therefore, researchers have modified alginate using physical or chemical approaches to enhance physical properties, biocompatibility, solubility and also to control the biodegradability of alginate-based materials. Crosslinking using ionic, covalent, photo and enzymatic approaches are one of the preferred methods for modifying the properties of alginates and its derivatives. Crosslinking binds the individual polymer chains with one another to form a network that enhances mechanical properties and stability. Among the different crosslinking approaches, ionic crosslinking provides biomaterials with limited stability whereas biomaterials with high mechanical stability can be prepared by covalent crosslinking. Although a wide variety of crosslinking chemicals and approaches are available to make alginate suitable for various applications, the methods used, properties and applications of the cross-linked materials vary significantly between studies. There are very few reports that have compared and evaluated the benefits of using different crosslinking approaches and the properties and applications of cross-linked alginate. In this review, the various methods of crosslinking alginates, their advantages, and limitations have been reviewed with particular emphasis on medical applications of alginate. The data for writing the review was obtained using search engines like Google scholar, Sci-hub and Sci finder and the keywords used include alginate, crosslinking, ionic, covalent, photo, enzymatic, biomedical applications.

Keywords: Alginate, Crosslinking, Ionic, Covalent, Photo, Enzymatic, Biomedical applications

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INTRODUCTION

Biomaterials are defined as materials that interact with biological systems to supplement or restore any tissue, organ or functions of the body [1]. Natural sources like cellulose, starch, and proteins have regularly been used in biomedical applications (e. g., tissue engineering scaffolds, sutures, and prosthetics). Although natural materials are used in medical applications, these materials have several limitations such as weak mechanical properties, limited resistance to aqueous conditions and constraints to be made into various shapes. Compared to natural materials, artificial polymers, ceramics, and metal alloys provide better performance and reproducibility [2-4]. Although synthetic materials can provide exceptional mechanical properties and can be made to specific requirements, these materials are non-biodegradable and in many instances cause cytotoxicity and immunological reactions.

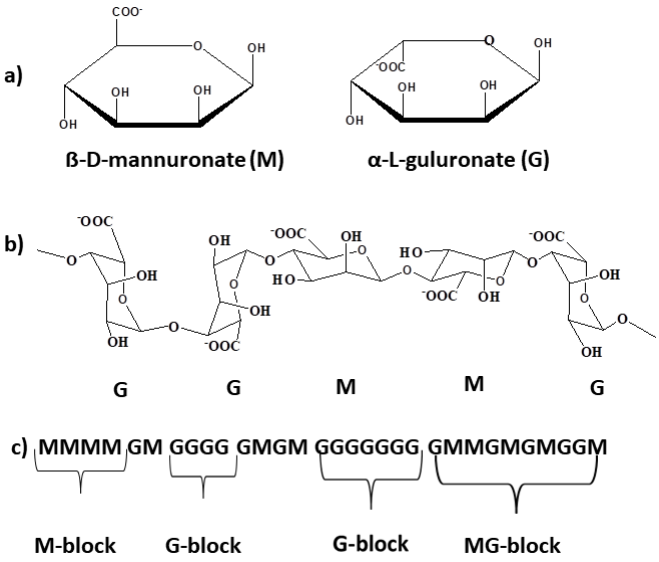

Fig. 1: (a) Schematic representation of alginate showing the structure of mannuronate (M) and guluronate (G) respectively. 1(b) shows the chair conformation 1(c) shows the sequence of M block and G blocks arrangement in alginate [13]. Reproduced with permission from wiley

Alginate is a naturally occurring anionic polysaccharide which is known to be the structural constituent of marine brown algae *Phaeophyceae* and *Quatrano.* It can be extracted from both bacterial and algal species and hence, the composition, yield and other parameters of alginate rely on the species, source of the copolymer used and the method of extraction employed [5-8]. As a biopolymer, alginate is non-immunogenic, non-toxic, biocompatible and biodegradable. Under natural conditions, alginate exists as the salts of sodium, calcium, magnesium, etc (Na^+, Ca^{2+}, Mg^{2+}, $Sr^{2+}$and $Ba^{2+}$of alginic acid) [9, 10]. In terms of structure, alginates are unbranched binary copolymers that are linked by β-D-mannuronic acid (M) in addition to α-1-guluronic acid (G) residues. Alginate is also considered to be a block copolymer consisting of orthomopolymeric regions of M and G termed M-and G-blocks, respectively, interspersed with regions of the alternating arrangement of (MG) block (fig. 1). The properties of alginate from a particular seaweed are dependent on both the M/G ratio and the block distribution within an alginate molecule [11]. Further, the monomer sequence (M and G) can also vary between different algal species [12].

In terms of applications, alginates are used in most industries, predominantly agri-food, textiles, cosmetic, medical and pharmaceutical [14, 15]. Since it is very convenient to prepare alginate biomaterials at low pH and temperature necessary for the encapsulation of sensitive biomolecules like proteins and nucleic acids, there is a high potential to develop new biomaterials based on alginates [16, 17]. However, there are several limitations that restrict the application of alginate. Hence, there is a trend to develop "value-added" alginates, by performing derivatization reactions on the polysaccharide's backbone.

There are plenty of free hydroxyl and carbonyl groups spread all along the polymer backbone and hence alginate provides a good opportunity for various chemical modifications. Modification of alginate can be performed using various approaches such as chemical bonding with synthetic biopolymers, coating the surfaces of alginate with micro-or nanospheres made from biocompatible artificial polymers, crosslinking with physical or chemical reagents, hydrophobization, a variation of guluronic/mannuronic ratio, etc. Further, chemical methods can be combined with biochemical techniques if required. This, in turn, allows for the alteration of alginate properties such as solubility, affinity for specific proteins, hydrophobicity etc. Before choosing any modification, solubility and reactivity are the two key parameters that should be considered. Alginates can dissolve in aqueous, organic or mixed organic solvents. Table 1 indicates the solubility of alginates in different solvents when the concentration of alginate was 15 mg/ml [18]. Although alginates dissolve in various solvents, the type of solvent used for derivatization should be chosen carefully since the type of solvent affects the properties and the substitution pattern [19].

Table 1: Solubility of alginate in different solvents at a concentration of 15 mg/ml [18]. Reproduced with permission from American chemical society

	H_2O	EG	DMAc	DMF	DMSO	DMAc/ LiCl	DMF/ TBAF	DMSO/ TBAF	DMAc/ TBAF	DMI/ TBAF
Alginic Acid	_	_	_	_	_	_	_	_	_	_
Na-alginate	+	_	_	_	_	_	_	_	_	_
TBA-alginate	+	+	_	_	_	_	+	+	+	+

(+) completely soluble and (-) partially soluble or insoluble; EG-Ethylene Glycol; DMAc–Dimethyl acetamide; DMF-Dimethyl formamide; DMSO-Dimethyl sulphoxide; DMI-1,3-Dimethyl-2-imidazolidinone; TBA-tetrabutylammonium; TBAF-tetrabutylammonium fluoride

Further, modification of alginates is not only dependent on the solvent but also on the location of the carbon and substituent groups. The two secondary OH positions (C_2 and C_3) or at the COOH C_6 position can be used for modification. In addition, the reactivity of the alginates is reliant on the type of functional groups and very selective to the type of chemical modification. Variation in reactivity between the functional groups can be an advantage to alter alginate selectively for specific applications.

Among the various chemical/physical modifications of alginate, crosslinking has been widely used due to the flexibility, ability to achieve desired properties, convenience, and relatively low cost. Fig. 2(a) shows the various methods of crosslinking alginate and examples of reagents used. In this review, we provide a comprehensive overview of the various types of alginate crosslinking. The approaches adopted facilitated the improvement of the properties of the alginate. The advantages and limitations of the various crosslinkers used to improve the properties of alginate have been discussed. Under the different crosslinking approaches, we have discussed the various forms of alginate cross-linked as separate sections. Critical evaluation of the pros and cons of each method of crosslinking is also provided at the end of each section.

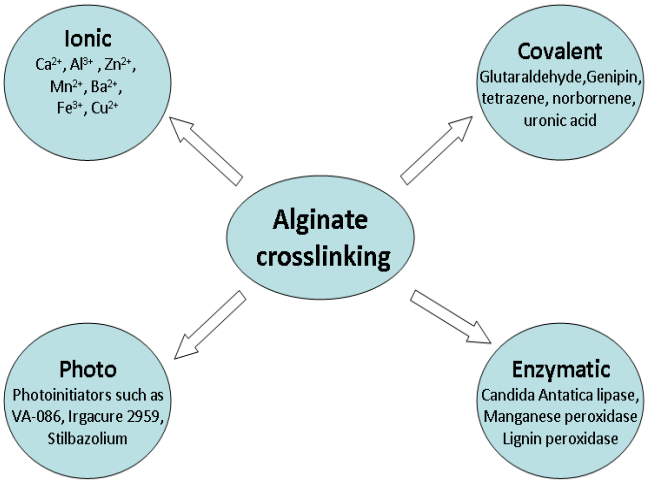

Fig. 2(a): Schematic depiction of methods of crosslinking alginate

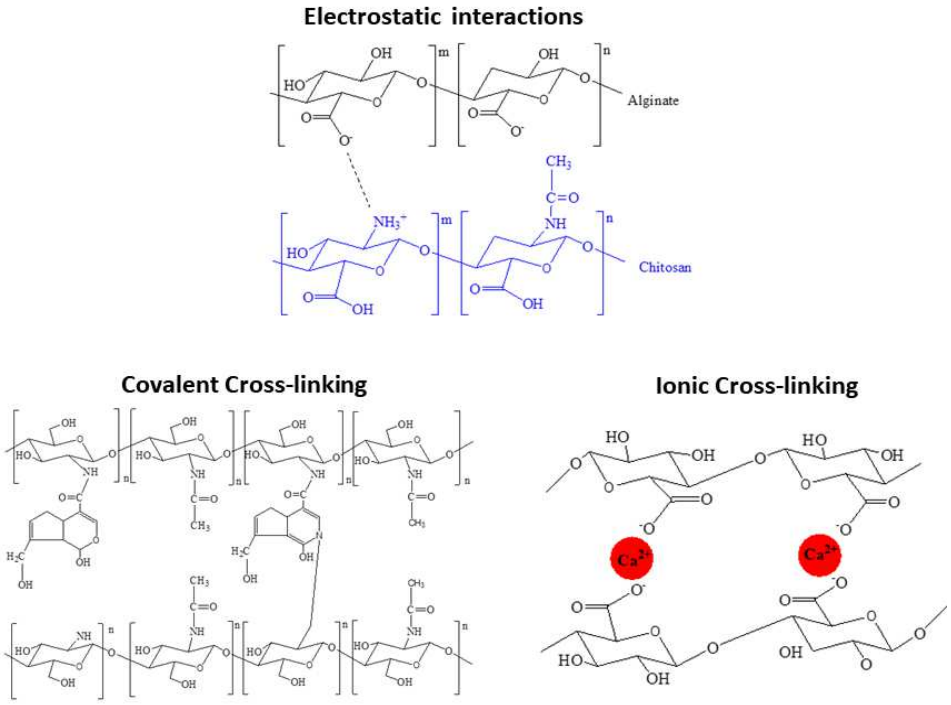

Fig. 2(b): Scheme showing the electrostatic interaction taking place during the synthesis of Chitosan-Alginate membranes along with their multilayer rearrangements ahead of covalent and ionic cross-linking [20]. Reproduced with permission from the royal society of chemistry

Ionic crosslinking

Ionic crosslinking is one of the simplest and most straightforward methods of crosslinking alginate. Ionic crosslinking has been used to develop alginate films, fibers, hydrogels, nano, and microparticles. In the presence of cations, G blocks present in alginate chains tightly associate at the junctions. Alginates that possess high G lead to stronger gels. However, the affinity of alginate also plays a major role in determining their crosslinking efficiency. The affinity constant for gelling gives us an overview of the spatial homogeneity of the crosslinking procedure together with information concerning mechanical stability. Reduction in the affinity of alginates towards divalent ions was found to be in the order: Pb>Cu>Cd>Ba>Sr>Ca>Co, Ni, Zn>Mn [21]. Although calcium does not possess the highest affinity, it is the most preferred divalent cation used to crosslink alginate. Alginate crosslinking by calcium can be done using 2 approaches, the diffusion technique, and the internal setting technique. The former process involves diffusion of alginate solution from outside reservoir whereas, the pH or the solubility of ion source helps in the release of ions in the latter method. The carboxylate groups that are negatively charged create the largest section of titratable sites along the alginate backbone and are the key to crosslinking and also to metal binding mechanisms. Regions of alginate chains loaded with G subunits form zigzag structures with cavities of appropriate size having specificity to Ca^{2+} due to their geometric order. Many oxygen atoms (darkened circles) also participate in this arrangement (fig. 3). Since Ca^{2+}aligns itself in the guluronate block structure, like eggs in an egg box, this arrangement is called an "egg-box" model shown in fig. 3. Such an arrangement provides high efficiency and the desired level of crosslinking.

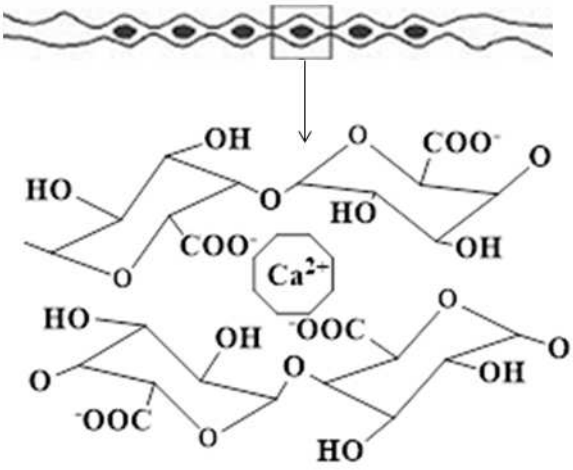

Fig. 3: Schematic representation of the egg box model for the crosslinking of alginate [22]. Reproduced with permission from Elsevier

Films

To enhance aqueous strength and mechanical properties, Cathell used an aerosolized spray of $CaCl_2$ solution along with EDTA as the calcium chelator for producing highly cross-linked alginate [23]. This procedure allows for controlled exposure of the polymer to the calcium-containing solution. Even after 30 min of immersion, the films that were cross-linked did not demonstrate any significant change in dimensions which proved that the films had excellent water stability and were strongly resistant to aqueous related thickness changes.

To evaluate the efficiency of crosslinking, the refractive index (RI) and thickness of cross-linked alginate films were measured after exposing them to various metal ion solutions. Table 2 shows the changes in the refractive index that occurs when calcium alginate films are exposed to metal ion solutions. Thickness changes in films were prominent for chromium. Hexavalent chromium caused considerably larger change (-19.1 nm) in thickness whereas trivalent chromium caused a positive change of 14.7 nm suggesting that the interaction of the two valencies of chromium with alginate films differs from one another. A similar trend was seen for lead-containing alginate films also. A maximum RI of 0.057 was seen for lead acetate compared to 0.045 for lead nitrate. This difference was considered to be the reason for the moderate change in the physical dimension of films but a relatively large shift in optical properties. The changes in reflectance of the alginate films were observed upon immersion in 50 ppm ionic solutions. Modifications in the optical properties were due to the reaction between the lead cation and alginate. The ability to control the optical properties are considered to be desirable in sensing an analyte in liquid media [24, 25].

Table 2: Changes in the thickness and RI (Δn) of alginate films after immersing in 50 ppm ionic solutions [23]. Reproduced with permission from American chemical society

50 ppm solution	Δ(thickness) (nm)	Δn	Reflectance max shift (nm)
Chromium(VI) oxide	**-19.1±1.0**	-0.002±0.007	**-46.6±0.4**
Mercury(II) nitrate	**7.2±0.6**	**-0.002±0.001**	**-13.4±2.9**
Lead(II) nitrate	**-4.8±1.8**	**0.045±0.015**	**-8.3±0.5**
Cadmium(II) chloride	-2.8±1.6	0.025±0.009	**-5.5±0.9**
Arsenic(III) oxide	-1.6±0.7	0.002±0.0020	-4.4±2.0
Water	-0.8±2.6	-0.001±0.003	0.5±0.5
Sodium nitrate	0.2±1.1	-0.003±0.002	-2.1±0.9
Cobalt(II) acetate	0.5±0.7	0.000±0.007	**-6.0±0.4**
(Disodium hydrogen) phosphate	0.8±1.6	**-0.007±0.002**	0.4±0.6
Sodium chloride	0.9±0.6	**-0.006±0.001**	-0.2±0.4
Sodium sulfate	1.4±1.4	-0.005±0.002	-0.2±0.5
Lead(II) acetate	**2.8±0.9**	0.057±0.002	**8.5±0.4**
Nickel(II) sulfate	**3.5±1.0**	0.002±0.001	**6.4±0.4**
(Sodium) acetate	**4.0±0.6**	**-0.005±0.002**	0.4±1.7
Chromium(III) nitrate	**7.2±0.4**	**-0.010±0.007**	**16.0±0.9**
Chromium(III) chloride	**14.7±0.9**	0.007±0.010	**32.1±0.8**

Variables in bold type denote statistically significant changes based on "t" tests.

Using the solvent evaporation technique, Dong *et al.* prepared cross-linked alginate films blended with gelatin for inhibited drug release applications [26]. The crosslinking agent was $Ca^{2+}$and the model drug was ciprofloxacin hydrochloride. Influence of factors such as the relative amount of alginate to gelatin, the quantity of ciprofloxacin hydrochloride, pH and ionic strength of the release solution, cross-linking time with Ca^{2+}on release properties of films was studied. Compared to 20 and 80 wt% blend films, 50 wt% films had the highest tensile strength. In addition to crosslinking variables, the condition of the release medium such as pH and ionic strength were found to affect the properties of the films. For instance, the drug discharge rate attained within 24 h was 100%, 100%, 77.6%, and 52.4%, for the films in the Ca^{2+} solution after 0, 5, 15 and 30 min, respectively. Longer the crosslinking process, slower was the drug discharge due to the higher level of crosslinking [27, 28]. These ionically cross-linked alginate/gelatin films were found to be suitable for drug delivery systems [29, 30].

Esser aimed to find the most suitable method to evenly distribute calcium ions in alginate films to achieve controlled degradation, sufficient flexibility, and stability [31]. Biocompatible, antiadhesive and mechanically stable alginate films were prepared by 3 different methods, namely (i) inner gelation, (ii) film drawing with cross-linking initiated by spraying with lactic acid and (iii) film drawing with inner cross-linking. The desired level of swelling and erosion of the films could be obtained by regulating the quantity of calcium in the films [30]. The films obtained were further modified with different plasticizers to alter mechanical parameters such as flexibility and load. It was found that the incorporation of glycerol as plasticizer increased the length to 150% of the initial length and hence made the films smoother and flexible. However, glycerol is highly hydrophilic and makes the films sticky. The films were reported to be suitable for wound dressing and also as secondary barrier films.

Drugs metoclopramide hydrochloride and cisapride monohydrate were loaded on to alginate matrices using salts such as calcium chloride dihydrate, barium chloride dihydrate, and aluminium chloride hexahydrate as cross linkers [30]. Regular, lower surface, inverted, combined and upper surface crosslinking procedures were adapted to crosslink alginate films. The type of crosslinking technique influenced the exterior morphology and consequently diffusion parameters of the films. It was shown that the crosslinking of the matrix film was an interfacial phenomenon and the level of cation transfer relies on size, hydration and valency of the cations used. Fig. 4 depicts the mechanism by which calcium and barium cations bond to alginate in a planar 2-D manner and with trivalent aluminium in a 3-D manner. Extended crosslinking could be observed throughout the aluminium cross-linked film because crosslinking can concurrently happen in two distinct planes leading to a compressed structure and hence slow drug release.

Additionally, the discharge from the matrices indicated that the solubility and the dimension of the molecules were critical factors in the release phenomena. Among the different types of crosslinking used, it was observed that the percent drug release was maximum when the lower surface crosslinking method was adapted [32].

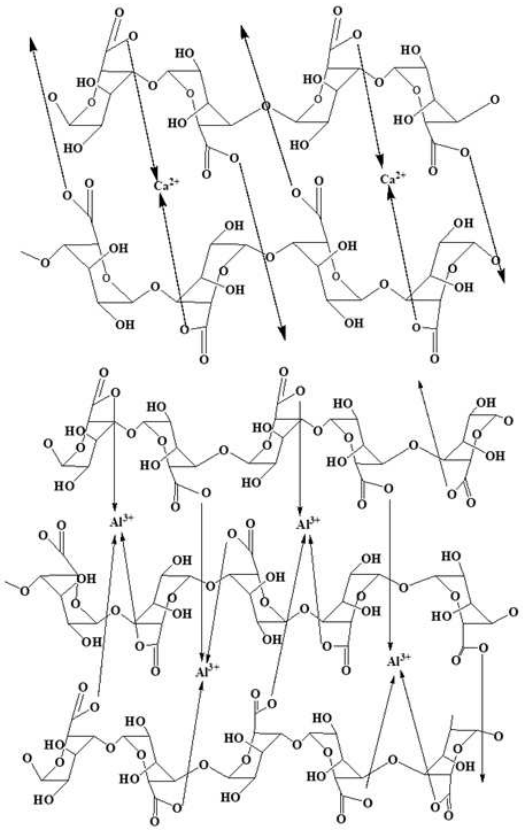

Fig. 4: Expected alginate crosslinking mechanism involving calcium, aluminum cations [30]. Reproduced with permission from Elsevier

Blends of alginate and phosphorylated chitin (P-chitin) films were developed and cross-linked with 4% $CaCl_2$ solution by Jayakumar *et al.* [32]. The ability of the blend films to adsorb various metal ions was studied. Batch kinetics of alginate/P-chitin blend films for Ni^{2+}, $Zn^{2+}$and Cu^{2+} ion adsorption was conducted. It was seen that with increasing contact time of alginate/P-chitin blend films, the amount of Ni^{2+}, $Zn^{2+}$and Cu^{2+}adsorbed (mg/g) increased [33, 34] and reached equilibrium at 5 h and 4 h for $Ni^{2+}$and Zn^{2+}, respectively. It was concluded that these novel alginate/P-chitin blend films may have the potential for environmental applications like removal of Ni^{2+}, $Zn^{2+,}$ and $Cu^{2+}$from water and wastewater. These films may also be useful for the delivery of pharmaceuticals, as substrates for tissue engineering and many other medical applications [16, 35].

Machida-*et al.* examined differences in diverse types of ion-cross-linked (Fe^{3+}, Al^{3+}, Ca^{2+}, $Ba^{2+,}$ and Sr^{2+}) alginate gel films substrates for culturing cells [36]. The affinity of human dermal fibroblasts to alginate films was studied by culturing cells on the surface of the films. Charge analysis revealed that all films had negatively charged surfaces with a modest distinction between the five different ions used for crosslinking. It was found that surface wettability [37] and morphology [38, 39] were the significant aspects of ionically cross-linked alginate films which determined the serum protein adsorption capacity and hence their cell compatibility. It was seen that cells on Fe-, Al-and Ba-alginate films showed higher growth among the films. Adsorption of serum proteins on the exterior of Ba alginate films was more favorable compared to Fe and Al, which in turn affected the functioning of the cells. The extent of cell proliferation on alginate films was proportional to the amounts of adsorbed proteins [40].

Alginate was cross-linked by Mn^{2+}, Zn^{2+}, $Ca^{2+,}$ and Al^{3+} and a comparison was done to evaluate their crosslinking efficiency. Properties like radiometric profiles, thickness, mechanical and water vapor permeation (WVP) of alginate mulching films and effects of ionic crosslinking were investigated by Liling *et al.* [41]. With Mn^{2+}, Zn^{2+}, and Ca^{2+}(but not Al^{3+}), the water vapor permeability of the films reduced and the tensile strength increased. The maximum elongation, tensile strength, and light transmission were found for Ca^{2+}cross-linked samples compared to the other divalent ions. However, increasing Ca^{2+}concentration caused a decrease in WVP along with light transmission, whereas the WVP of films increased steadily with crosslinking time. The crosslinking time did not show any significant influence on light transmission ($p>0.05$). The degree of crosslinking, along with the transmittance of the films, remained more or less stable with increasing crosslinking time [42]. In another study, alginate films cross-linked by 2% (w/v) calcium chloride were studied for their potential as agricultural mulching films. These cross-linked alginate thin films with a thickness of 0.011-0.014 mm had a high tensile strength of 40 MPa and light transmittance of 88.04 % and low water vapor transmittance of $3.631 \times 10^{-}$[11] g/ms Pa. These properties were considered to be suitable for mulching applications.

Remunan-Lopez and Bodmeier obtained chitosan glutamate and sodium alginate films through casting and cross-linked the films with tripolyphosphate (TPP) and calcium chloride ($CaCl_2$) [43]. The films were permeable to water vapor but were water-insoluble. It was inferred that the water vapor transmission rate of chitosan films decreased linearly with increasing concentration of the crosslinking agent [44]. Compared to uncross-linked films, the moisture uptake of dry cross-linked films was low and varied between 15-20 % since the majority of the hydroxyl groups would be used for crosslinking. Puncture strength improved with increasing $CaCl_2$ concentration but only up to 3-5%. It was suggested that by choosing the appropriate tripolyphosphate concentration, the mechanical, swelling and drug diffusion parameters of the films could be controlled [45, 46].

Alginate films with magnetic properties developed by incorporating nanoparticles of maghemite was studied by [47] for their effect of Ca on water solubility, particle release, and swelling properties. Surface management of the magnetic nanoparticles through citrate ions was first done to limit interactions of alginate with carboxylate groups and to stabilize them in neutral pH. A boost in water-resistance of alginate films was observed owing to the formation of a thick complex by cross-linking alginate with Ca ions [43, 48]. Based on this observation, it was suggested that the

swelling ratio was a good measure to quantify the extent of crosslinking [43]. After 10 min of immersion in $CaCl_2$, the complete conversion of sodium alginate to calcium alginate was seen. The magnetic particles did not significantly modify the ion switch over process but the sodium counter ions adsorbed on the particles can be replaced by calcium inducing an increase in the amount of calcium within the film which in turn could lead to the formation of a firmly cross-linked structure. Alternatively, the shift of symmetric COO^-peak to an elevated frequency and a raise in intensity of this FTIR peak in the region of 700-1200 $cm^{-1}$was observed and considered to be a sign of crosslinking.

The presence of guluronic acid units and 'their effects on the physical properties of calcium cross-linked alginate films was explained by Russo [49]. Residues of guluronic acid brought about an appreciable transformation in the physical parameters of the films [15]. Cross-linked samples showed a reduction in glass transition temperature and β relaxation which was detected by performing a dynamic-mechanical experiment. The decrease in physical parameters like glass transition temperature and β relaxation was explained in terms of increased free volume through the crosslinking process. The film thickness enhanced but a reduction in permeability after crosslinking was observed and this was considered to be the reason for the increase in free volume and hence better crosslinking [50].

Effect of using different proportions (0-40% v/v) of ethanol as co-solvent to ionically crosslink alginate with Ca^{2+} in a solution of $CaCl_2$ was studied [51]. The films were designated as Ca-SAE0, Ca-SAE10, Ca-SAE20, Ca-SAE30, Ca-SAE40 where 0,10,20,30 and 40 represents the percentage of ethanol. It was noted that with increasing ethanol proportion, the amount of calcium in the films improved and the values were 75, 76, 82, 79 and 49 mg/g for Ca-SAE0, Ca-SAE10, Ca-SAE20, Ca-SAE30, Ca-SAE40 films, respectively. There was an enhancement in the form, dimension, exterior homogeneity and mechanical parameters of the film mainly due to reduced swelling [52]. The characterization studies showed that ethanol does not affect the film's uniformity [48]. It can be deduced that when ethanol percentage exceeded 30% v/v, there was a reduction in Ca^{2+}crosslinkingdegree. A green, simple and efficient process for the preparation of calcium alginate films, which showed good surface appearance plus mechanical parameters for pharmaceutical products was possible through this approach. The surface appearance of the films showed that when the proportion of glycerol in the calcium cross-linked alginate films increased, the films became smoother and homogenous. The outcome of ethanol content on tensile strength (TS) and elongation (E) at the break of Ca-SA alginate films in desiccated and wet states were studied. A modest rise in the TS and a slight reduction in the %E of the dry films were observed on addition of ethanol. Particularly, the film's TS value in the dry state increased from 119 to 135 MPa when the ethanol proportion was increased from 0 to 40% v/v, while the E% decreased from 8.76 to 7.32%. This was suggested to be due to the occurrence of calcium-cross-linked "egg-box" connection zones. Films cross-linked using $CaCl_2/H_2O/C_2H_5OH$ solution had higher physical crosslinking points (caused by chain entanglement) compared to films cross-linked using $CaCl_2/H_2O$ solution. As a result, the TS of the films improved and E% of the films reduced when crosslinking was done in the presence of ethanol.

Roh synthesized alginate-carrageenan (Al–Ca) complex films and cross-linked them with $CaCl_2$ [53]. The pore dimension of the films was reliant on the Al-Ca ratio and studies showed that a 6:4 ratio was the most suitable to achieve the desired mechanical strength of alginate–carrageenan chains. With increasing carrageenan content, the extent of swelling increased [54] whereas pore volume decreased. When the alginate ratio was increased further, the permeabilities of glucose and dextrans for Al-Ca films also improved. The most flexible film (% elongation of 20) was formed when the ratio of alginate to carrageenan was 6:4.

Mohanan and Vishalakshi prepared films from two hydrophilic natural polymers, namely, sodium alginate (NaAlg) and gelatin (Gltn) in the presence of glycerol using Ca^{2+}as crosslinker [55]. The films exhibited a pH-dependent swelling behavior with an equilibrium swelling ratio of 130-480% in the neutral medium at 27 °C [56]. Least swelling was observed in pH 1.2 whereas maximum swelling was observed at pH 7.0. Film composition and preparation conditions influenced the swelling capacity and the rate of swelling [40]. When alginate films were cross-linked with 0.5M $CaCl_2$ for 10, 30 and 60 min (NG1, NG2 and NG 3, respectively) it was observed that the number of crosslinks increased with increasing concentration of $CaCl_2$ and time of contact of the films with the crosslinking medium. Higher the time of contact, lower was the swelling of the films i,e NG1>NG2>NG3. The films proved to be good candidates for biomedical applications especially as stimuli-responsive polymer materials for controlled release of drugs since the films were made from biocompatible materials and had good swelling ratios [57].

Table 3: Gives a comparison of the mechanical parameters of alginate films. Ca alginate film with glycerol as a plasticizer showed a minimum strength of 1.89 MPa due to the plasticizing effect. Also, Ca alginate film formed by internal crosslinking method exhibited a maximum strength in the range of 39.7-52.9 MPa and elongation at break of 2.3-4.4 %

Films	Other processing conditions/Additives	Tensile strength (MPa)	Elongation at break (%)	Reference
Na–alginate	By spraying	23.16	32.16	[58]
Ca-alginate	By spraying	23.11	27.35	[58]
Ca-alginate	With glycerol as plasticizer	1.89	17	[59]
Ca-alginate	With sorbitol as plasticizer	65.9	2.5	[60]
Ca-alginate	With oregano essential oil	31.1	3.7	[60]
Ca-alginate	Without voids	12.03	1.939	[62]
Ca alginate	With voids	2.892	3.129	[62]
Ca alginate	By internal crosslinking	39.7-52.9	2.3-4.4	[61]

Hydrogels

The rate of degradation of alginate in the body is very slow and takes place in a disorderly manner caused by the liberation of strands of high molecular mass. To overcome this, the degradation of partially oxidized alginate was reported by Bouhadir [63]. Partially oxidized alginate capable of forming hydrogels were synthesized by ionic cross-linking with calcium [64]. The partially oxidized alginates degraded over time to yield low molecular weight oligomers, with the speed of degradation depending on the pH plus temperature of the solution. The partially oxidized alginate hydrogels were utilized to improve cartilage-like tissue development *in vivo*.

Sprayed hydrogel dressings (SHD) were synthesized by combining polyvinyl alcohol (PVA) along with sodium alginate (SA) as artificial and biopolymeric components, respectively. Here, PVA and SA were cross-linked with boric acid and calcium chloride, respectively to evaluate the likely hood of this combination to support wound healing. The schematic depiction of SHD of PVA/SA cross-linked with $CaCl_2$ and boric acid is shown in fig. 5. In another study, a novel idea of spontaneous film dressing (SHD) was reported by Kumar [65]. *In vitro* properties like degradation, microbe penetration assay and water absorption ability were investigated. Also, biological parameters like protein absorption and haemocompatibility were studied [66]. It was said that the quantity of crosslinker was crucial for the preparation of sprayed films with desirable properties. These dressings were considered to be ideal for curing wounds because of their biocompatibility, excellent swelling and almost 100% biodegradation [17].

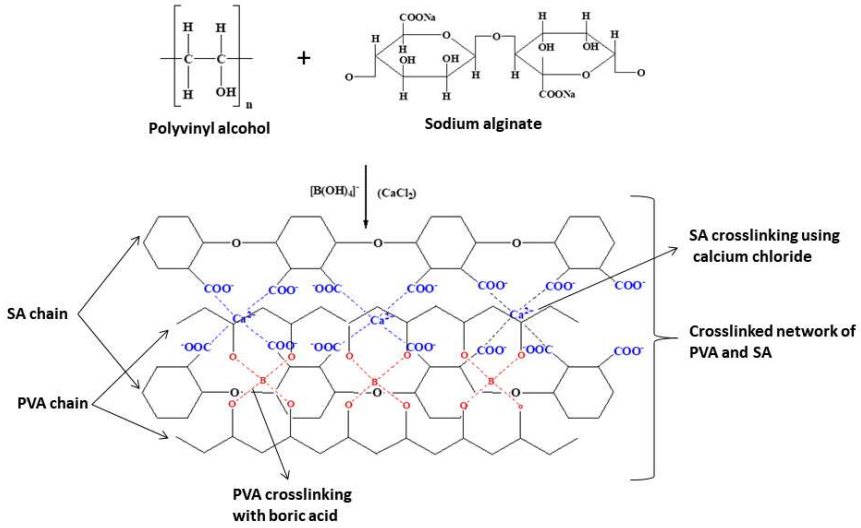

Fig. 5: Schematic depiction of the synthesis of cross-linked SHD film of Polyvinyl Alcohol/Sodium Alginate) PVA/SA through crosslinking with $CaCl_2$ and boric acid [65]. Reproduced with permission from Wiley periodicals, Inc

Alginate hydrogels with different proportions of L-guluronic acid (G) and D-mannuronic acid (M) residues were fabricated and cross-linked with ferric ions. Machida-Sano evaluated the appropriateness of the hydrogel as a culture substrate for human dermal fibroblasts [67]. High G Fe containing alginate showed less variation in gel parameters during immersion in terms of swelling as well as polymer content compared to high M Fe–alginate gel [68] which were altered due to loss of ions. It was suggested that cross-linkage involving ferric ions and alginate could be varied depending on alginate composition [69] and hence the gel stability varied.

The homogeneity and efficacy of two different cross-linking processes, namely the conventional air-jet dropping method and the ''crystal gun'' method to crosslink alginate microcapsules with Ba^{2+}were compared by Manz [70]. Advanced ^{1}H NMR imaging using paramagnetic Cu^{2+}as contrast agent was utilized to authenticate the cross-linking process. Quantitative and qualitative information regarding the homogeneity of the crosslinking process was obtained. Two-and three-dimensional pictures along with plots of the spin-lattice relaxation period T1 of microcapsules cross-linked by Ba^{2+}exposed to external Cu^{2+}were obtained. It was established that the crystal gun method was more proficient than the air-jet dropping method for crosslinking alginates.

The consequence of crosslinking dried (<5% moisture) paracetamol containing alginate granules through calcium chloride solutions was investigated. Factors such as solution temperature, calcium concentration, stirring pace and time used throughout cross-linking along with physical parameters of the dried granules were studied [71]. It was seen that rapid crosslinking takes place at elevated concentrations of $CaCl_2$ compared to low concentrations [72-74]. Treatment conditions controlled the yield, early release, drug entrapment and extent of cross-linking [45,75]. In a similar study, the result of drug entrapment on factors like calcium ion concentration (6.02-12.4%) temperature (25-45 °C), time (1.5-5.5 min) and speed of reaction was studied. Noteworthy interactions were observed between solution temperature, stirring time, and stirring speed. Lowest drug release was observed for granules prepared under high solution temperature, high stirrer speed and high stirring time.

Kinetics of cross-linked and thermally set alginate gels wherein calcium ions were the crosslinkers were studied by Chavez [76]. At constant temperature, the sharp surface interface depiction was utilized to explain the kinetics. The concentration of the two gel systems gelatin-alginate and agar-alginate were 6.179 x $10_a^{-5}$and 6.370 x10_b^{-5} (^{a,b} Porosity = 0.9525 and 0.982, respectively). The diffusion coefficient values for the individual components in each system was1.79 x10^{-6} for gelatin and±0.220x 10^{-6} for alginate in the gelatin-alginate gel system, and for agar–alginate gel system the values were 1.673 x10^{-6} for agar and±0.074x 10^{-6} for alginate. The cation size for calcium ions was large compared to sodium ions [21] and the gel matrix formed both by agar and gelatin contributed to the low diffusivity of the two gel systems. The "q" (Moles of calcium ion used in the reaction per volume of unreacted solid (mole/cm^3) values were almost the same since alginate gel concentration and stoichiometry were the same for both systems but the marginal variation among the two was credited to the carriers.

Sodium alginate was utilized as a polyelectrolyte to demonstrate the aggression behavior in the solution obtained after partial protonation of carboxylate groups in alginate molecules by Cao [77]. Sodium alginate assemblies were formed in the aqueous solution using 2, 2-(ethylenedioxy)-bis(ethylamine) as a cross-linker and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide as the coupling agent. Protons released from the reaction of $K_2S_2O_8$ with water at 70 °C cause partial protonation of carboxylate groups in sodium alginate chains. This further leads to the configuration of alginate assemblies with a core-shell structure. Such partially cross-linked alginate assemblies were pH-sensitive [56] and were capable of changing into hollow structures at high pH. The usage of block or graft copolymers as the precursors to prepare assemblies and capsules can be avoided using this technique. Also, this approach provides a well-designed exterior for a successive chemical reaction at the surface (e. g., for binding biomolecules and for surface grafting) which would be helpful in biomedical applications [78, 79].

Blends

Giridhar prepared biodegradable blends of sodium alginate (SA) with lignosulphonates (LS) [80]. The blends were cured by barium chloride as the crosslinker to achieve selective permeation and strong crosslinking. To check the possibility of using these blends for controlled drug delivery for the gastrointestinal tract, the SA: LS blends (80:20) were subject to swelling studies at pH of 1.2 and 7.4 [81]. Crosslinking by barium chloride increased resistance to water and degradation was seen over a series of temperatures. Table 4 shows the crystallinity values for PE-PS, PP-PS, and their pure polymers. It can be seen from the table that the crystallinity decreased in blends compared to their pure polymers [83].

Table 4: Crystallinity values for PE-PS and PP-PS blends [82]. Reproduced with permission from elsevier

Sample	X_{PE} (%)	X (%)	Sample	X_{PP} (%)	$X_{PP}(\alpha)$ (%)	X_{PP} (β) (%)	$X_{PP}(\gamma)$ (%)	X (%)
PE	41	41	PP	50	63	23	14	50
PE80PS20	33	26	PP80PS20	32	71	29	-	26
PE60PS40	24	14	PP60PS40	26	70	30	-	14
PE50PS50	25	12	PP50PS50	39	63	37	-	19
PE40PS60	27	11	PP40PS60	58	47	53	-	23
PE20PS80	22	4	PP20PS80	45	43	56	-	9

X_{PE}, Crystallinity of polyethylene in a blend, X, Total crystallinity of a blend, $X_{PP,}$ Polypropylene Crystallinity in a blend, $X_{PP}(\alpha)$, Weight fraction of α phase in the crystallinity of polypropylene, X_{PP} (β) Weight proportion of the β phase in the crystallinity of polypropylene, $X_{PP}(\gamma)$ Weight fraction of the γ phase in the crystallinity of polypropylene.

Beads

Crosslinking sodium alginate with carboxymethyl guar gum(CGG) using $BaCl_2$ was reported by Bajpai [83]. Initially, the derivatization of guar gum was performed to obtain CGG and then the polymer solution containing sodium alginate along with CGG was supplemented to the gelation medium containing $BaCl_2$ solution. It was observed that as the crosslinking time increased, the stability of the beads increased and water uptake decreased. The biopolymeric beads obtained were sensitive to pH changes and stable up to 18 h in simulated intestinal fluid. Finally, it was concluded that these beads can be used to deliver drugs orally through the gastrointestinal tract.

Nanocomposites

Preparation of alginate with alginate-carbon dot (CD) nanocomposites with divergent bivalent and trivalent cross-linker ions was reported by Konwar [84]. Alginate was cross-linked with metal ions like Ca^{2+}, Ba^{2+}, Cu^{2+}, $Fe^{3+}$and $Al^{3+}$and their equivalent carbon-dot nanocomposites prepared by means of solution casting. It was seen that films cross-linked by metal ions with bulky size (Ba^{2+}) [85] exhibited very high tensile strength and higher thermostability. Fig. 6 depicts the loading of carbon-dots in the cross-linked alginate system. The presence of the carbon dots leads to elevated absorption in the UV region suggesting that the films could be used as UV blockers. Although higher strength and UV resistance could be achieved, the presence of 2% CD's in the Ca^{2+}, $Ba^{2+}$and Al^{3+}cross-linked films reduced UV transmittance to 4, 5 and 7 % respectively. For Fe and Cu, the transmittance value reduced to 5 and 6% but with enhanced UV blocking.

Nanofibers

Jiang prepared polyelectrolyte complex (PEC) membranes of cationic chitosan and anionic sodium alginate having fibrous structure using the freeze-drying method [86]. Chitosan plus sodium alginate were blended in a series of concentrations and frozen at varying temperatures. Freeze-dried fiber membranes were investigated for their inter-molecular interaction, morphology, and biocompatibility. The swelling behavior studies exhibited that in an aqueous medium, PEC membrane cross-linked with glutaraldehyde showed pH as well as ionic strength-dependent swelling [81] which might have a possible application in tissue engineering or drug controlled release whereas the fiber structure exhibited swelling in aqueous media. Besides, chitosan–sodium alginate fibers showed improved cell adhesion and proliferation compared to pure chitosan. This signified that nanofibers of the natural polyelectrolyte complex were suitable for tissue engineering or as drug carriers [87, 88]

Covalent crosslinking

The common approach in cross-linking alginate is to use divalent ions as described previously. Although ionically cross-linked alginate-based materials are extensively used in various fields, this method has many drawbacks such as limited long term stability, destabilization, and rupture of structure (hydrogels) when cross-linked with monovalent ions. To overcome these limitations, chemical crosslinking is done to create stable networks and achieve desired properties. Covalent crosslinking is one such method that has advantages like improved mechanical properties and stability of the cross-linked materials.

Microspheres

Glutaraldehyde crosslinking of alginate and hyaluronate, two biomaterials that possess pH-responsive characteristics, were done for exenatide encapsulation [89,90]. It was established that compounds loaded by alginate or hyaluronate could be protected in the stomach but released in the small intestine as a result of the pH differences. The microspheres could be kept compact in acidic solution but would be swollen in alkaline condition and therefore mimic the situation in the stomach and small intestine, respectively. However, alginate and hyaluronate together were not able to self-organize due to the repulsions between them. Cross-linking of alginate with hyaluronate was considered to provide better interaction between the two polymers and also imparted pH-responsive behavior. Cross-linked microspheres of alginate and hyaluronate loaded with exenatide were capable to deliver the drug and lower plasma glucose levels under *in vivo* conditions [91].

Blends

For the discharge of naproxen sodium (NS), blends of poly (vinyl alcohol) with sodium alginate were synthesized and cross-linked with glutaraldehyde (2.5%v/v) [92]. Blending led to a lowering of the drug release rate from the beads and was proportional to the increased NaAlg content. A maximum of 84% drug release was seen for a PVA/NaAlg blend ratio of 1/2 and a drug/polymer ratio of 1/4. An enzyme lipase extracted from *Mucor javanicus* was held in hybrid gel beads of alginate-silica by means of instantaneous cross-linking with glutaraldehyde [93]. The beads were set by in situ hydrolysis and polycondensation of Tetramethoxysilane (TMOS) in alginate solution in the presence of glutaraldehyde followed by gelation in $CaCl_2$ solution. Compared to those of the enzymes entrapped in the simple alginate beads or in the alginate-silica hybrid, cross-linked beads showed higher enzyme activity. The enzyme leakage markedly decreased from the hybrid beads [94] but the action and activity recovery was enhanced with immediate cross-linking by glutaraldehyde. The effective interface of enzyme molecules with the micro-sized silica particles and modification of bead morphology by glutaraldehyde was observed.

Films

Organic solvents acetic acid (AA) and isobutanol (IB) form close boiling mixtures when used with water and hence their dehydration is a challenge. Sodium alginate is a polymer that is hydrophilic in nature and can be made into membranes having potential applications in dehydrating aqueous organic solutions. Badiger prepared thin-film composite membranes of sodium alginate, cross-linked the membrane with a 2,4-toluene diisocyanate (TDI) and glutaraldehyde for the dehydration of IB and AA/water blends by means of pervaporation (PV) [95]. To assess polymer-liquid

interactions, sorption studies were conducted at equilibrium in pure liquids with binary organic-water mixtures of diverse compositions. For 4 h, ion exchangeability and degree of crosslinking were 0.784 and 78.6 % respectively, indicating that most of the functional groups were cross-linked. Sorption studies revealed that the membranes possess a superior affinity toward water than organic solvents. The tensile property and elongation of the membranes developed were observed to be in close agreement with the previously reported literature. Alginate-membranes exhibited an ability to dehydrate solvents up to a purity of 99 %.

Bekin studied the dielectric characteristics and electro responsive behavior of interpenetrating polymer network (IPN) films prepared from sodium alginate/poly(acrylic acid) (SA-AA-GA) and cross-linked by glutaraldehyde (GA) [96]. The presence of poly (acrylic acid) and crosslinking of sodium alginate by GA reduced the dielectric constant plus conductivity. The dielectric constant of SA-AA-GA films was inversely related to the frequency. The same effect was seen in SA-GA films but in a narrower range. The dielectric behavior was ascribed to the crosslinking of SA by GA, which made the polarization of SA molecules difficult [97, 98]. Also, the compact structure produced due to the higher AA content of the films decreased the mobility of ions and hence the conductance in SA was higher compared to SA-AA and SA-AA-GA films.

In order to enhance the cellular adhesion parameters of polyelectrolyte multilayer (up to 10) films and rigidify them by reducing their gel-like nature, chemical crosslinking by genipin was done by Hillberg [99]. Effects of genipin crosslinking on the cell adhesion parameters of blend films containing chitosan (CH), hyaluronate (HA) and alginate (Alg) were studied. Each one of the films was affected differently by crosslinking. $[CH–HA]_{10}$ CH was slightly thicker (350–450 nm) and extremely viscoelastic, while $[CH–Alg]_{10}$CH films could be developed to thicknesses of 100 nm. Differences in thickness were attributed to the various growth regimes of the polyelectrolyte systems. $[CH–HA]_n$CH cross-linked films were nonadhesive, suitable for the culture of pre-osteoblasts and fibroblastic skin cells. Crosslinking of the films resulted in a boost in cell attachment, which became more evident when 44 mmol genipin was utilized to cross-link the 3 and 5 bi-layer films (thinner films). For 10 bi-layer films, the cell attachment was evident for all concentrations of genipin. Finally, cross-linking [CH–Alg]n CH films were found to enhance pre-osteoblast and rat fibroblastic skin cell adhesion, particularly for high bi-layer numbers and also when higher concentrations of cross-linker were used.

The effect of dialdehyde alginate (ADA) as a crosslinker on the chemicophysical characteristics of gelatin films was considered by Boanini [100]. Aldehyde groups of alginate created from partial oxidation interacted with the e-amino set of lysine or hydroxylysine side groups of gelatin to form a crosslink. At low concentrations of ADA, the extent of crosslinking of the gelatin films was quite modest, reaching a maximum value of 23%. The extent of swelling and amount of gelatin released in solution was reduced by the presence of ADA and this led to an improvement in mechanical characteristics of the films. It was found that the addition of Ca^{2+}reduced the degree of crosslinking between ADA and gelatin because Ca^{2+}reduces the amount of alginate aldehydic groups available for crosslinking. With increasing gelatin and ADA concentration, there was a rise in Young's modulus, E and stress at break, σ_b values ($p<0.05$) whereas deformation at the break, $\acute{\varepsilon}_b$ did not show any trend with respect to ADA. In the case of GEL5, the occurrence of calcium showed a significant improvement in Young's modulus whereas a slight reduction in modulus was seen in GEL10 and GEL15. Table 5 shows the denaturation temperature (T_D) along with denaturation enthalpy (ΔH_D) for different films in wet and dry conditions. Due to an enhancement in the number of covalent crosslinks which break exothermically and a reduction in the number of hydrogen bonds that breaks endothermically, there was a decline in ΔH_D values of wet gelatin films. The increase in triple helix structures of gelatin films was also responsible for increased thermal stability.

Table 5: Mechanical properties, denaturation temperature (TD) and denaturation enthalpy, (ΔH) values of films [100]. Reproduced with permission from elsevier

Sample	Deformation at break (%)	Stress at break (MPa)	Young's modulus (MPa)	Dry		Wet	
				T_D peak (°C)	ΔH (Jg^{-1})	T_D peak (°C)	ΔH (Jg^{-1})
GEL5	233±20	0.7±0.4	1.4±0.5	97±1	27±1	38±1	24±1
GEL5-ADA1	177±30	1.7±0.4	2.7±0.8	97±1	29±1	38±1	21±1
GEL5-ADA3	195±25	2.9±0.4	3.8±0.7	96±1	28±1	39±1	21±1
GEL5-ADA1Ca	204±39	2.6±0.7	3.9±0.6	96±1	28±1	39±1	24±1
GEL5-ADA3Ca	206±17	3.8±0.3	5.4±0.8	95±1	27±1	41±1	23±1
GEL10	203±22	1.2±0.3	2.0±0.2	94±1	29±1	37±1	24±1
GEL10-ADA1	280±42	2.2±0.3	2.6±0.3	96±1	30±1	39±1	20±1
GEL10-ADA3	265±30	2.7±0.3	2.9±0.3	97±1	29±1	40±1	21±1
GEL10-ADA1Ca	308±49	2.1±0.2	2.0±0.2	94±1	30±1	39±1	23±1
GEL10-ADA3Ca	241±13	2.6±0.2	2.6±0.4	95±1	28±1	39±1	20±1
GEL15	306±49	2.5±0.3	2.5±0.3	97±1	28±1	38±1	25±1
GEL15-ADA1	275±17	3.1±0.1	3.3±0.6	97±1	29±1	39±1	22±1
GEL15-ADA3	232±18	3.4±0.3	4.0±0.5	96±1	26±1	39±1	21±1
GEL15-ADA1Ca	284±21	2.8±0.1	2.7±0.3	94±1	29±1	39±1	21±1
GEL15-ADA3Ca	230±12	3.3±0.2	3.7±0.1	96±1	26±1	38±1	21±1

A hydrophilic film was prepared using carbodiimide as a crosslinker by Xu [54] with the potential to act as protective coatings for hydrophobic membranes and in membrane distillation and osmotic distillation processes. By film immersion method, alginate-carrageenan (AL-CG) blend films were prepared and film preparation conditions such as pH, non-solvent(ethanol) content, the concentration of crosslinking medium and relative amounts of polymer blends were optimized to obtain high water sorption capability. A 20 wt% of carrageenan in the blend film, 60 vol% ethanol in the crosslinking medium and 120 mmol water-soluble carbodiimide (WSC) at pH 4 provided the optimum crosslinking.

Hydrogel

Currently available tissue-engineered skin has many drawbacks such as difficulty in handling and not being customizable [101]. To succeed over these limitations, Yanez synthesized a printable biological alginate hydrogel containing self-crosslinking agents for wound dressing applications [102]. Aldehyde groups were created by oxidation of sodium alginate to facilitate the crosslinking process between modified alginate and gelatin without the use of crosslinkers. Later, these aldehyde groups were cross-linked with amino groups of gelatin in order to form a biodegradable hydrogel. Also, the thickness, gelling period and the extent of crosslinking of alginate with respect to pH, level of oxidation, plus temperature were investigated. Using the TNBS assay, it was observed that when the concentration of gelatin increases, the extent of crosslinking decreases. Finally, it was stated that controlling alginate concentration and spatial dispensing via printing in a controlled environment permitted the production of wound dressings with tunable properties.

Alginate crosslinking via tetrazine-norbornene chemistry directed towards the formation of click alginate hydrogels was reported by Desai [103]. Covalently cross-linked alginate hydrogels lack chemoselectivity and hence are not preferred biologically. Therefore, aqueous carbodiimide chemistry was utilized to modify the alginate backbone. Here, tetrazene plus norbornene groups were introduced to polymer chains of alginate to form covalently cross-linked click alginate hydrogels without the need for external energy, crosslinkers, or catalysts. This ensures that cells can be encapsulated without any harm. In addition, the mechanical plus swelling parameters of these hydrogels can be altered by organizing the total polymer concentration and stoichiometry of the functional groups [104, 105].

In a similar approach, *N*-(3-Aminopropyl) pyrrole was attached covalently with alginate by way of carbodiimide-mediated activation for biosensor applications [74, 106]. The conjugation chemistry was designed in such a way that only 25-35% alteration of the molecule was possible and the remaining 70% of the carboxylic acid residues could crosslink with the calcium residues. UV spectroscopy characterization studies revealed that the inclusion of pyrrole led to specific interactions in the polysaccharide. The ^{13}C NMR results confirmed the conjugation of pyrrole to alginate through amide bond formation.

The newly developed pyrrole-alginate conjugate was used for the immobilization of polyphenol oxidase (PPO) on top of an electrode surface through physical entrapment ensuing from the gellification method and electrochemical polymerization of the pyrrole groups. The competence of this cross-linking technique (chemical and electrochemical) was examined by evaluating the quantity of enzyme liberated from polypyrrole-alginate and regular alginate. The results supported the primary task played by the *in-situ*–generated polypyrrole for the retention of immobilized enzyme molecules with the preservation of their activity. Also, the polypyrrole acted as an added polymeric binder that improves the gelling capabilities of the alginate gel and exhibited excellent permeability.

Photo/(UV) crosslinking

Photocross-linked materials have been preferred for biomedical applications. Aqueous macromer solutions possessing cells and/or bioactive factors can be delivered in a modestly invasive mode and then swiftly cross-linked in a physiological environment in situ, after short exposure to ultraviolet (UV) light. Further, molecules called photoinitiators that produce free radicals can be included to initiate the polymerization process to effectively convert the macromer solution into hydrogels.

Hydrogels

Following the above-mentioned approach, Jeon fabricated Arg-Gly-Asp(RGD) modified methacrylated alginate in a two-step reaction using standard carbodiimide chemistry [107]. Initially, sodium alginate of low molecular weight was synthesized by irradiating protanal LF 20/40 at a gamma dose of 5 Mrad. Later, the RGD sequence was supplemented to methacrylated alginate to obtain modified alginate hydrogels. To examine whether the peptide modification has an effect on mechanical parameters of photocross-linked hydrogels, strain-rate compression trials were performed. It was observed that stress-strain plots of RGD modified and unmodified hydrogels were similar in shape proving that peptide modification does not affect the cross-linked structure.

In a similar study, sodium alginate of low molecular weight was irradiated using Protanal LF 20/40 at a gamma dose of 5 M rad [108]. Next, the low molecular weight sodium alginate was treated with 2-aminoethyl methacrylate. These methacrylated alginate hydrogels were photocross-linked with 365 nm UV light at 8–20 mW/cm^2 for 8 min to form hydrogels. The swelling behavior, elastic moduli along with degradation rates of photocross-linked alginate hydrogels were controllable by varying the percentage of alginate methacrylation [109]. The prepared hydrogels were found to be biodegradable, exhibited low cytotoxicity and had excellent biocompatibility making them suitable for various biomedical applications.

Rouillard prepared 3D alginate-chondrocyte hydrogel scaffolds with high viability by photocrosslinking methacrylate modified alginate with the photoinitiator VA-086 [110]. Initially, methacrylate-alginate was created by replacing secondary alcohols on the alginate backbone with groups of methacrylate using anhydride chemistry. Next, under exposure to ultraviolet light, the methacrylate-alginate solution was photo-cross-linked in the presence of a photoinitiator to initiate polymerization of the methacrylate groups leading to the formation of crosslinks. The results from VA-086 were compared with other photoinitiators like Irgacure 2959 for solubility, ultraviolet exposure, and photoinitiator cytotoxicity on the feasibility of chondrocytes in 2D culture [111]. It was found that the IRG 1870 and V-044 were cytotoxic and inappropriate for tissue engineering applications while IRG2959 and VA-086 were the most promising.

Higham examined ionic crosslinking of alginate upon ultraviolent irradiation using in situ dynamic rheologies [112]. A combination of alginate with calcium carbonate ($CaCO_3$) particles and a photoacid generator (PAG) led to the formation of hydrogels [113, 114]. Free calcium ions for ionic crosslinking were released due to photolyzation of PAG upon UV irradiation to form egg box junctions with adjacent alginate chains. The viscous and elastic moduli during gelation were monitored as a function of the exposure time, the intensity of UV irradiation, the concentration of alginate, and the ratio among alginate and calcium carbonate. It was found that as the intensity of the UV radiation increased, the time required for gelation decreased [115]. Since higher UV intensity photolyzes a larger fraction of PAG molecules, a larger concentration of Ca^{2+}ions that would initiate ionic crosslinking are released. The minimum concentration of alginate required for gel formation was 2 % wt/vol. The gel modulus increased with increasing alginate concentration due to increased accessibility of G blocks required for crosslinking.

Using reductive cation exchange method bruchet treated iron (III) cross-linked alginate hydrogels with calcium salts and sodium ascorbate which resulted in the reduction of iron (III) to Iron (II) that were further replaced by Ca^{2+}resulting in homogenous, patterned ionically cross-linked alginate hydrogels [116]. Another alternative method was chosen where the cation exchange was performed by the photochemical reduction in the presence of $CaCl_2$ as a sacrificial photoreduction [117, 118]. The likely hood of photochemical patterning of Iron (III) cross-linked hydrogel followed by the photochemical reductive exchange was demonstrated. The homogenous hydrogels produced due to photochemical reduction where Ca^{2+}cations bind polyguluronate domains were in the "egg-box" fashion. It was established that ionically cross-linked alginate hydrogel can behave as a positive photoresist intermediate for the preparation of patterned calcium alginate hydrogel. Also, iron (III) cross-linked hydrogels served as a *negative* photoresist intermediate.

Coatings

Encapsulation of glucose oxidase enzyme was done along with photo-crosslinking of diazo-resin (DAR) coatings on microspheres of alginate [119]. To know the competence of nanofilm coatings with crosslinking, the activity in addition to physical encapsulation of the trapped enzyme was studied over 24 w. It was observed that for nanofilms containing layers of polyelectrolyte, cross-linked as well as uncross-linked DAR/PSS-coated spheres preserved 50% of their original activity subsequent to 4 w. It was seen that the rate of leaching of the drug glucose oxidase reduced after crosslinking. Thus, enzyme arrest, along with stabilization, was achieved by using easy alterations to the layer-by-layer self-assembly technique [120-122].

Capsules

One of the drawbacks of using polyelectrolyte multilayer(PEM) for drug release systems is the uncontrolled release properties and kinetics [120, 124]. Drug release could be controlled by crosslinking the PEM. Compared to conventional chemical procedures, the photo-crosslinking process is roughly 16 times faster. Hu 2015 demonstrated a method of fast photo-crosslinking chitosan-alginate PEM capsules which allows efficient lowering and diffusion across the PEM walls [121]. The results showed that the rate of anticancer drug, doxorubicin release was lower in photocross-linked capsules compared to non-cross-linked capsules suggesting their suitability for controlled drug delivery and other biomedical applications [125].

Scaffolds

Coates demonstrated the utility of photocross-linked alginate hydrogels containing interpenetrating hyaluronic acid chains [126]. Hyaluronic acid was supplemented to the photocross-linked scaffolds, which improved gene markers and showed promising potential for cell-based cartilage repair therapies. El-sayed prepared photo-cross-linkable styryl-pyridine modified alginate (ASP-Alg) using UV radiation and characterized the modified alginate using various techniques [127]. The ASP-Alg membranes were cross-linked via [2+2π] cycloaddition reaction after UV irradiation. The crosslinking reaction took place between casted (ASP-Alg) membranes and photoactive styryl and pyridine moieties. At regular time intervals, the UV-visible light spectra of the irradiated membrane were examined to monitor crosslinking density and kinetics. The results were found to fit the second-order kinetic model, indicating that crosslinking occurred via a bimolecular [2+2π] cycloaddition reaction [128]. Swelling and biodegradability studies showed that the rise in the crosslinking density to be due to lowered swelling ratio as well as degradation rate [129].

Enzymatic crosslinking

Enzymes are proteins that accelerate chemical reactions. High extent of regioselectivity, enantioselectivity, and chemoselectivity are the main advantages of using enzymes in various chemical and biochemical reactions. The greatest importance of enzymes lies in their ability to catalyze the synthesis of products in high enantiopurity. These advantages of enzymes are often limited by their short shelf-storage life and their cumbersome recovery and re-use. Enzymes have also been extensively studied for crosslinking biopolymers and bioproducts including alginate and alginate derived materials.

Beads

Zhang cross-linked enzyme-alginate suspension using calcium cation, to synthesize beads with porous structure and later immobilized *Candida antarctica* lipase B (CALB) in an alginate carrier [130]. The action of the enzyme-alginate composite was established in aqueous phase by enzymatic hydrolysis reaction of p-nitrophenol butyrate. Parameters such as pH, temperature, embedding and lyophilizing time on the reactive behavior were studied. It was found that the enzyme-alginate composite activity was maintained up to 6 repeated cycles. In a similar study, Zhang cross-linked enzyme *Candida antarctica* lipase B(CALB)–alginate suspension in the presence of $Ca^{2+}$to form beads with porous structure [131]. The activity of the beads was studied by the enzymatic hydrolysis reaction of P-nitrophenol butyrate. It was noticed that the action of the enzyme–alginate beads could be retained up to 5 repeated cycles without marked deactivation.

Table 6: Comparison between the different techniques of crosslinking

Crosslinking type	Ionic	Covalent	Ultraviolet	Enzymatic
Advantages	• Simple • Nontoxic	• Improved mechanical properties • More stable	• Minimally invasive • In situ crosslinking happens swiftly • Mild reaction conditions required	• Regioselectivity • Enantioselectivity • Chemoselectivity
Disadvantages	• Limited long term stability	• Usage of toxic reagents • Removal of unreacted chemicals	• Exposes cells to UV light • Usage of chemical photoinitiators • Usage of organic solvents	• Lacks long term operational stability • Short shelf-storage life • Cumbersome recovery and reuse

Combination of methods of crosslinking

A combination of various methods of crosslinking alginates has been reported by many authors to succeed over the drawbacks of using any of the single methods discussed previously [132, 133].

Films

The invention of a self-standing multilayered arrangement based on biopolymers has been of increasing interest for potential biomedical applications. However, their utilization has been limited because of their gel-like properties. Silva reported the combination of covalent and ionic cross-linking by means of natural as well as non-cytotoxic cross-linkers like genipin and calcium chloride ($CaCl_2$) [20]. Combining both types of cross-linking improved the mechanical characteristics whereas water uptake decreased [134]. The ionic cross-linking of multilayered chitosan (CHI)-alginate (ALG) films gave rise to self-supporting membranes with interesting properties, such as improved mechanical strength, calcium-induced adhesion with shape memory ability. The use of $CaCl_2$ also offered the likelihood of reversible switching of all these properties by easy immersion in a chelating solution. Here, the improvement of the mechanical parameters, shape-memory capability and the tendency for induced-adhesion is credited to the ionic cross-linking of the multilayers. There was an enhancement in Young's modulus (E) and tensile strength (σ_{max}) after crosslinking with genipin. Also, a rise in E and $\sigma_{max.}$ after immersion of the ionically cross-linked membranes in EDTA were seen. The mechanical parameters were fully recovered proving the reversible/switchable properties of these membranes. The results substantiate that CHI-ALG multilayers have the shape-memory ability because they have the capacity to memorize one or multiple temporary shapes and are capable to go back from this temporary shape to their permanent shape upon introduction to an external stimulus, such as hydration.

Hydrogels

Kong demonstrated a useful method to control the physical parameters of a broad range of hydrogel-forming polymers [135]. It was hypothesized that by adjusting molecular weight(MW) distribution of polymers, a control over degradation and elastic moduli(E) could be achieved [136]. Using this approach, hydrogels were synthesized from both ionically and covalently cross-linked alginates and later partially oxidized (1% uronic acid residues), low [molecular weight (MW) _ 60 000 g/mol] and high MW alginates (MW _ 120 000 g/mol). E was comparable to high MW alginate gels when the fraction of low MW alginates was increased to 0.50. The rate of degradation was faster irrespective of the crosslinking mode. This outcome

was credited to a quicker separation among cross-linked domains upon breakages for the low MW alginates. The biodegradable oxidized dual cross-linked hydrogels allowed the development of novel bone tissues from transplanting bone marrow stromal cells.

Industrial employment of alginate hydrogels is limited because of the low structural steadiness of the beads [137]. Cha prepared an interpenetrating polymer network (IPN) through a permutation of ionic crosslinking of methacrylic alginate with calcium ions and subsequently photocrosslinking methods to enhance the durability of the beads [133]. The resultant methacrylic alginate exhibited superior strength, strain, stiffness, and stability [107]. Also, the yeast cells encapsulated in these beads exhibited more metabolic activity in ethanol production compared to those in calcium cross-linked alginate gel beads. Overall, it was shown that the beads could be used for encapsulation devices with improved structural toughness for an extensive array of prokaryotic and eukaryotic cells used in biochemical and industrial processes. Some advantages of this method were improved rigidity of the gel and avoiding chemicals that can be potentially cytotoxic to the encapsulated cells. Using this approach, it was demonstrated that neither the UV-initiated crosslinking process nor the decreased gel permeability was harmful to the cells, as exhibited with the minimum difference of cell viability among the cells in alginate gel beads and those in IPN-MA gel beads. Also, IPN-MA gel beads had mechanical properties that were similar or higher than that of gel beads made using previously reported cross-linking methods.

Wang fabricated two new types of dopamine modified alginate gel beads reinforced using titanium(IV) coordination and self-polymerization of dopamine (crosslinker) along with the usual Ca^{2+}crosslinking [138]. Characterization studies revealed that dopamine was covalently attached preferentially to the mannuronic acid residues of alginate. Alginate beads reinforced with titanium (IV) coordination [Ca–Ti(AlgDA)] were synthesized by cross-linking with $Ti^{4+}$and Ca^{2+}. Reinforced alginate beads (Ca–AlgPDA) which were covalently cross-linked, were prepared by self-polymerization of dopamine and cross-linking with Ca^{2+}. Compared to those of Ca–Alg beads, the swelling of Ca–AlgPDA and Ca–Ti(AlgDA) were both subdued, plus the mechanical parameters were improved 3 times. The beads were utilized for the immobilization of alcohol dehydrogenase (YADH). Fig. 7 illustrates the optical images and schematic structural formulas of Ca–Alg beads (a and d), Ca–Ti(AlgDA) beads (b and e) and Ca–AlgPDA beads (c and f). The immobilization efficiency of Ca–Ti(AlgDA) and Ca–AlgPDA reached up to 100% and 89%, respectively, both notably higher than that of Ca–Alg (67.4%). Stabilization of the immobilized YADH in Ca–AlgPDA and Ca–Ti(AlgDA) toward pH, storage, and recycling were enhanced compared with those immobilized in Ca–Alg.

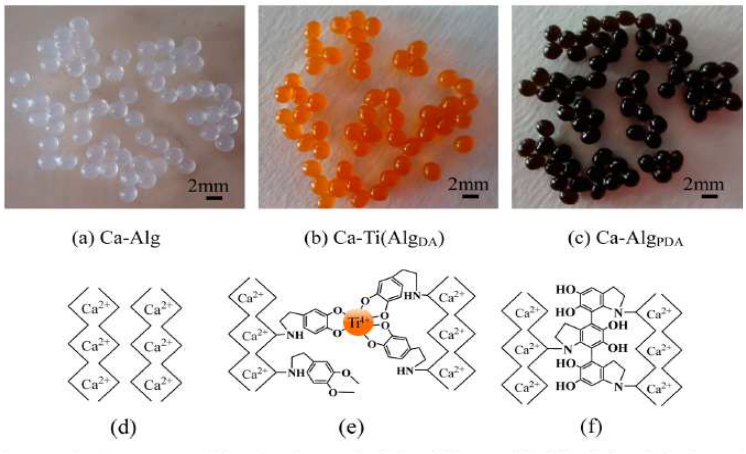

Fig. 7: Optical images and schematic structural formulas of Ca–Alg beads (a and d), Ca–Ti(AlgDA) beads (b and e) and Ca–AlgPDA beads (c and f) [138]. Reproduced with permission from American chemical society

Wen prepared novel biopolymer hydrogels that had interpenetrating polymer networks (IPN) composed of gelatin and alginate by a combination of enzymatic plus ionic crosslinking [139]. Using rheological techniques, the gelation procedure of the hydrogels was examined and the formation of dual networks; one gelatin network cross-linked by transglutaminase (TG), and another alginate network cross-linked by calcium ions was observed during crosslinking. It was revealed that the permutation of these types of crosslinking improved both tensile and compressive strength [20]. Preliminary studies have also revealed that the hydrogels can sustain cell adhesion and spreading. It can be observed that the majority of the cells were well spread on the gels. The quantity of cells per unit area between pure gelatin hydrogel and IPN gel containing alginate did not illustrate any major differences. Finally, at the end of 5 d, the growth of all the fibroblasts on hydrogels with a diverse concentration of alginate had reached 90-95%.

Photocross-linked alginate hydrogels draw extensive attention in tissue engineering due to their elevated controllability and firmness but also due to their high hydrophilic characteristics which reduce cell adhesion. Yin fabricated a hydrogel with a dual crosslinking network which involves initial photocrosslinking the basic backbone and later ionic crosslinking [140]. Ionic crosslinking involved the incorporation of variable amounts of magnesium ions (Mg^{2+}). It was observed that the ratio of Mg^{2+}ions can control various physicochemical properties of the alginate hydrogel such as surface structure, composition, swelling ratio, ion release along with elastic modulus [141]. Also, a particular amount of Mg^{2+}appreciably enhanced the attachment plus the spreading of osteoblasts on the hydrogels. Cell adhesion to the surfaces of Mg-0 and Mg-1 was 3.50±1.40% and 4.25±0.50% respectively showing that there was no difference among them ($p>0.05$). In the case of Mg-10 and Mg-100, there was a noteworthy raise in cell adhesion ($p<0.05$). Particularly for Mg-100, cell adhesion was nearly 15.69%. Thus Mg^{2+}incorporated photocross-linked alginate hydrogel showed potential as scaffold for bone tissue engineering.

Hertzberg reported the ionotropic gelation of alginate with bivalent cations such as Ca^{2+}along with photo-induced gelation of polyvinyl alcohol (PVA) containing photosensitive stilbazolium (SbQ) groups [142]. The diffusion properties and probable lethal effects were evaluated. The PVA-SbQ/alginate mixtures were utilized like carriers in a denitrification procedure with constant feeding of unsterilized water medium. Under these conditions, the PVA-SbQ mixture showed a longer life than natural alginate. Samorezov combined ionic and UV crosslinking to fabricate dual cross-linked hydrogels for achieving increased stiffness and decreased swelling compared to the individual ones [143]. The differences between shear storage, moduli, and swelling of many types of hydrogels were studied. It was seen that dual-crosslinking led to hydrogels with higher shear storage moduli compared to either calcium-or UV-crosslinking alone. There was enhanced cell proliferation on top of the surface of dual cross-linked

hydrogels compared to the calcium cross-linked hydrogel [144]. Photomasks were utilized to synthesize patterned hydrogels with clear cut spatial control over regions that were only calcium cross-linked against dual cross-linked. Also, dual crosslinking helped to spatially control cell attachment and proliferation using cell adhesion ligands.

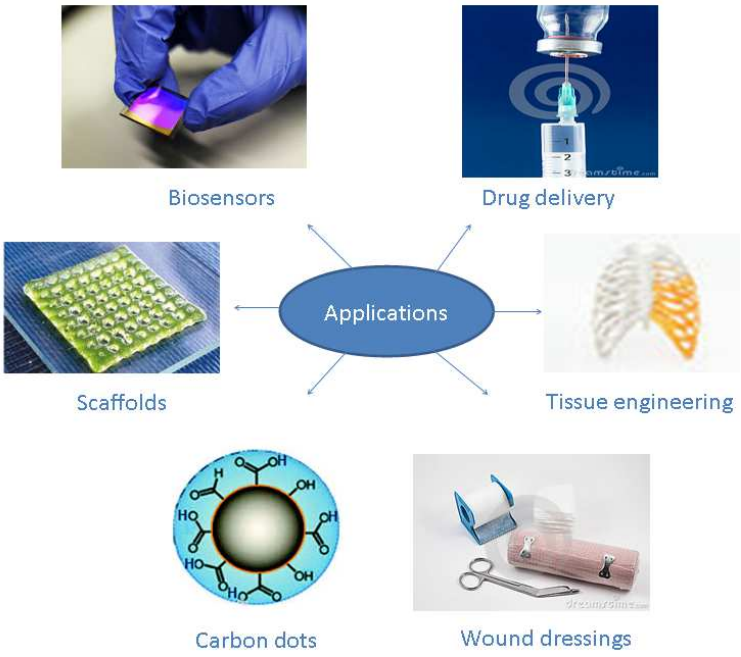

Fig. 8: Schematic representation of possible applications of cross-linked alginate

CONCLUSION

Alginates are versatile biopolymers with diverse applications. They are specifically suited for medical applications due to their biocompatibility and ability to be made into various shapes and sizes. Crosslinking has been the primary approach to modify alginate and make it suitable for desired applications. Films, fibers, hydrogels, beads made from alginate have been cross-linked using ionic, covalent, enzymatic and combination of these approaches. Covalent cross-linking provides alginate structures with good mechanical properties whereas enzymatic crosslinking would be preferable for medical applications. UV crosslinking in combination with other crosslinking has shown good potential to increase the stability of alginates. Although conventional chemicals such as glutaraldehyde and various ionic solutions have been used to effectively crosslink alginate, there is a need to develop highly efficient and green crosslinkers. Since it may be difficult to achieve desired properties using a single crosslinker, a combination of crosslinking and chemical/physical approaches may be necessary. Information on the various types of crosslinkers and their merits and limitations are presented here. Crosslinking provides multiple opportunities and the reader has to make a judicious choice of the methods and approaches depending on the intended application.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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