Pharmacological, Structural, and Drug Delivery Properties and Applications of 1,3-β-Glucans

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ABSTRACT: 1,3-β-Glucans are a class of natural polysaccharides with unique pharmacological properties and the ability to form single- and triple-helical structures that can be formed into resilient gels with the application of heat and humidity. The pharmacological capabilities of 1,3-β-glucans include the impartation of tumor inhibition, resistance to infectious disease, and improvements in wound healing. Curdlan is a linear 1,3-β-glucan that has been used extensively to study the nature of these helical structures and gels, and Curdlan sulfates have found ongoing application in the inhibition of HIV infection. 1,3-β-Glucan gels have been used in food science as stabilizers and encapsulating agents, in nanoscience as scaffolds to build nanofibers and nanowires, and in drug delivery to form nanoparticles and create helical micelles encapsulating polynucleotides. 1,3-β-Glucans are beginning to have enormous significance due to their dual nature as structure-forming agents and pharmacological substances, and research is especially focused on the application of these polymers in animal nutrition and drug delivery.

KEYWORDS: 1,3-β-glucans, Curdlan, schizophyllan, helix, freeze−thaw, antitumor, infection, nanostructure, nanoparticle, drug delivery, polynucleotide

■ INTRODUCTION

1,3-β-Glucans. Natural polysaccharides are an abundantly available resource from which to obtain unique properties applicable to a wide variety of industries. Cellulose is perhaps the most well-known example, with uses in paper manufacturing, membrane technology, textiles, and numerous food applications, and its nanocrystalline form is being used in high-strength polymer composites and novel bandage materials to speed wound healing.[1] Other notable examples are starch, xanthans, and others that are regularly used as freeze−thaw stabilizers, thickeners, and gelation agents in food science,[2] chitosan from shrimp shells with mucoadhesive properties that can facilitate ocular drug delivery,[3,4] and alginates from kelp that form hydrogels suitable as scaffolds for model extracellular matrices[5] or protein delivery vehicles that avoid protein denaturation during gelation.[6]

1,3-β-Glucans are a class of glucopyranose polysaccharides with (1,3) glycosidic linkages (Figure 1) and varying degrees of (1,6) branching obtained from fungal[7] or microbial sources.[8] An illustration of the fungal cell wall adapted from electron micrographs of *Candida albicans*[9] demonstrates the natural presence of β-glucans in fungi (Figure 2). 1,3-β-Glucans form helical structures that may be prompted to gel with the addition of heat and have a unique ability to increase host immunocompetency. Reported pharmacological effects include antitumor activity,[10−12] infection resistance,[13,14] cholesterol reduction,[15,16] and wound healing.[17−19]

The formation of 1,3-β-glucan helical domains may be utilized for many applications or, provided the polysaccharide concentration is high enough, allowed to continue to the formation of a macroscopic gel. The gelation profile is dependent on the degree of branching due to the effect of C(6) branching on helix

packing.[20] Curdlan, a linear 1,3-β-glucan, has been a good model for the study of 1,3-β-glucan helical structures as it lacks the interference of periodic branching.[21,22] Because the exact properties such as gelation profiles, solubility, and degrees of branching differ so drastically among the family of 1,3-β-glucans, discussion of the physical properties of these polysaccharides will follow the microbial 1,3-β-glucan Curdlan as a model. Subsequent discussion of applications will focus on the general family of 1,3-β-glucans. The unique properties of 1,3-β-glucans have led to a variety of applications including the formulation of food gels for consumption or to improve stability and nutrition,[23,24] direct therapeutic application,[25,26] encapsulation and controlled release of various bioactive species,[27,28] and application as helical scaffolds for nanostructure formation.[29,30]

■ PHYSICAL PROPERTIES OF CURDLAN 1,3-β-GLUCANS

Overview. Curdlan was first discovered as a resilient gel-forming polysaccharide bearing β-glycosidic linkages that was biosynthesized from the soil bacterium *Alcaligenes faecalis* var. *myxogenes* in the mid-1960s.[31,32] Curdlan was found to be a linear 1,3-β-glucan that was insoluble in water but soluble in alkaline solutions. Experimentation with the gelation characteristics of Curdlan began shortly afterward.[33,34] Alkaline solutions inhibit hydrogen bonding between C(2) hydroxyls, inhibiting helix formation and leaving the random coil state. Commercial alkali treatment leaves most available Curdlan powder <30% crystalline with a prevalence of a mixture of random coils and some
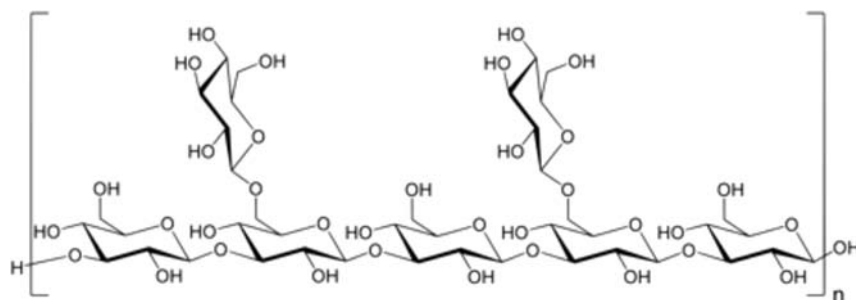
Figure 1. Schematic of a 1,3-β-glucan. 1,3-β-Glucans consist of glucopyranose monomers joined by glycosidic ether linkages between C(1) and C(3) in the glucopyranose rings wherein the hydroxyl groups on C(1) and C(3) form the β configuration and branch points often occur periodical in the (1,6) configuration.
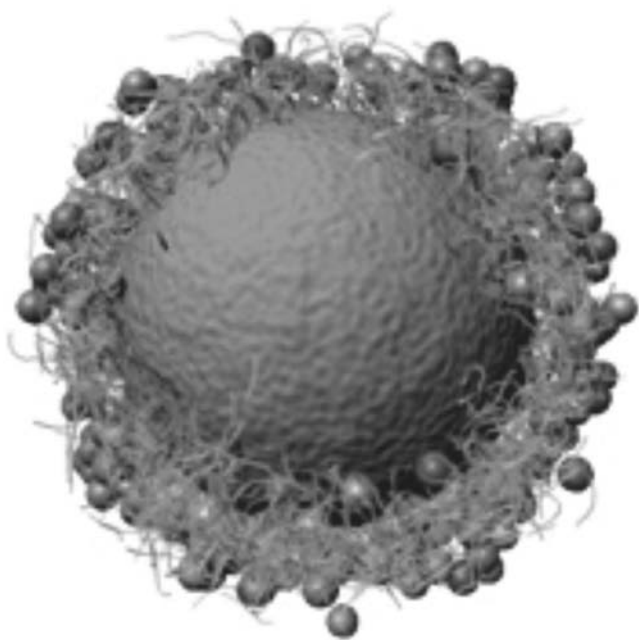

Figure 2. Schematic of a fungal spore. Adapted schematic (from ref 9) of the fungal cell wall containing a composite of chitin and β-glucans with interspersed mannoproteins (spheres) surrounding the phospholipid membrane.

single and triple helices.[35] This partially crystalline native form may be thermally treated in water to induce hydrogen-bonded crystallinity and the incorporation of water molecules into helical structures to form a hydrogel. This hydrated, crystallized form may then be dehydrated to further improve crystallinity and reswelled at will.

Helical Conformations in Curdlan. Helical polysaccharide structures held together by O(2) hydrogen bonding in 1,3-linked polysaccharides were hypothesized in 1968.[36] As stated previously, Curdlan may form both single-helical and triple-helical structures with some disagreement in the literature as to the relative populations of these species.[37] Single helices form via intrachain hydrogen bonding between O(2) hydroxyls on glucopyranose residues.[38] Gelation of Curdlan causes single helices to rearrange hydrogen bonds to form interstrand hydrogen bonding and hence the formation of the triple helix.

X-ray diffraction uncovered the first evidence of the triple-helical structures in gelled and dehydrated Curdlan. A right-handed triple-helical model was assigned with six glucose residues

per turn and a monomer advance of 2.935 Å (Figure 3a,top).[21] A model projection of the Curdlan triple helix is shown in Figure 3b (top) with the six glucose residues (two from each single helix) in each turn.[38] The periodic crystal of Curdlan shows four triple helices associating via O(4) and O(6) hydrogen bonding to produce longer range order (Figure 4). The unit cell would be drawn as a parallelogram from the center of each of the four helices, showing the presence of six glucose residues in the unit cell. It is of interest to note that the hydroxymethyl groups on C(6) decorate the outside of the triple helices, not affecting the internal structure but being critical for longer range order. For applications then, functionalization at the C(6) position would be inconsequential to the triple helices themselves but may change the overall crystallinity and gelation profile.[21]

Investigation of hydrated Curdlan in comparison to the dehydrated form began in 1983. The crystalline arrangements were similar between the two forms except for the dimensions of the unit cell showing an increased fiber repeat parameter, indicating a loss of symmetry and an increase in the volume of the unit cells in the hydrated state. The increased volume was suggestive of the incorporation of significant amounts of water into the triple helix. Approximately 18−36 molecules of water were found per unit cell that all contributed significantly to the hydrogen bonding of the helical structures.[22]

Although these studies are generally considered to be conclusive as to the crystallographic forms of Curdlan, there still exists some controversy. The presence of two additional hydrogen-bonding schemes has been postulated. Besides the originally proposed interstrand hydrogen bonding (Figure 3a, top), the association of single helices without interstrand hydrogen bonding, instead relying on van der Waals forces to maintain the triple helix, has been found to be favorable via simulation based on an improvement in the linearity of the H-bond, despite an unfavorable increase in bond length (Figure 3a, bottom right). A third H-bonding scheme that maintains interstrand bonding to hold the triple helix together but requires O(2) H-bonds with adjacent atoms has also been proposed, ultimately requiring a left-handed helix instead of the traditional right-handed helix (Figure 3a, bottom left). Considering heat of formation and bond energy, the new hydrogen-bonding structures were thermodynamically favored in simulation.[38] The existence of these new hydrogen-bonding structures has not yet been verified experimentally, but they offer insight into the transition of bonds that may occur during crystallization as single helices aggregate and rearrange hydrogen bonds to form triple helices.

Current Understanding of Curdlan Gelation. Curdlan gels have been studied since 1967 wherein gelation was observed

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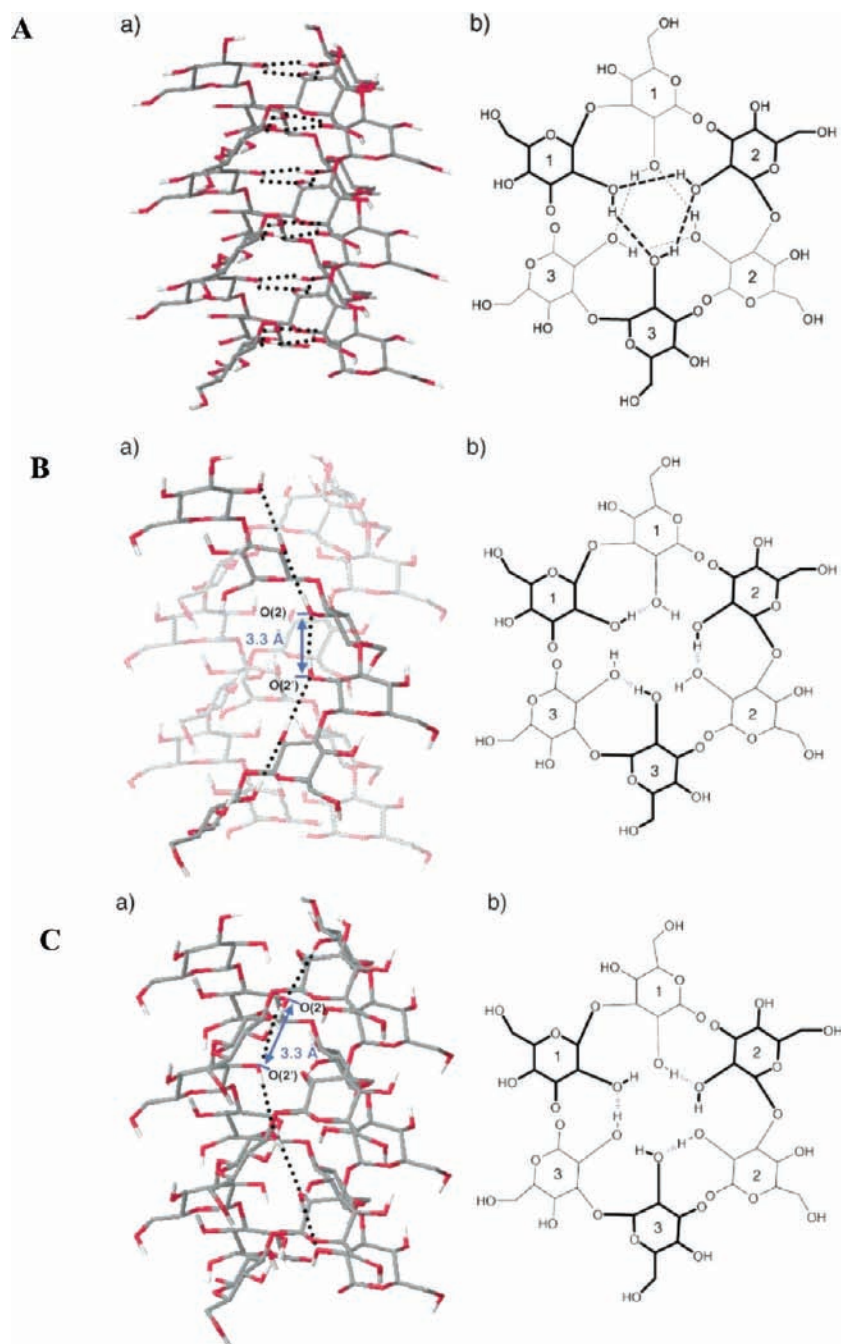dx.doi.org/10.1021/jf200964u |*J. Agric. Food Chem.* 2011, 59, 6813–6828

Figure 3. Triple-helical hydrogen bonding schemes for Curdlan: (A) original right-handed triple helix with interstrand H-bonding; (B) left-handed triple helix with interstrand bonding; (C) right-handed triple helix with intrastrand H-bonding; (a) model of triple-helical formation; (b) $X-Y$ projection of helical strands. Reproduced with permission from ref 38. Copyright 2004 Wiley.

above 55 °C when heated from aqueous or dilute alkaline solutions.[34] Curdlan gels may be obtained by simply hydrating Curdlan under heating or by neutralization from alkali solution.[35] The general methods for the gelation of Curdlan follow (Table 1).

Gelation is normally carried out from 10% (w/v) suspensions in water, dilute alkaline solution, or buffer[39] or with 10% solutions in DMSO that are extruded into methanol and dried before heating under humidity.[21] Gelation can be separated into two distinct regimens. Treatment between 55 and 80 °C induces the formation of a low-set gel. Low-set gels may dissociate upon

cooling, but they may be further treated to strengthen the gel into the next regimen. Treatment between 80 and 145 °C induces the formation of a thermally irreversible high-set gel that will not melt and cannot be further crystallized.[39] Gelation is also possible by neutralizing a 0.1 M NaOH solution of Curdlan with 0.1 M HCl and purifying the gel suspension by dialysis.[40] Dissolution of crystalline Curdlan may be carried out using DMSO, a good solvent for Curdlan, that disrupts hydrogen bonds or by increasing alkaline concentration.[41,42] The gelation mechanism of Curdlan has been heavily investigated in the literature (Table 2).
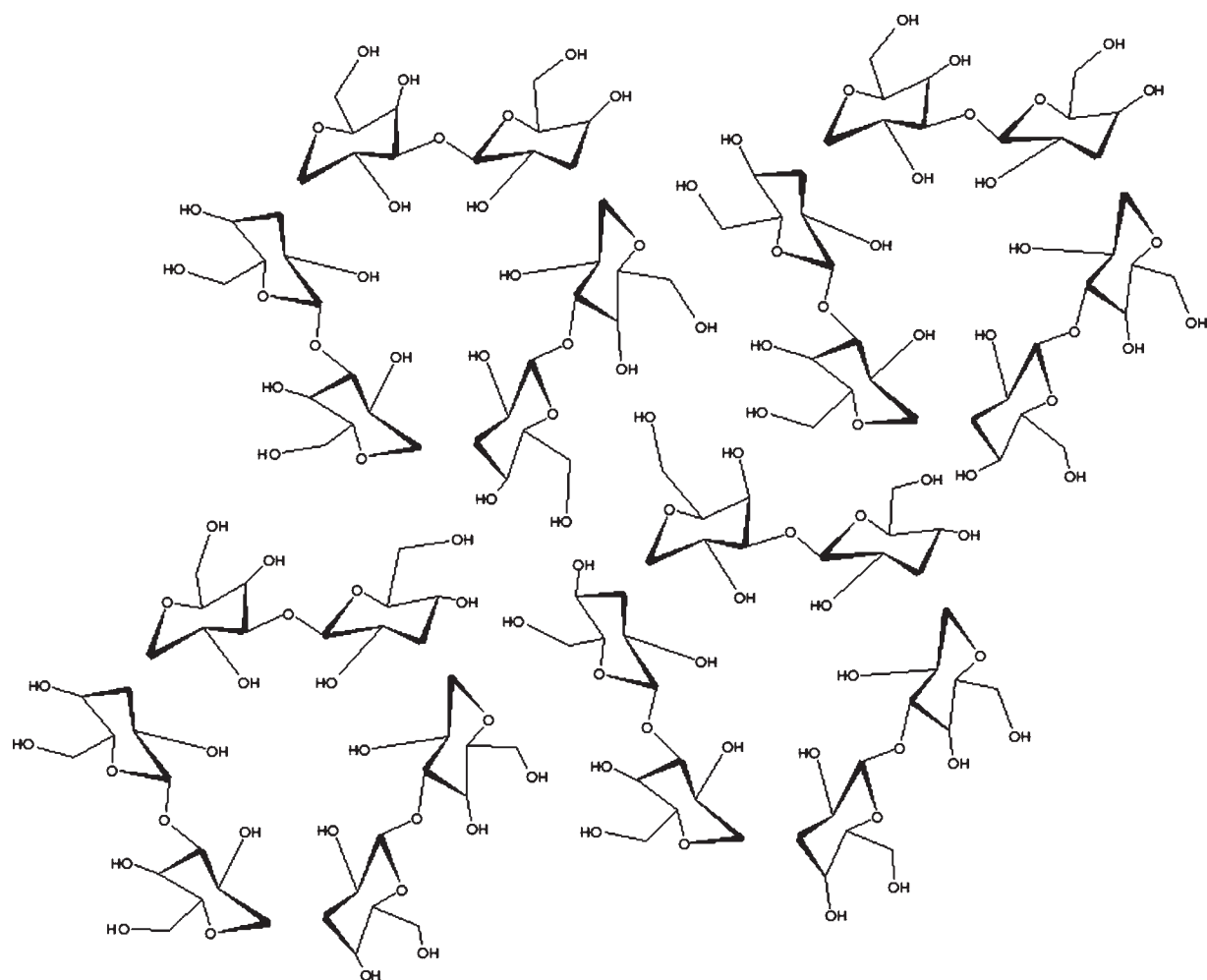
Figure 4. Periodicity in highly crystalline Curdlan: four triple helices held together by hydrogen bonding between O(4) and O(6) oxygens.

Table 1. Methods of Curdlan Gelation

type	crystallinity	method
native	<30%	N/A
Curdlan hydrate	high	55−145 °C in humidity or from suspension neutralization of alkaline solution
dry Curdlan	very high	dry Curdlan hydrate

^{13}C nuclear magnetic resonance (NMR) has been used to observe the proportions of single to triple helices in Curdlan gels formed from aqueous and dilute alkaline suspensions. Significant line broadening for the six carbon peaks between 60 and 100 ppm occurred due to the lack of chain mobility in the triple-helical structures (Figure 5, left) with these peaks sharpening slightly as the alkaline concentration was increased and then drastically sharpening around 0.22 M NaOH, indicative of the formation of random coils (Figure 5, right). This behavior is consistent with studies on the loss of crystallinity in alkaline solutions.[42] It was reasoned that the entirety of the ^{13}C signal was due to single-helical structures due to the immobilization of chains in multiple-helical networks, so it was concluded that Curdlan gels might contain more significant populations of single helices.[37]

Table 2. Characterization Methods for Curdlan

method	parameter(s)	ref
nuclear magnetic resonance	^{13}C peak intensity and width	37
electron microscopy	direct observation	40
differential scanning calorimetry	enthalpy change	43
attenuated total reflection infrared spectroscopy	infrared band shifts	39
rheology	storage modulus	35
nuclear magnetic resonance relaxometry	chain mobility	35
atomic force microscopy	direct observation	45
single-molecule force spectrsoscopy	interaction forces	46

Electron microscopy (EM) has been used to obtain images of the gelling behavior of Curdlan. SEM images revealed 30 Å fibrils in 100−200 Å bundles with micrographs clearly showing the similarity in helical conformations formed by heating to low-set gels and neutralization, both forming rope-like Curdlan fibers (Figure 6A,B). Images of samples heated to 90 °C after heating at 60 °C (Figure 6C) demonstrated that low-set gels may be further crystallized to high-set gels. Reheating at higher temperatures (120 °C) resulted in a return to the less ordered structure
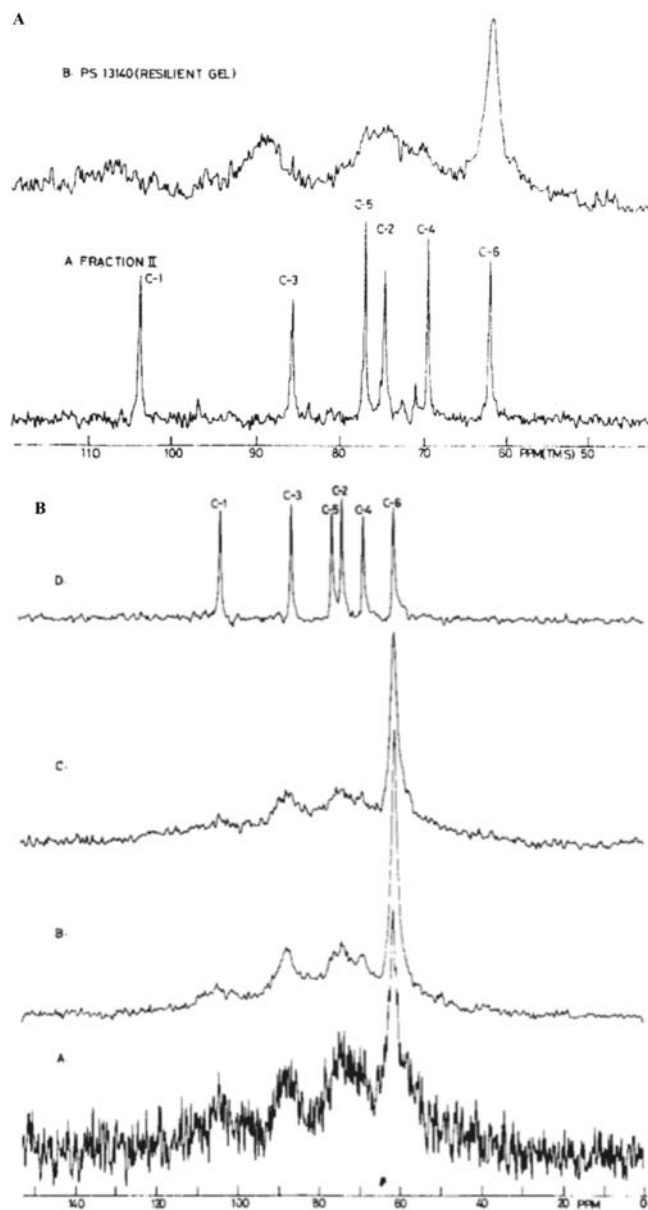
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Figure 5. Peak broadening and effect of alkaline concentration on the ^{13}C NMR spectra of Curdlan. The six ^{13}C NMR peaks of Curdlan show significant broadening when moving from native Curdlan (A, bottom) to the resilient gel (A, top), and the peaks sharpen when exposed to increasing concentrations of NaOH solution (B): (A) aqueous suspension; (B) 0.06 M; (C) 0.19 M; (D) 0.22 M. Reproduced from ref 37. Copyright 1977 American Chemical Society.

(Figure 6D). The formation of the low-set gel was proposed to be primarily composed of hydrogen bonds that initially break when heated to release microfibrils and then reorganize to rebuild greater crystallinity. High-set gels were believed to gain additional strength from hydrophobic interactions.[40]

Differential scanning calorimetry (DSC) demonstrated the temperature-induced gelation of Curdlan in an aqueous suspension. Endothermic swelling was observed at 56 °C, highest crystallization at 142 °C, and melting of the gel at 154 °C (Figure 7). Thus, low-set, thermally reversible gels that may be further crystallized are created in the temperature range of 60–80 °C, containing small amounts of triple helices, and high-set,

thermally irreversible gels are created at temperatures up to 150 °C with much higher crystallinity.[43]

Attenuated total reflectance infrared spectroscopy (ATR-IR) allowed observation of reversible band shifts assigned to the formation of hydrogen bond networks with water that were observed after only 5 min at 30 °C. Under heating, the band at 1110 cm^{-1} shifted at 55.9 °C, verifying the DSC characterization gelation scheme. High-set gels were produced at 95 °C with <10% reversibility possible as monitored by the ratio of the peaks at 1080 and 1045 cm^{-1} with low-set gels showing much more reversibility.[39]

Rheological studies measuring the storage modulus of Curdlan gels throughout the gelling temperature range demonstrated that there is an initial increase in storage modulus before the low-set gel temperature as single helices aggregate followed by a decrease as these helices break hydrogen bonds and then a gradual increase as the helices reorganize to form more crystalline triple-helical structures.[35] This result confirms the initial breaking of bonds observed in the formation of the low-set gel.[40]

NMR relaxometry studies further confirm this result by measuring chain mobility during temperature ramping, showing a characteristic decrease in chain mobility as the single helices aggregate, followed by an increase as they melt, and a decrease as triple-helix crystals are formed. It was also demonstrated that holding temperature in the gelation regimen initiated an annealing of triple-helix crystals with the implication that the terminology of discrete low-set and high-set gels is perhaps misleading as a continuum of states exists.[35]

AFM studies have been carried out on both Curdlan and branched 1,3-β-glucans, allowing the observation of fiber dimensions.[44,45] Three nanometer thick fibers were observed for Curdlan that formed network structures of fibers on mica from 0.01 M NaOH suspensions (Figure 8, left). Variation of the height confirmed the heterogeneity of Curdlan at this lower alkaline concentration. Higher concentrations of alkali (1 M NaOH) demonstrated the thickness of fibers falling to 0.5 nm in some places, but the network structure was still intact in places, suggesting incomplete solubility even at high concentrations of NaOH (Figure 8, left). The observation of network structures even at high alkaline concentrations suggested that the thermal gelation mechanism involved partial breaking off of random coils from parent fibers that form triple helices to cross-link the parent fibers (Figure 8, middle, right). The previously observed increase in hydrogen bonding followed by bond breakage before triple-helix assembly suggests that collapsed random coils might form intramolecular hydrogen bonds to single helices that then associate with other single helices, break intrastructural hydrogen bonds, and then re-form interstrand hydrogen bonds to create triple-helical structures. Hydrophobic interactions then also play a key role in holding the triple helices together.[45]

Curdlan has been investigated with single molecule force spectroscopy (SMFS) to elucidate the transitions that occur during increased concentrations of sodium hydroxide. Experimentation on 0.5 M NaOH solutions of Curdlan demonstrated the stretching of single Curdlan chains, indicative of random coils of Curdlan in alkaline solutions of this concentration. A helix–coil transition was observed in 0.1 M NaOH with a 60 pN force proposed to be required to begin the unwinding of triple-helical structures. At 0.2 M NaOH, it was believed that the observed phenomenon was the unwinding of single helices from duplex structures, as the unwinding force was only 40 pN. This transition from triple helices to single helices and then random coils
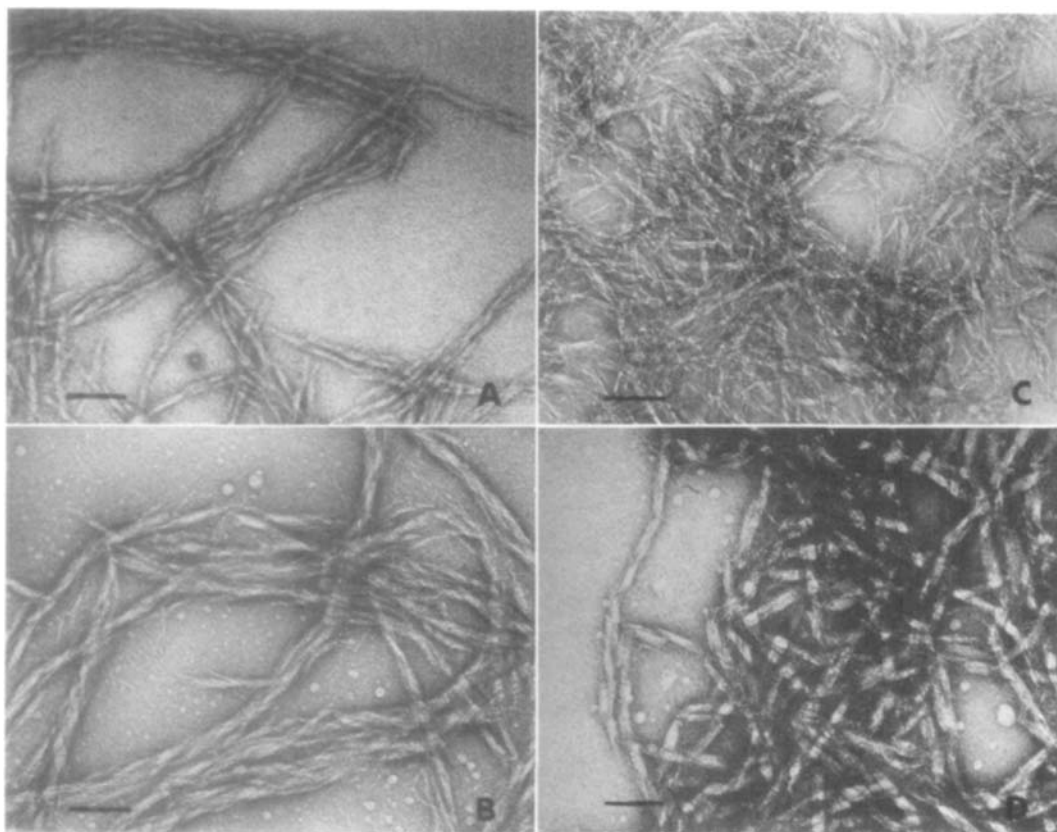
Figure 6. Electron micrographs of Curdlan gels: (A) Curdlan gelled by neutralization shows 100−200 Å bundles with (B) heating to low-set gel at 60 °C improving crystallinity, (C) reheating at 90 °C showing further increases into the high-set regimen, and (D) reheating at 120 °C showing a decrease in crystallinity. Reproduced with permission from ref 40. Copyright 1979 Oxford University Press.
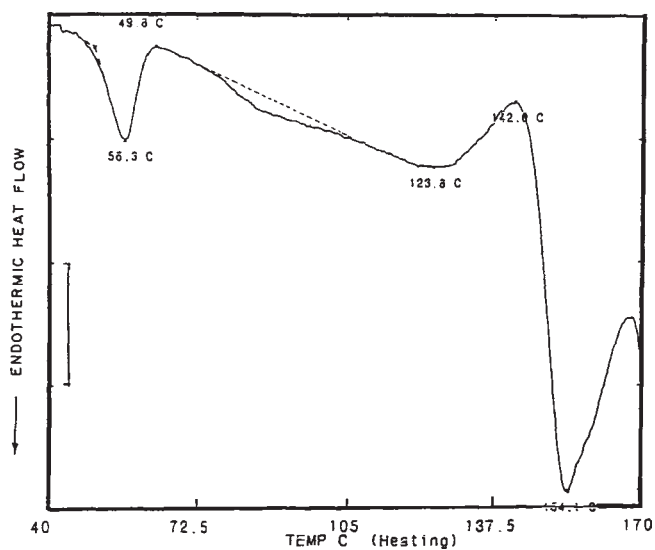

Figure 7. Differential scanning calorimetry study of Curdlan. DSC of a 4% aqueous suspension reveals an initial endothermic event at 56 °C as Curdlan is heated to a low-set gel and another event at 142 °C when Curdlan reaches its highest point of crystallinity before melting. Reproduced with permission from ref 43. Copyright 1991 CRC.

from 0.19 to 0.24 M NaOH agreed well with prior predictions and elucidated a possible intermediate duplex state between triple helix and random coil that may be involved in gelation.[46]

Despite the extent of characterization efforts, the exact mechanism for gel formation of Curdlan is disputed. Some harmony is materializing from the use of more visual techniques such as AFM, explaining some of the otherwise strange bond breakages and formations observed via rheology and NMR studies. Despite this, AFM studies have introduced confusion of their own by showing the presence of random coil networks even at very high alkaline concentrations. The possible mechanism of cross-linking of these fibers by the increasing populations of single and triple helices to create Curdlan gels is a promising lead. It is the prevalence of complex mixtures of random coils, single helices, and triple helices at different alkaline concentrations and temperatures that has made the elucidation of the mechanism of Curdlan gelation a moving target as characterization techniques improve to gain a better understanding of these populations.

■ BIOACTIVITY OF 1,3-β-GLUCANS

The interaction of 1,3-β-glucans with immune cells generates a potent pro-inflammatory effect consisting of stimulation of the production of cytokines, increased phagocyte and lymphocyte proliferation, oxidative burst, and phagocytosis against opsonized tissues. This pro-inflammatory effect has led to a number of therapeutic applications using 1,3-β-glucans to impart tumor inhibition, disease resistance, and wound healing. In addition, β-glucans from oats and barley that contain some 1,3-β linkages among a predominance of 1,4-β linkages are known to be effective in lowering low-density lipoprotein (LDL) cholesterol. The importance of the physical properties of β-glucans in
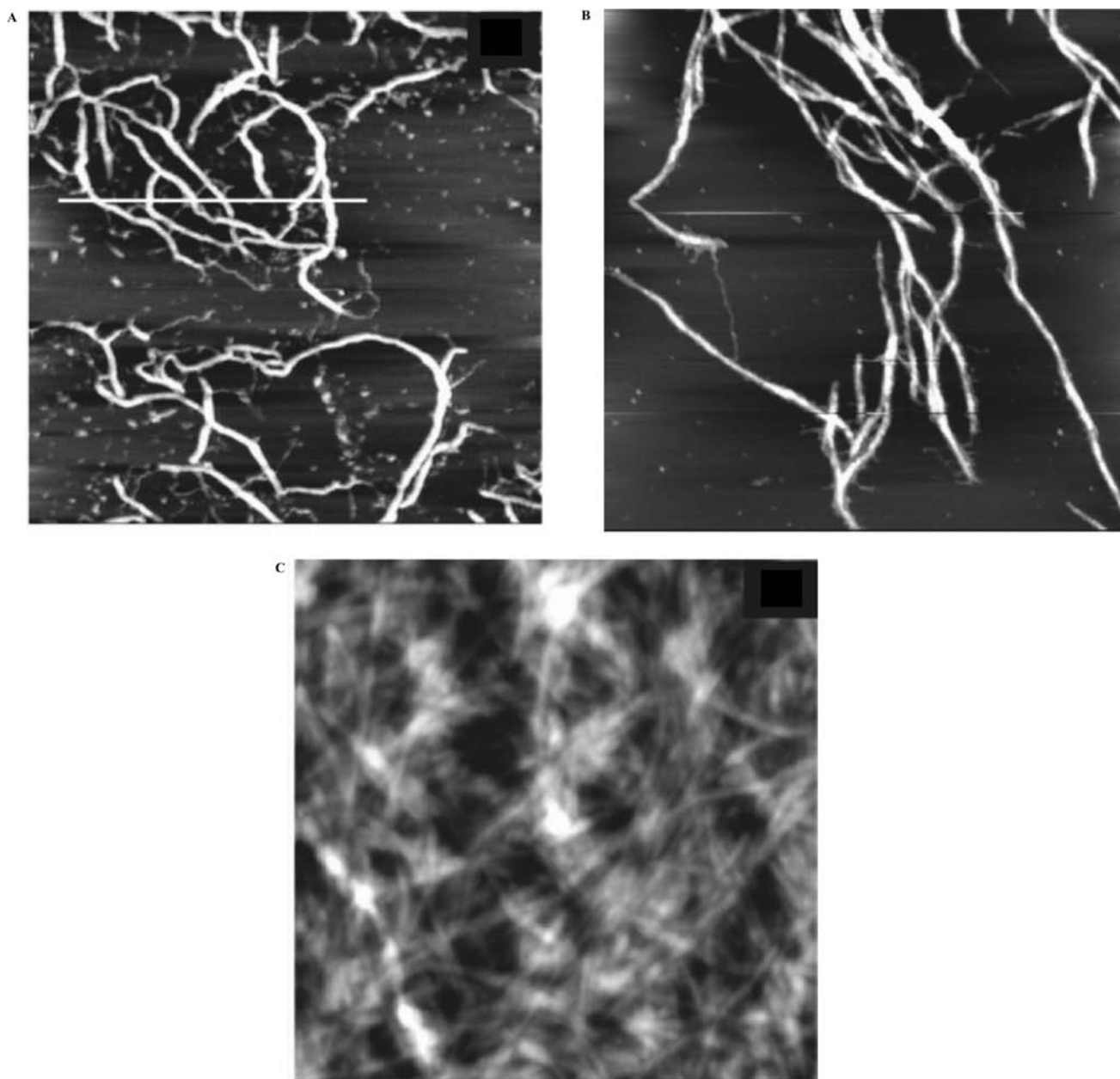
Figure 8. Atomic force microscopy scans of Curdlan gelation: (A) 1 M NaOH solution of Curdlan demonstrates the continued presence of a network structure with 1 nm microfibrils extending from parent fibers; (B) 0.1 M NaOH suspension of Curdlan that was heated at 90 °C for 4 h showing the heat-induced dissociation of previously observed microfibrils; (C) bulk-gel precursor from the same suspension demonstrating the formation of densely cross-linked networks attributed to cross-linking by the dissociated microfibrils. Reproduced from ref 45. Copyright 2005 American Chemical Society.

determining these results is under clinical investigation. 1,3-β-Glucans interact with the human body using a number of different pathways to accomplish their pharmacological and nutritional effects. The most studied pathways are presented here.

Activation of Alternative Complement Pathway. Zymosan, a fraction of sterilized cell wall from *Saccharomyces cerevisiae* containing 70% β-glucans, was known to be an initiator of the alternative complement pathway since the discovery of the properdin pathway in 1954, forming an active complex with properdin that allowed the consumption of C3b, complement cascade, and the destruction of the pathogen.[47] Interaction with C3b initiates the formation of membrane attack complex (MAC) and the generation of opsonins, among many other processes.

Kinetic studies on the formation of zymosan—properdin complexes were carried out, and the involvement of factor B in the process of C3 and C5 consumption was demonstrated.[48] Later, it was verified that neutral 1,3-β-glucans could yield a prominent activation of the alternative complement pathway, ruling out the hypothesis that zymosan activation occurred due to the presence of phosphate groups in the unpurified fraction.[49]

Interaction of 1,3-β-Glucans with Phagocytes and Lymphocytes. The antitumor activity of zymosan was originally believed to be due to a lipid fraction until it was demonstrated that isolated polysaccharide fractions of zymosan decreased the half-life of colloidal carbon 10 times when administered to mice by generating hyperplasia and hyperfunction in immune cells.[50]

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The cellular membrane receptor on phagocytic macrophages for the binding of 1,3-β-glucans was the focus of a large volume of research, with the candidates being a complement receptor or Dectin-1, both of which were found to be dependent on iC3b opsonization, the proteolytically deactivated form of the complement protein C3b. It was demonstrated in 1986 that complement receptor 3 (CR3) was responsible for the binding of opsonized zymosan.[51] In 1996, it was demonstrated that macrophages bearing CR3 were capable of attacking any iC3b-opsonized pathogen in the presence of 1,3-β-glucans.[52] The complement protein C3b normally coats pathogens, marking them for detection by complement receptor 1 (CR1) or activation of the complement cascade. The deactivation of C3b to iC3b is a regulatory mechanism to avoid unnecessary immune activity. Because iC3b is recognized by CR3 in the presence of 1,3-β-glucans, CR3 and the iC3b opsonin play a critical role in innate immunity toward fungal and microbial pathogens, mediated by the presence of 1,3-β-glucans in these pathogens. Independently delivered 1,3-β-glucan could then yield a targeting of cancer cells with surface coatings of iC3b.[52]

Using murine models, the roles of CR3 and Dectin-1 were compared for the binding of unopsonized zymosan. It was demonstrated using fluorescent labeling that CR3 deficiency was inconsequential in murine models.[53] Further evidence for this result was provided in 2003 when the production of a cytokine, tumor necrosis factor α (TNF-α), was used as a measure of receptor efficacy when macrophages were retrovirally transduced to produce large amounts Dectin-1. The transduced macrophages bound more unopsonized zymosan and showed a dose-dependent secretion of TNF-α.[54]

In the most recent studies with human neutrophils, no phagocytosis of unopsonized zymosan was observed when CR3 was blocked, no zymosan binding was possible in CR3-deficient humans, and the efficacy of respiratory burst was inconsequential with Dectin-1 primed zymosan compared to plain zymosan. It was concluded that although Dectin-1 plays an essential role in mediating the interaction of 1,3-β-glucans with immune cells in murine models, functional CR3 was required for the efficient binding and response to both opsonized and unopsonized zymosan in humans.[55] Figure 9 shows two elements critical to the structure of CR3, the β-chain that interacts with a 1,3-β-glucan and the α-chain that interacts with an iC3b opsonin. The binding of 1,3-β-glucans to the β-chain initiates cell stimulation as well as a conformational shift in the α-chain that allows CR3 to bind iC3b-opsonized pathogens (Figure 10).[56]

Although most studies focus on the binding of 1,3-β-glucans to CR3 or Dectin-1 on phagocytes, the primary form of activity of many 1,3-β-glucans such as lentinan is interaction with and stimulation of T-cell and natural killer cell activity to promote acquired immune response and lysis by other means such as the release of perforins and granzymes. Lentinan also interacts with T-cells and natural killer cells to promote proliferation of the cells themselves or proliferation with desirable IL-2 cytokine receptors.[57,58] Very recently, increases in lymphocyte proliferation were observed as a result of all 1,3-β-glucan treatments, with Curdlan at concentrations up to 800 μg/mL yielding the greatest effect.[59] Thus, more research into the effects of 1,3-β-glucans on lymphocyte populations is necessary to expand the current understanding of pharmacological effects.

Pharmacological Effects of 1,3-β-Glucans. 1,3-β-Glucans stimulate the production or induction of a variety of pro-inflammatory mediators and cytokines including TNF-α, interferon-γ

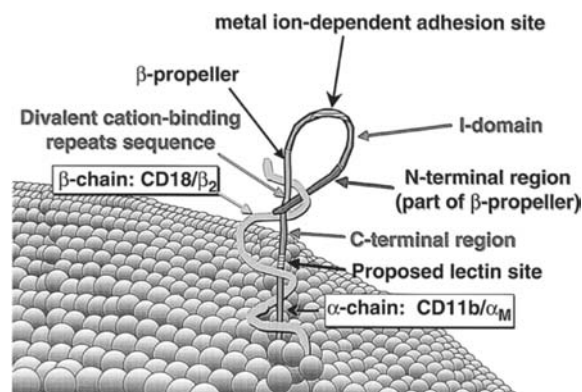

Figure 9. Schematic of the domains of complement receptor 3. Complement receptor 3 consists of the β-chain, which interacts with 1,3-β-glucans, and the α-chain that experiences a conformation shift, allowing the recognition of iC3b opsonins. Reproduced with permission from ref 56. Copyright 1999 International Journal of Immunopharmacology.
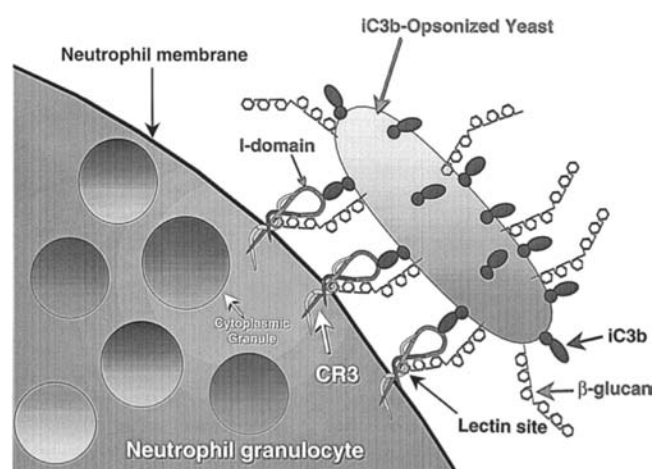

Figure 10. β-Glucans initiate a conformational shift to allow response to iC3b ppsonin. Under normal circumstances, CR3 dual interactions with 1,3-β-glucans and iC3b allow for the recognition of fungal pathogens, but this system has been leveraged for cancer therapy. Reproduced with permission from ref 56. Copyright 1999 International Journal of Immunopharmacology.

(IFN-γ), granulocyte-macrophage colony stimulating factor (GM-CSF),[60] inducible nitric oxide synthase (iNOS), macrophage inflammatory protein 2 (MIP-2), and a host of interleukins (IL-1Iβ, L-8, IL-10, IL-12, IL-4, IL6).[59] This stimulation was demonstrated to occur at the mRNA level with MIP-2 and TNF-α similar to lipopolysaccharide (LPS) stimulation.[61,62] The production of these pro-inflammatory mediators is linked to activation of nuclear factor-κB (NF-κB). A recent study has highlighted the critical role of a phospholipase C enzyme (PLC-γ2) and a calcium ion flux in the intracellular signaling resulting from receptor activation.[63]

Oxidative burst is an innate immune response to pathogens external to or internalized by phagocytes that is characterized by the release of reactive oxygen species (ROS). Different types of ROS include peroxides and peroxynitrites formed from superoxide anions and nitric oxide that are responsible for peroxidation of lipids and proteins and the cytotoxicity of microbes. Curdlan was shown to effectively mediate the induction of iNOS

and production of nitric oxide in vitro in rat macrophages.[61] Very recently, a variety of 1,3-β-glucans were shown to stimulate the production of ROS in differing amounts dependent on branching and chain length.[59]

Phagocytosis of pathogens such as opsonized cancer cells in response to 1,3-β-glucan stimulation has been studied much less than the corresponding oxidative burst and cytokine-producing pharmacological effects in the recent literature. However, good theories exist as to the mechanism of this behavior.[56] Elimination of pathogens or cancer cells can also occur by acquired cellular immune pathways involving cytotoxic T-cells or natural killer cells as was initially demonstrated with lentinan[57] and later demonstrated with most 1,3-β-glucans.[59]

The pharmacological effects of 1,3-β-glucans are heavily dependent on the specific conformation and structure of the polysaccharide. When macrophage activation by 1,3-β-glucans was studied in vitro, macrophage activation was observed with the addition of 10−100 μg/mL and enhanced by pretreatment with sodium hydroxide, dimethyl sulfoxide, or zymolase to produce the single-helix conformation. In addition, longer 1,3-β-glucans were found to be necessary as shorter Laminarin oligosaccharides showed much less activity.[62] These findings were consistent with early results suggesting that the single helix was critical[37,64] and that shorter 1,3-β-glucans could not produce iC3b recognition in CR3.[52] The effect of molecular weight is also significant in LDL cholesterol reduction, with low molecular weight oat β-glucans having insignificant results.[15] In the most recent studies, these effects along with the effects of branching continue to be investigated.[65] Despite the fact that the removal of branching was the only way to generate an immunomodulatory response from pachyman,[10] other studies suggest that a complex branching structure in fungal 1,3-β-glucans may be more effective.[12] No firm conclusion yet exists in the literature pointing to the most potent conformation and structure of a 1,3-β-glucan for therapeutic usage, but it is generally accepted that the single helix is critical and higher molecular weight polysaccharides are superior.

■ PHARMACOLOGICAL APPLICATIONS OF 1,3-β-GLUCANS

Cancer Inhibition. Clinical studies on the 1,3-β-glucan lentinan for use in treating neoplastic diseases began in 1983.[66,67] Lentinan has been used for cancer immunotherapy in Japan since 1986 for the treatment of lung, gastric, and cervical cancers[25] and is currently receiving significant attention in clinical trials focusing on combination therapies with radiofrequency ablation (RFA) and transcatheter arterial chemoembolization (TACE) treatments for hepatocellular carcinoma patients.[68,69] In the early studies on lentinan with subcutaneous implantation of sarcoma 180 into mice, intraperitoneal injection of 1−4 mg/kg lentinan over 10 days yielded a tumor inhibition of 99.6%, and inhibition ratios of 96% were also found with pachymaran, the synthetically debranched form of pachyman.[10] A number of theories on the nature of the antitumor effects have arisen in the literature. The effects of lentinan and pachymaran are thought to rely heavily on T-lymphocytes as the removal of the thymus or treatment of mice with antilymphocyte serum (ALS) virtually eliminated antitumor effects.[57] This was verified in 1992 when lentinan was found to increase natural killer cell activity and the proliferation of T-cells with IL-2 receptors.[58] Experiments with bovine serum albumin (BSA) showed that denaturation of protein

helices could be linked to the antitumor mechanism of lentinan as only those polysaccharides that denatured the protein showed antitumor activity.[70] The generation of new serum proteins was also thought to be linked to the T-cell-mediated effects of lentinan.[71] 1,3-β-Glucan derivatives such as pachyman, pachymaran, and corresponding derivatives have been found to activate the alternative pathway of complement, involving factor B, as opposed to the T-cell-mediated route.[49]

Throughout the literature, other 1,3-β-glucans showing similar effects against solid sarcoma 180 were discovered. In the early studies in the 1970s, scleroglucan was found to produce inhibition ratios of 90.4% at a dose of 2.5 mg/kg, and Curdlan was found to inhibit tumors from 99 to 100% with doses ranging from 10 to 20 mg/kg.[11] In 1985, a branched 1,3-β-glucan, grifolan, was found to inhibit solid sarcoma 180 up to 97.9%.[72] In 2001, a complex branched 1,3-β-glucan from *Agaricus blazei* was found to inhibit up to 99.3% of tumor growth.[12] The robust antitumor effects of various 1,3-β-glucans have led to the modern use of 1,3-β-glucans as adjuvants to monoclonal antibody treatments. Human tumor xenografts from melanoma, epidermoid carcinoma, breast carcinoma, metastatic lymphoma, and daudi lymphoma were affected positively by combination treatments of 1,3-β-glucans and antibodies, yielding tumor inhibition and survival rates greater than those of individual treatments.[73] A recent review considers the topic of combined treatments for cancer immunotherapy in more detail.[26]

To improve the usability of 1,3-β-glucans, a significant amount of work has gone into the development of water-soluble derivatives that retain antitumor efficacy. Early work with the development of water-soluble carboxymethylpachymaran showed tumor inhibition ratios up to 99.6% in solid sarcoma 180 with doses of 5 mg/kg, equal with lentinan. Interestingly, the use of the water-soluble derivative allowed for the use of other injection routes besides intraperitoneal injection to obtain the same result.[74] Other 1,3-β-glucans including hydroxymethylpachyman and hydroxypropylpachyman showed up to 100% inhibition at 5 mg/kg in solid sarcoma 180.[74] In 1979, various water-soluble carboxymethylglucans were synthesized that also showed high antitumor activity.[75] Carboxymethyl Curdlan in particular is used extensively in modern applications. Water-soluble glucosyl, sulfethylated, and sulfopropylated Curdlan derivatives also retain antitumor activity.[76,77] All of these results suggest that solubility of the 1,3-β-glucan is not a critical factor for antitumor activity.

Infection Resistance. Because 1,3-β-glucans cause cytokine production, oxidative burst, immune cell proliferation, and increases in phagocyte and lymphocyte activity, 1,3-β-glucans have been investigated for use in increasing overall host immunocompetency. In 1978, it was demonstrated that mouse survival rates for immune challenge with staphylococcal infection increased dramatically, with 3% mortality observed in 1,3-β-glucan-treated mice versus 30% in the control group.[78] More recently, oral administration of 1,3-β-glucans to promote resistance to fungal and bacterial infection has been attempted with a full pharmacokinetic characterization demonstrating the survival rate for *Staphylococcus aureus* bacterial and *Candida albicans* fungal challenge increasing by 50% in mice through oral administration.[14]

This ability to impart infection resistance has been of growing interest with a growing volume of work attempting to increase immunocompetency in fish and pigs. In 1991, Curdlan, inulin, Krestin, laminarin, lentinan, schizophyllan, scleroglucan, yeast glucan, and zymosan were demonstrated to improve survival rate by 40−50% toward infectious challenge with *Edwardsiella tarda*

and *Aeromonas hydrophila* by *Cyprinus carpio* L. carp at doses of 5−10 mg/kg.[13] A later study found similar results with tilapia and grass carp toward infection with *Aeromonas hydrophila*.[79] Resistance to infection with *Streptococcus suis* bacteria in weaning pigs was negative in 1995,[80] but, more recently, increased levels of TNF-α were observed in weaning pigs and an interesting synergistic effect with vitamin C supplements was postulated.[81] In addition, a positive resistance to LPS challenge was observed.[82] The most recent in vivo studies showed a resistance to intestinal colonization by enterotoxigenic *Escherichia coli* (ETEC), fewer bacteria in feces, and less severe diarrhea.[83] Recent in vitro studies confirm the stimulating capacity of a variety of 1,3-β-glucans, demonstrating that porcine leukocytes show increased cytokine and ROS production and increased proliferation in response to 1,3-β-glucans.[59] It is now clear that the differences in results obtained over >20 years of research into improving resistance to infection in various species are highly dependent on the specific 1,3-β-glucans used, their concentration, and their conformation during administration.

Viral Resistance. Similar to work in resistance to bacterial and fungal challenge, sulfated 1,3-β-glucans have been tested for the ability to impart resistance to viral infection to malaria, herpes simplex virus (HSV),[84,85] and HIV. In early studies, Curdlan sulfate completely inhibited HIV virus infection in MT-4 cells at 3.3 μg/mL as characterized by the lack of virus-specific antigens.[86] These results were consistently verified,[87−89] leading to ongoing clinical trials. Malaria infection was resisted in the presence of Curdlan sulfate in vitro through an inhibited fusion mechanism.[84] More recently, sulfated 1,3-β-glucans have imparted resistance to infection by HSV through a suggested electrostatic binding to the viral surface that inhibits interaction with host cells.[85] This mechanism of viral interaction was studied with experiments between Curdlan sulfate and polylysine, causing the formation of electrostatic cross-linking to produce clear, strong gels. It was believed that this electrostatic interaction between anionic Curdlan sulfate and cationic polylysine may mimic an interaction between Curdlan sulfate and a cationic glycoprotein (gp120) in the viral envelope of HIV, blocking interactions with CD4 receptors.[90] This blocking mechanism has been extensively studied in experiments on HIV inhibition.[91−93]

In 1994 it was confirmed that Curdlan sulfate interacts with gp120 on HIV-1, which affects the cell−virus fusion mechanism, but that there was no interaction between Curdlan sulfate and CD4 because pretreated cells were vulnerable to infection. This blocking mechanism was supported by evidence suggesting inhibitory action before any viral genetic replication.[91,92] This result was later confirmed with additional evidence that Curdlan sulfate inhibits TNF-α production, which led to T-cell apoptosis caused by gp120 interaction with CD4.[89] An additional mechanism may lie in the mediation of β-chemokines and cytokines by Curdlan sulfate, with demonstrations that MIP-1α, MIP-1β, and MCP-1 were all inhibited, whereas RANTES and IL-16, two cytokines known to have anti-HIV activity, were enhanced.[94]

The synthesis of Curdlan sulfates in various conformations and their incorporation into other systems have received attention as well.[95] Azidothymidine (AZT), a nucleoside analogue that inhibits viral reverse transcriptase, has been chemically bonded to Curdlan sulfate through an alkylene linker, allowing enzymatically triggered release of AZT.[86,96] An additional opportunity follows the recent research around fullerene derivatives as anti-HIV agents.[97] Water-soluble Curdlan sulfate−C$_{60}$ conjugates have been synthesized that combine the anti-HIV activity of Curdlan sulfate and fullerenes.[98] Alongside new conjugates and delivery vehicles, research is also working on improving the synthesis of Curdlan sulfates. A growing amount of work exists on the development of easy functionalization mechanisms by "click chemistry".[99−101] Some of the most recent work has investigated the use of ultransonication for sulfation of Curdlan with impressive results showing 4-fold increases in the degree of substitution. However, molecular weights were decreased significantly.[102]

Wound Healing. Wound healing effects of 1,3-β-glucans have been studied extensively as a growing application. Suggested effects of 1,3-β-glucans related to wound healing are improved transport of macrophages to the wound site[103] and improved collagen deposition.[18] The effect on collagen deposition was originally thought to be indirect through the stimulated release of growth factors from macrophages, but a recent study has shown that insoluble glucan from zymosan, laminarin, and glucan phosphate can interact, sometimes only partially, with normal human dermal fibroblasts (NHDF). This binding stimulated NF-κB activity and IL-6 mRNA expression.[104] The discovery of increases in NF-κB activity and direct binding to NHDFs has stimulated investigation into the ability of 1,3-β-glucans to induce collagen synthesis. Type I and III collagen biosynthesis was substantially improved with treatment with 1,3-β-glucans in recent studies by measurement of procollagen mRNA and hydroxyproline levels.[93] Consistent with these findings, β-glucan collagen matrix (BGC) was evaluated in the treatment of pediatric burns with observed reduction in pain and required analgesic, reduction in the necessary number of wound dressing changes, and improvements in healing and cosmetic appearance.[105]

■ STRUCTURAL APPLICATIONS OF 1,3-*B*-GLUCANS

The ability of 1,3-β-glucans to form helical structures that can be gelled with the application of heat and humidity has generated a fast-growing volume of research on the use of these polysaccharides as structural agents to provide scaffolding for the formation of macroscopic and nanoscale structures.

Food Gels. The gelation mechanism of Curdlan has been heavily utilized in food science after the U.S Food and Drug Administration (FDA) approved Curdlan for addition to food in 1996.[106]

Nakao investigated the gel strength and syneresis of Curdlan gels at room temperature and when frozen and formed into strawberry- and honey-flavored gels, soy milk noodle-like gels, and mixtures with other gelling agents demonstrating that Curdlan had higher gel strength after thawing than carrageenan, agar−agar, and konjac. The addition of waxy corn starch and sucrose reduced syneresis of the gels.[23] Combination of Curdlan and xanthan hydrogel complexes was also found to be the most stable in terms of viscosity, heat stability, and gel strength through multiple freeze−thaw cycles.[107]

Curdlan has been investigated as an additive to reduce fat content in donuts with the addition of 1% of Curdlan to the donut batter resulting in a reduction of 5.6% in total fat content and 9% in oil uptake during frying. Curdlan was also found to inhibit moisture loss.[24] This research has also been expanded to the frying of other products, namely, akara from cowpea flour, where Curdlan reduced fat content by 10% in akara with mixtures of 1% Curdlan and 20% soybean flour while maintaining texture as long as the composite flour was sufficiently moisturized prior to use.[108]
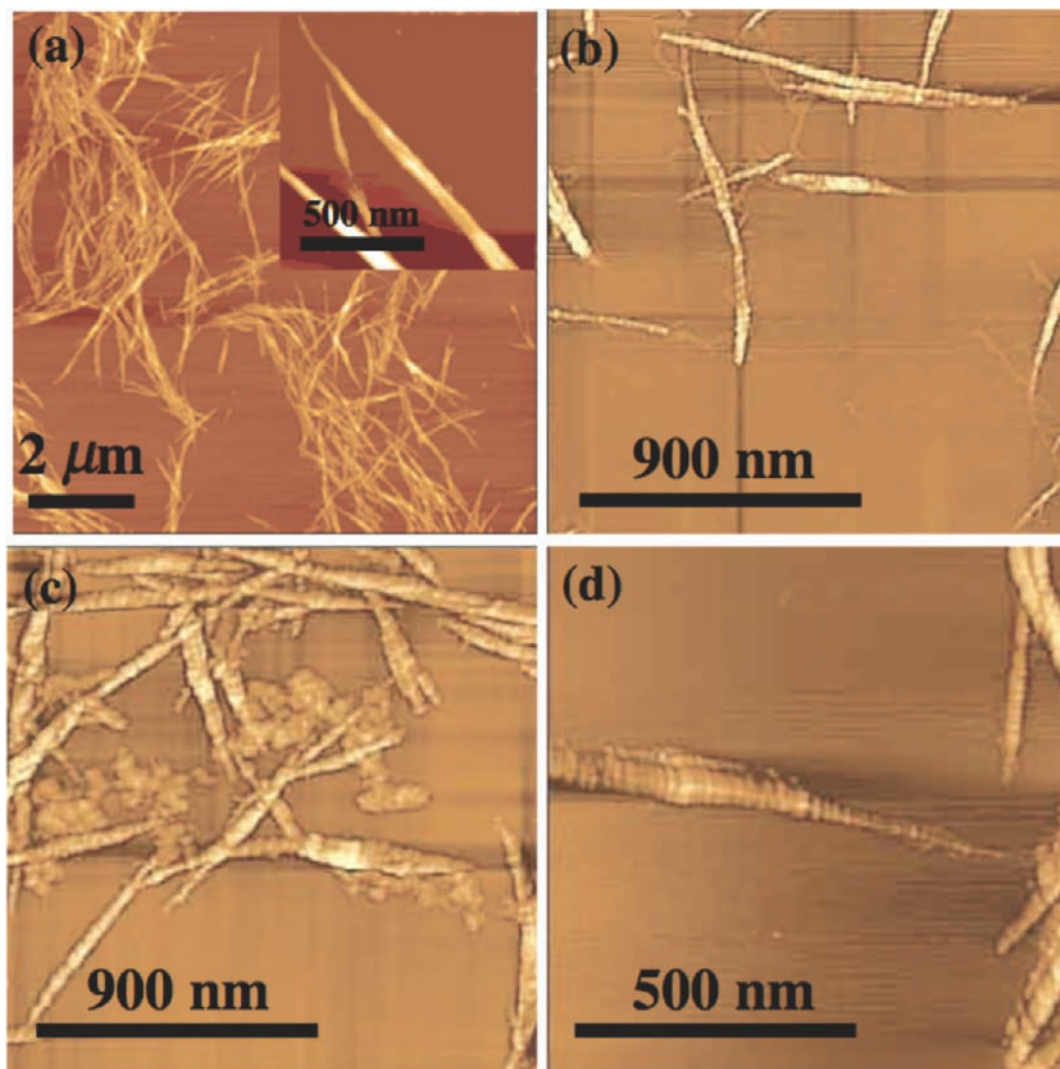
Figure 11. Helical encapsulation of SWCNTs as seen through AFM: (a) SWCNTs; (b) schizophyllan encapsulation; (c) Curdlan encapsulation; (d) magnified view of schizophyllan encapsulation showing helical oblique striations. Reproducd with permission from ref 112. Copyright 2004 Chemical Society of Japan.

Curdlan has been investigated as a fat substitute in low-fat meat products with "false-fat" created from a mixture of 3% Curdlan, 10% microcrystalline cellulose, and 1% modified tapioca starch. The false-fat was incorporated into sausages (20%) and showed lubricity, viscosity, mouthfeel, and appearance as determined by static viscoelasticity measurements comparable to those of sausages made of 20% pork fat.[109]

The isolation of environmentally sensitive pigments has been an important issue in food science. Curdlan has been used to encapsulate and release pH-sensitive blackberry anthocyanins. Spraying of a suspension of ~5% Curdlan and anthocyanin extract into a soybean oil bath and recovering capsules by filtration proved to be an effective means to stabilize the dye.[110]

Environmental Gels. Heavy-metal removal from substances is often carried out with activated carbon particles that contain porous internal structures capable of adsorbing metal contaminants. Suspending activated carbon particles in Curdlan gels demonstrated the removal of copper, manganese, lead, and cadmium from oriental herbs with the pore size adjustable on the basis of the Curdlan/carbon ratio.[111]

Nanostructure Formation. The helix-forming capability of 1,3-β-glucan is attractive for nanoscience applications in which structures with dimensions or conformations suitable to interact with single helices are manipulated. The triple-helical structures of Curdlan and schizophyllan have been used to encapsulate single-walled carbon nanotubes (SWCNT) by renaturing of Curdlan and schizophyllan from a DMSO solution (by addition of water). Encapsulation of SWCNTs was justified by the presence of helical oblique stripes on the surface of SWCNT bundles through AFM (Figure 11).[112] Hierarchical superstructures have been assembled using these stabilized one-dimensional carbon nanotubes, utilizing ammonium- and sulfite-functionalized Curdlan backbones to first encapsulate SWCNTs and then stack the nanotubes into layers via alternating electrostatic interactions.[113] The structures formed may have utility in both SWCNT structure formation and the hierarchical assembly of bioactive polymers such as peptides or nucleotides.

The formation of ordered metal nanowires for application in nanoelectronic circuitry has been an ongoing challenge in nanotechnology. Historically these nanowires have been constructed
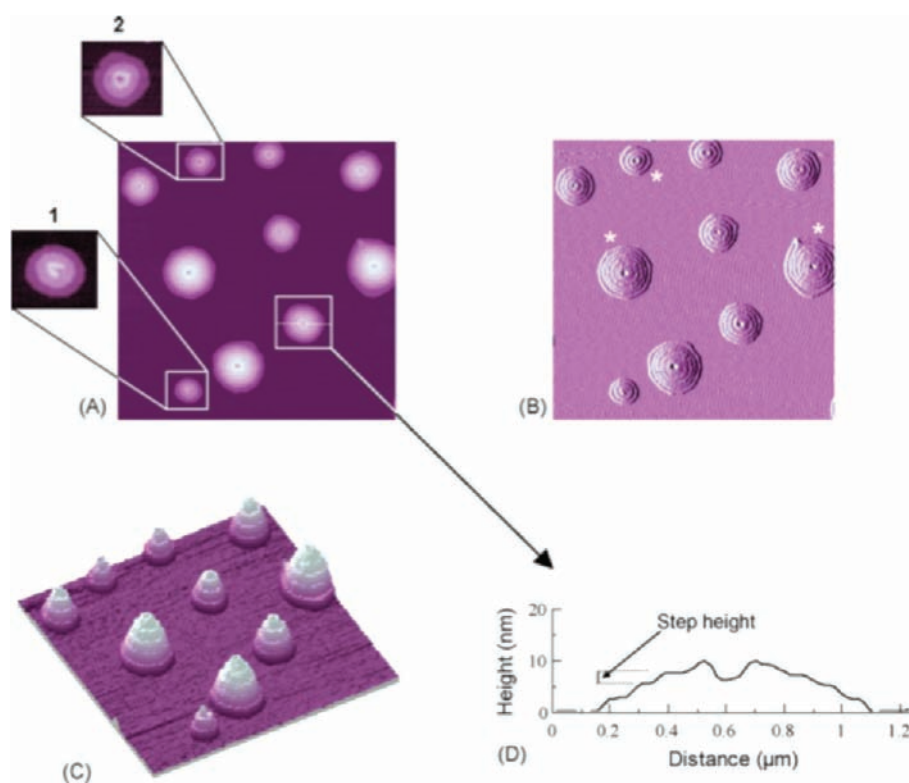
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Figure 12. Conical-terraced assemblies of Laminarin as seen through AFM: (A) AFM height image; (B) AFM amplitude image; (C) 3D construction from AFM; (D) cross-sectional height scan showing the presence of a pore in the center. Reproduced with permission from ref 117. Copyright 2007 Elsevier.

using surfactant assemblies or porous nanostructures to direct growth, but recently there has been interest in schizophyllan as a scaffold or templating material for Au nanowires. Mixing Au nanoparticles in water and schizophyllan in DMSO allowed the precipitation of schizophyllan helices containing 1D arrangements of Au nanoparticles.[114] This finding could be used to create helical micelles containing any sort of nanoparticle including those designed to deliver therapeutic substances, creating functional hierarchical assemblies. The templating effects of schizophyllan have been used in the development of a number of other systems as well including silica,[115] oligosilane,[30] and porphyrin nanofibers,[29] and in situ polymerizations.[116] Other 1,3-β-glucans are also currently under investigation for templating and scaffolding properties including laminarin, which has recently been formed into terraced cone-like structures (Figure 12).[117] These advancements in templating technologies utilizing 1,3-β-glucans provide a more practical and cost-effective method to scaffold nanostructures than DNA templating and provide important pharmacological properties in the process.

In addition to nanostructure templating, 1,3-β-glucans are currently being used chemically in nanoparticle synthesis. Carboxymethyl Curdlan was used a "green" biopolymer reducing agent in the synthesis of silver nanoparticles. Light scattering and TEM demonstrated that 40−80 nm particles were formed within 10−15 min by reduction of silver ions in a CM-Curdlan solution.[118]

Liquid Crystals. The ability of 1,3-β-glucans to gel when dialyzed against calcium chloride solution has been utilized to create liquid crystalline gels with interesting refractive index gradients and other optical properties.[119] This system has been studied in terms of the effects of Curdlan molecular weight[120] and used as a model in the liquid crystal gelation of DNA.[121] Using creative experimental setups, these liquid crystalline gels have been developed in the form of polymer beads as well, opening up application in oral drug delivery.[122]

DRUG DELIVERY APPLICATIONS OF 1,3-B-GLUCANS

The combined pharmacological and structure-forming capabilities of 1,3-β-glucans are finding application in drug delivery, wherein both of these properties may be utilized simultaneously in a single system.

Drug-Impregnated Gels. Gel encapsulation of indomethacin, prednisolone, and salbutamol sulfate in Curdlan gel suppositories has been the hallmark example of Curdlan application in this area, with suppositories allowing drug diffusion in the lower rectum, avoiding first-pass clearance in the liver compared to other suppository systems that immediately dissolve and deliver drug into the colon and consequently the hepatic portal vein.[123] Dry tablet encapsulation of theophylline by spray-drying Curdlan/theophylline solutions was also demonstrated with good pharmacokinetics.[124]

Nanoparticle Drug Delivery. Nanoparticle drug delivery approaches have been successful with the synthesis of solid lipid nanoparticles consisting of cacao butter and Curdlan. Curdlan was believed to coat cacao butter nanoparticles when introduced in an ammonium hydroxide solution. The stabilized nanoparticles were loaded with verapamil, which was quickly released within 12 h due to its high solubility in the lipid core.[125] Solid lipid nanoparticles consisting of glyceryl caprate coated with

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Curdlan encapsulating doxorubicin have also been developed with encapsulation yields of 2.8%, particle size of <200 nm, and stability after 1 year of frozen storage.[126]

Carboxymethylated Curdlan (CM-Curdlan) has been conjugated to sulfonylurea to create a grafted polymer with a hydrophilic backbone and hydrophobic sulfonylurea branches capable of forming 181 nm nanoparticles encapsulating *all-trans*-retinoic acid (ATRA) and releasing 50% of the drug with first-order kinetics.[27] Curdlan copolymers have been developed with the conjugation of CM-Curdlan to cholesterol to encapsulate epirubicin via amphiphilic copolymer self-assembly initiated by probe sonication. A broader distribution in cells over the bare epirubicin was observed with no cytotoxicity.[127]

Polynucleotide Complexes. In the past 10 years, the development of polysaccharide—polynucleotide complexes has been of growing interest and especially so with 1,3-β-glucans that form hydrogen-bonded helical structures similar to the hydrogen bonds formed by polynucleotide chains in DNA. Nanoscale complexes formed between soluble 1,3-β-glucans and polynucleotides could be used therapeutically to provide a potent polynucleotide delivery system that also bears the pharmacological properties of 1,3-β-glucans. In 2000, water-soluble 1,3-β-glucans showed the formation of a polynucleotide complex as seen through circular dichroism. Insoluble 1,3-β-glucans such as Curdlan could not be used as they precipitated before any nanoscale complex could form.[128] Shortly after this finding with Curdlan, the polynucleotide complex was successfully formed by first carrying out a hydrolytic cleavage of the backbone to reduce the molecular weight.[129] More recently, the addition of solubilizing carbohydrate appendages through click chemistry to Curdlan has been an alternative route in utilizing Curdlan to bind poly-(C).[130] Using a similar chemical scheme, a positive ammonium group was grafted onto Curdlan to render it water-soluble and capable of including a polynucleotide. In this instance, electrostatic interactions with the guest macromolecule were thought to assist the complexation.[131] The formation of the Curdlan—-poly(C) complex was studied through semiempirical molecular orbital calculations, demonstrating the presence of new hydrogen bonds between the host and guest and a unique deformation of the ribose sugar in the polynucleotide.[28] In addition, it has also now been demonstrated that the parallel arrangement of the two macromolecule chains in the complex is favored.[132] Most of the work in this area has utilized homogeneous polynucleotides, as this has been found to be the only type of polynucleotide capable of forming helical complexes. Recent work has generated the first potential applications of these findings with the development of a homogeneous polynucleotide-appended antisense oligonucleotide to which a poly(ethylene glycol) (PEG) grafted schizophyllan copolymer could bind in a helical complex. The PEG grafts improved cellular uptake and reduced lysosomal degradation, allowing for successful demonstration of inhibited cell growth through polynucleotide delivery.[133,134]

FUTURE RESEARCH

1,3-β-Glucans have enormous potential in a wide variety of fields due to their unique helix- and gel-forming capacity and potent pharmacological properties. As the literature simultaneously characterized the pharmacological effects and discovered the helical arrangements formed by these polysaccharides, two streams of research could be clearly identified, the first being the discovery of potential use of these polysaccharides in direct immunotherapeutic intervention for the treatment of cancers, healing of wounds, and impartation of microbial, fungal, and viral resistance, and the second being the use of the helical gel structure beginning with the creation of food gels and now growing deeper into the development of nanostructures and encapsulation and release of active therapeutics. These two clearly defined streams will coalesce with a greater volume of research dedicated to systems that utilize the pharmacological and structure-forming capabilities of 1,3-β-glucans.

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