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reviews

Controlled Release Biopolymers for Enhancing the Immune Response

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Abstract: Controlled release of biologically active compounds in the context of drug and vaccine delivery is an important area of research with broad implications in many areas of medicine. In particular, the challenges of oral delivery are of specific interest to reduce the cost and potential health risks related to parenteral administration of pharmaceuticals and vaccine formulations. We discuss the biological activities of two biopolymers, β-glucans and emulsans, both of which offer significant potential for individual formulations related to drug impact, while in combination offer synergistic opportunities in terms of formulation and delivery. β-Glucans have been established as potent immunomodulatory and biologically active compounds with application in a wide range of disease systems. The emulsan family of biopolymers also has significant potential in vaccine and drug delivery based on recent studies. Each of these biopolymers offers exciting opportunities to modulate biological responses via control of chemistry and physical properties achieved during biosynthesis or postsynthesis modifications. When combined into a delivery system for controlled release, synergistic outcomes may be achieved that offer new and exciting opportunities as described in the present paper. These outcomes represent the combined improvements of solubility in physiological environments and immunomodulation due to the specific chemistry and structures involved. Overall, this approach provides a new direction in controlled release wherein the biomaterial carrier, in this case emulsan, and the drug, in this case β-glucan, play an active role both in biological activation as well as in delivery profiles.

Keywords: Emulsan; β-glucan; controlled release; immunopotentiation; drug delivery; *Acinetobacter*

Introduction

The innate immune system is the first line of defense against attack, whether from parasites, bacteria, or viruses. The organism must correctly recognize the type of infection and respond with appropriate counter measures which can involve a spectrum of responses from biochemical attack, a cellular response by phagocytic cells, to the induction of the acquired immune system. The recognition of nonself in the innate immune system is mediated largely by specific cellular receptors which are able to identify molecules called pathogen-associated molecular patterns (PAMPs). One class of macromolecules that is able to trigger the PAMP-mediated response is biopolymers, like glucans and lipopolysaccharides, which are able to play a role in the enhancement of the immune system and may potentially have a role in vaccination. The ability to modulate these responses, via

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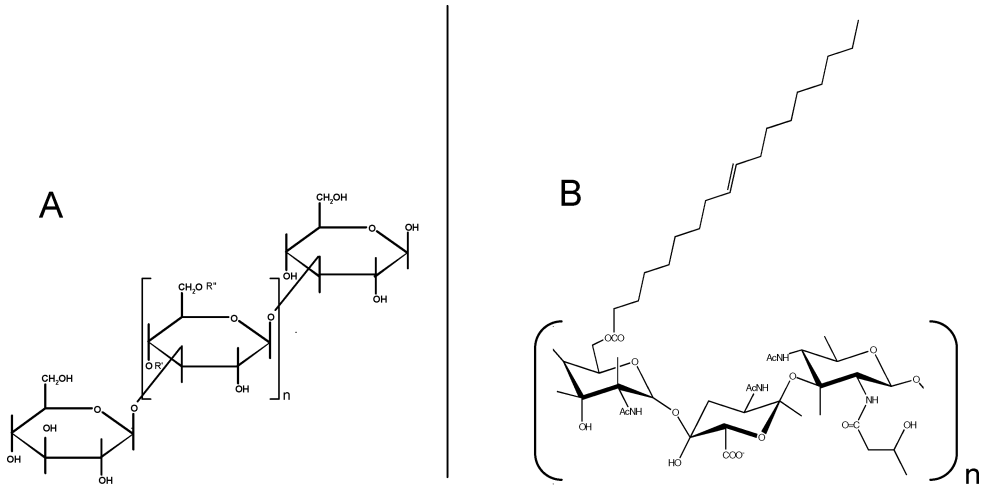

Figure 1. (A) Generic structure of β-glucans. Typical examples follow: R′ = H in Curdlan, laminarin, and schizophyllan, R′ = glucose residues in barley glucans, R″ = H in barley glucan and Curdlan, and R″ = glucose residues in laminarin, *Saccharomyces* β-glucan, and laminarin. (B) Generic structure of emulsan with a three-amino sugar repeat and fatty acid side chains.

biopolymer engineering to alter chemistry or molecular weight, is a major focus of our research efforts.

Most β-glucans contain (1→3)-β-D-glucopyranosyl units with (1,6)-β or (1,4)-β side chains with varying distributions and lengths depending on the biological sources (Figure 1). β-Glucans have several characteristic advantages over other biopolymers. These include availability across several genera, simple purification protocols, lower molecular variability, neutral charge in aqueous solution, high stability at various temperatures and pH levels, long half-life in vertebrates (due to the lack of β-glucanases), lack of allergenic response, and absence of inhibitory or competing anti-β-glucan antibodies in mammals. In addition, since β-glucans are not found in higher organisms, they trigger the innate immune response.[1] Humans do not possess β-hydrolases, so β-glucans remain in the bloodstream and lymphatic system and gradually are deposited in the liver and/or spleen over a few months without structural change until oxidative degradation by phagocytes or secretion through glomerular filtration.[2,3] Degradation of β-glucans can occur in the human intestine and is related to the presence of β-glucanases in the local microbial flora.[4] Because of this, molecules able to reduce or inhibit β-hydrolase activities could play a relevant role in drug formulation to preserve the β-glucan molecule integrity and biological activity. Administration of β-glucans to mammals has been reported to be beneficial in the context of some pathologies and in antimicrobial and anticancer therapies where they induce a required immune response.

Emulsan is an anionic exopolysaccharide produced from a variety of hydrocarbon sources, including fatty acids and ethanol, by the Gram-negative bacterium *Acinetobacter venetianus* strain RAG-1, previously classified as *Acinetobacter calcoaceticus*[5,6] (Figure 1). The polysaccharide main chain contains three amino sugars, D-galactosamine, D-galactosaminouronic acid, and a dideoxydiamino hexose in a 1:1:1 ratio.[7,8] The polymer has *O*-acyl- and *N*-acyl-bound side chain fatty acids ranging in chain length from 10 to 22. These fatty acid substituents constitute 5−23% (w/w) of the polymer. The emulsan amino groups are either acetylated or covalently linked by an amide bond to 3-hydroxybutyric acid.[8] The combination of the hydrophilic anionic sugar main chain repeat units with the hydrophobic side groups leads to the amphipathic behavior of emulsan and, therefore, its ability to form stable oil-in-water (o/w) emulsions.

The emulsan family of polymers represents a novel class of adjuvant candidates. While the emulsans share some similar structural features with lipopolysaccharides (LPSs), they are a distinct family of acylated exopolysaccharides. The most obvious distinctions between emulsans and LPSs

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are the differences in molecular weight, the variability in fatty acid side chain lengths and types, and the degree of substitution with fatty acid side chains.[9] Utilizing direct biological synthesis for control and manipulation of complex structural features offers a new path to a family of compounds that cannot be attained by current organic chemistry approaches. This process can be exploited to generate emulsans with modified acyl fatty acid groups (for example, chain lengths, unsaturation or saturation, hydroxylation, and fluorination have all been demonstrated) on the polysaccharide backbone, thereby generating an essentially unlimited suite of adjuvant candidates within this family of related polymers that may induce variable immune modulation, both in the degree and in the nature of the response.

Emulsan preparations have served as effective adjuvants, as demonstrated using several systems. Emulsan-induced antibody titers against classical hapten carriers were shown to be similar to levels obtained using complete Fruend's adjuvant,[10] and emulsan-based formulations for intranasal delivery also stimulated significant titers against hapten conjugates (B. Panilaitis et al., unpublished results). In models of Lyme disease, botulinum intoxication, and *Yersinia pseudotuberculosis*, emulsan-based vaccines protected the animals from lethal and/or pathology-inducing challenge doses (B. Panilaitis et al., unpublished results).

Oral medication is one of the most common routes for drug release because it is noninvasive and less harmful than parenteral delivery and is recommended especially in chronic diseases or when multiple serial doses are required.[11] In addition, oral drug administration offers a gateway for delivery through the gastrointestinal territory and for systematic delivery through access to the lymphatic system and blood stream. However, a major limitation for oral delivery of biomolecules is the digestive tract, particularly the stomach due to the harsh acid environment with pHs ranging from 1.0 to 3.5 (Figure 2). In addition, the presence of hydrolytic enzymes in the digestive tract significantly reduces the bioavailability of sensitive compounds used in pharmacology. Typical examples of compounds with reduced bioavailability are some antibiotics, therapeutic peptides, proteins like insulin, and some enzymes.[12,13] To overcome these problems, drug coating and adsorption are the prevalent alternatives.

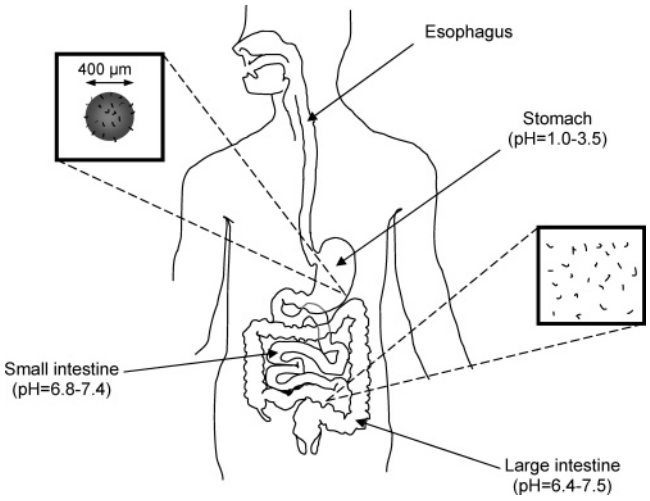

Figure 2. Oral release of Curdlan on emulsan−alginate gel microspheres in the human digestive tract.

In these strategies, polymeric carriers play a key role in providing for the drug a microenvironment that protects it from the harsh external environment. A range of synthetic polymers and copolymers have been used as coating or adsorbent agents. However, multistep processes are usually required to synthesize and/or modify these polymers. In addition, the presence of undesirable secondary products, sometimes toxic, requires additional purification steps which are expensive and time-consuming. As a result of these complications, the search for biocompatible, degradable, and environmentally sensitive or "smart" coating polymers that are relatively easily used has become desirable.

Chapter 1. Biomedical Applications and Properties of β-Glucans

Antimicrobial and Immunomodulatory Properties. β-Glucans are the most abundant components of fungal and yeast cell walls, but they are also widely distributed in algae, higher plants, and some bacteria (Table 1). Over the past decade, β-glucans have been studied because of their cholesterol-lowering activities, and their anticoagulant and antithrombotic activities which reduce the risk of cardiovascular diseases.[14−16] Also, the metabolic control of diabetes by β-glucans was previously reported.[17] In fact, β-glucans are biopolymers designated Generally Recognized As Safe

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Table 1. Typical Examples of Biologically Active β-Glucans

carbohydrate (common name)	main glycosidic linkages	sources
AC-1 glucan	β-(1,4) with branches	*Acetobacter xylinum*
barley glucan	β-(1−3), β-(1−4)	*Hordeum vulgare* (barley)
Curdlan	β-(1−3)	*Alcaligenes faecalis* var. *myxogenes*
glucan phosphate	β-(1−6)	*S. cerevisiae*
laminarin	β-(1−3)	*Eisenia bicyclis*
lentinan	β-(1−3), β-(1−6)	*Lentinus edodes* (Shiitake mushroom)
lichenan	β-(1−3), β-(1−4)	Icelandic moss
PGG-glucan	β-(1−3)	*S. cerevisiae*
pustulan	β-(1−6)	*Penicillium allahabadense* and *Umbilicaria* species
scleroglucan	β-(1−3), β-(1−6)	*Sclerotium glucanicum*
schizophyllan	β-(1−3)	*Schizophyllum commune*
SSG-glucan	β-(1−3)	*Sclerotinia sclerotiorum*

(GRAS) by the U.S. Food and Drug Administration (FDA) since 2003, and of late, the European Communities Commission designated a soluble β-glucan purified from *Saccharomyces cerevisae* as an orphan drug for treatment of oral mucositis in head and neck cancer patients.

However, the development of the beneficial biological activities of high-molecular weight (HMW) β-glucans as immunomodulators is still in its infancy. HMW β-glucans are considered as biological response modifiers (BRM) and were reported to be useful for the treatment of some bacterial, fungal, protozoan, and viral pathogen infections as well as certain types of tumors.[18,19] In particular, in vitro studies revealed that β-glucans or their derivatives possess relevant biological activities such as inhibition of parasites like *Plasmodium falciparum*, the malaria-causing agent,[20] and *Babesia bovis*, one the most significant protozoa in cattle disease.[21] Also, bacterial and parasitic infection resistance in mice was increased after oral administration or intraperitoneal doses of oat-derived β-glucan.[22] Administration of *S. cerevisae* β-glucans has been reported to increase the resistance against major microbial pathogens such as *Aeromonas hydrophila*, *Edwardsiella tarda*, *Streptococcus* sp., *Vibrio anguillarum*, *Vibrio salmonicida*, and *Yersinia ruckeri*, in fishes of economic interest such as African catfish (*Clarius gariepinus*),[23] Atlantic salmon (*Salmo salar*),[24] brook trout (*Salvelinus fontinalis*),[25] carp (*Cyprinus carpio*),[26] rainbow trout (*Oncorhynchus mykiss*),[27] and yellowtail (*Seriola quinqueradiata*).[28] The physiological mechanisms of enhanced bacterial resistance mediated by β-glucans in Atlantic salmon, catfish, and rainbow trout were attributed to the enhancement of humoral as well as nonspecific cellular defenses such as the bacterial killing activity of macrophages and phagocytosis.[27,29,30] In addition, enhanced complement activity, an increase in the level of cytokine synthesis, and production of oxygen free radicals by macrophages were detected in fish after treatment with β-glucans.[31−33] β-Glucans also induced an augmented specific immune response in Atlantic salmon and catfish when administered prior to vaccination against *Aeromonas salmonicida* and *Edwardsiella icta-*

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luri.[29,34] In previous work, cells of *Blastomyces dermatitidis*, a yeast well-known as a β-glucan producer, were opsonized and facilitate complement deposition, suggesting that β-glucan in the cell surface is accessible to antibodies.[35] A novel β-glucan-conjugated vaccine was reported to successfully prevent vaginal infections of *Candida albicans* in rats and lethal systemic infection of *Aspergillus fumigatus* in mice.[36] Interestingly, *Aspergillus* and *Candida* spp. are phylogenetically very distant and harbor different cell wall compositions, but both synthesize β-glucans as cell surface polymers. Moreover, in vitro experiments have demonstrated an increase in the macrophage killing activity of *Candida* sp. by anti-β-glucan antibodies, and a correlation of the decline of anti-β-glucan antibody titer with the presence of mycosis.[37] These results confirm previous findings of other research groups.[22,38,39] It is important to note that the fungicidal activity of β-glucans is relevant since fungal infections are more than 10% of nosocomial infections and are of significant concern in immunocompromised patients.[40] However, the complete mechanism of β-glucan biological activities is extremely complex and still under investigation. Additional beneficial biological activities described for β-glucans were the inhibition of AIDS viruses HIV-1 and HIV-2.[41,42]

Anticancer Properties. Tumoricidal activity of β-glucan has been attributed to the activation of the complement mechanism involving complement receptor 3 (CR3, also named αMβ2 integrin, Mac-1, or CD11b/CD18) present in myeloid and natural killer (NK) cells and also in selected leukocytes. CR3 is a heterodimeric molecule composed of the α_m (CD11b) and β_2 (CD18) chains. CR3 is an adhesive structure with different binding domains. The CD11b chain possesses an I domain, which recognizes iC3b-opsonized particles, intercellular adhesion molecules, and extracellular matrix proteins, and the C terminus, a lectin-like domain, harboring a binding site for carbohydrates like β-glucan.[43,44] The trigger of CR3 for phagocytosis or cytotoxic degranulation of tumor cells requires the binding to the receptor of both β-glucan and iC3b (opsonized cells). When tumor cells are resistant to complement-dependent cytotoxicity (CDC), the use of antitumor antibodies able to attach iC3b to the cell surface in conjunction with β-glucan generates the activation of complement and has been probed to be a promising alternative in cancer therapies. In particular, laboratory studies using animal models showed Curdlan, an unbranched linear β-(1→3) glucan, prevents colon aberrant crypt foci induced by 1,2-dimethylhydrazine in rats[45] and demonstrated anticancer activities against sarcoma 180 in mice and tumor in endothelial cells.[46,47] Oral delivery of β-glucans enhanced the antitumor activities of several monoclonal Abs against mouse breast carcinoma, and Lewis lung carcinoma, melanoma, and neuroblastoma in humans.[48−52] Cancer immunotherapy using monoclonal Abs combined with the use of β-glucan adjuvants exhibits the characteristics

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of a targeted attack based on surface tumor molecular markers. Such specificity of targeting is an important advantage when compared to nonspecific chemo- and radiotherapeutic cancer treatments.

Mechanism of Activity. Several β-glucan receptors which mediate the complex biological responses of this class of polymers have been identified. Among them is lactosylceramide (LacCer, Galβ1-4Glc-ceramide, or CDw17) which belongs to the glycosphingolipid (GSL) family. LacCer has been identified as an important signaling intermediate for the regulation of proliferation and adhesion in mammalian cells.[53] Also, LacCer has been identified as a receptor for β-glucans and has been shown to recognize a variety of microbes.[54] Binding of β-glucans with the LacCer receptor induces the activation of NF-κB, enhancement of neutrophil oxidative burst, and production of macrophage inflammatory protein (MIP-2) in alveolar epithelial cells.[55,56] In human monocytic cells, LacCer induces the expression of the platelet/endothelial cell adhesion molecule (PECAM-1 or CD31 antigen) and activates protein kinase C α and ϵ by translocating from the cytosol to the membrane; consequently, phospholipase A2 is activated, and as a result, increasing levels of homotypic and heterotypic adhesion and transendothelial cell migration can be observed.[57]

Scavenger receptors recognize LDLs (low-density lipoproteins) and fatty acids and are involved in mechanisms of inflammation and atherogenesis but also are able to recognize other molecules such as β-glucans.[58] Further experiments demonstrated that binding of β-glucan to the macrophage scavenger receptor protects against endotoxic shock and weakens the binding of LDL.[59,60] Since the uptake of LDL by macrophages is considered one of the initial steps in the formation of atherosclerotic lesions, the use of β-glucans to prevent atherogenic events could be an alternative therapy to be explored.

In humans, other receptors involved in the binding to β-glucans described in the literature are Toll-like receptors 2 and 6 (TLR-2 and TLR-6, respectively) which work cooperatively. TLRs are present in a variety of human cells, including the gastrointestinal tract and cell lineages involved in the immune response. TLRs recognize different types of PAMPs and were discovered through their homology to the Toll receptors of *Drosophila* which are relevant in antifungal defense.[61] Vertebrate TLRs are single-pass transmembrane proteins with an extracellular leucine-rich domain and an intracellular domain homologous to the cytosolic interleukin-1 receptor (IL-1) domain. Activation of TLR is mediated by adaptor proteins, like MyD88 and/or Mal, which participate in the signaling and induce the expression of nuclear transcription factor κB (NF-κB), and interferon response element 3 (IRF) which regulates IFN-β production. The subsequent cascade involves the transcription of proinflammatory cytokine genes.[62,63]

More recently, a major receptor for β-glucans, Dectin-1, was identified in mouse myeloid cells (macrophage, monocyte, and neutrophil lineages). Dectin-1 in concert with TLR is involved in the proinflammatory response to β-glucans. Dectin-1 is a C-type lectin-like receptor containing an extracellular carbohydrate-recognition domain, a stalk and

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transmembrane region, and a cytoplasmic tail having an immunoreceptor tyrosine-based activation motif (ITAM). When the β-carbohydrates, but not α-glucans, bind to the extracellular domain, a tyrosine residue is phosphorylated and starts the activation of transcription factor NF-κB, which also is enhanced by TLRs at the cell surface increasing the level of production of IL-12 and TNFα.[64,65] The human homologue to Dectin-1, named β-glucan receptor (βGR), is spliced in many forms, but only two isoforms (βGR-A and βGR-B), which vary only in the presence or absence of a stalk region, are able to recognize β-glucans.[66]

Another biological role of Dectin-1 was demonstrated in vitro by inducing the proliferation of CD4+ and CD8+ cells.[66,67] Dectin-1 is also upregulated by cytokines IL-4 and IL-13, which are involved in the induction of the Th2-type response which results in an alternative activation of macrophages.[68] Expression of Dectin-1 is influenced by many molecules, including cytokines and steroids, but the presence of high levels of Dectin-1 receptors in tissues like lung and intestine has been suggested to have a potential role as a surveillance receptor.[69] Consequently, oral delivery should be the most favorable route for enhancing and realizing the maximal immune response via this receptor.

Summary. β-Glucans are abundant in fungal and yeast cell walls, among other organisms, and have been under intensive study during the past decade for a variety of physiological impacts. While development of the beneficial biological activities of HMW β-glucans as immunomodulators is relatively new, a challenge remains for effective and sustained delivery of these polymers, such as for the treatment of pathogen infections and cancers. Mechanisms of action for this family of biopolymers have been elucidated, and several β-glucan receptors which mediate the complex biological responses have been identified. New options for optimizing the controlled delivery of these biopolymers should improve biological function and clinical outcomes, the issues addressed in Chapter 3.

Chapter 2. Biomedical Applications and Properties of Emulsans

Structural Tailorability. Previous studies of emulsan were directed at exploring the metabolic flexibility of the bacterium to incorporate exogenous fatty acids under a variety of culture strategies. These studies led to the observation that significant manipulation in both the composition and degree of fatty acid substitution could be achieved.[8,70−72] Thus, the idea that a family of acylated exopolysaccharides can be formed by a microbial production system has now been demonstrated. The bioengineering of the surfactant structure is fundamentally important since it shows that the acylation of the sugar backbone by the acyltransferase enzyme system can potentially take place with a wide range of acyl donor molecules to match a specified product performance. This leads to the concept of "tailorability" of these acylated exopolysaccharides.

Recently, on the basis of the earlier success with modulation of fatty acid content and levels, additional control of incorporation profiles of the fatty acids provided to *A. venetianus* was explored.[73] Therefore, mutants deficient in fatty acid utilization were sought, with the assumption that this would lead to further "tailoring" of structural profiles. Transposon mutagenesis of *A. venetianus* RAG-1 using a modified transposon, Tn10 (mini-Tn10PttKm),[74] was used as previously described.[75,76] Conjugation was found to be more effective than electroporation for the transformation of *A. venetianus* RAG-1 directly with the isolated plasmid pLOFPttKm containing the mini-Tn10PttKm transposon. Antibiotic selection was used to isolate transformants, and mutant selection was based on two phenotypic functions:

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(1) loss of β-oxidation pathways (to promote direct incorporation of exogenous fatty acids) and (2) loss of fatty acid synthesis. A lower level of fatty acid substitution was a general characteristic of these mutants. Data collected included fatty acid profiles, total fatty acids (degree of substitution), and the molecular weight of the polysaccharide backbone and emulsification properties.[73] In additional studies, fluorinated fatty acid esters were successfully incorporated into emulsan.[77] These products were characterized by ^{19}F NMR, and levels up to 8.5 wt % of total fatty acids per milligram of emulsan were found. The solution behavior of these modified polymers was evaluated by an emulsification assay, and the level was found to be significantly higher than that of native emulsan.

Stimulation of Murine Macrophages by Emulsan. As a first step in the exploration of immunological responses to emulsan, one of the emulsan structures, produced on an ethanol feed source, was characterized with macrophages. The effects with and without bound protein were assessed to elucidate differences. Removal of the protein resulted in the ability to attribute immunomodulatory properties specifically to the polymer and to rule out any effects of the contaminating protein without affecting the emulsification properties of the polymer, which may be important in the adjuvant activity of emulsan. To determine the immunomodulatory effects of emulsan, we first determined whether emulsan or deproteinized emulsan (apoemulsan) could stimulate macrophages to release TNFα. The results suggested that both the proteinated and deproteinated polymers could generate this response. These results set the stage for exploration of the relationship between structural features of the polymers and levels of immunomodulation.

This family of polymers has structural similarities to bacterial LPSs that suggested it might have pro-inflammatory activity. The polymeric nature of emulsan's polysaccharide backbone suggests that it might also share properties with chitin and chitosan derivatives that have demonstrated an ability to activate macrophages.[78−81] On the basis of the composition of emulsan and structural similarities to traditional microbial lipopolysacharides, the release of the pro-inflammatory cytokine, TNFα, was considered to be a suitable property to assay after stimulation of macrophages. Emulsan molecules present a significant capacity to activate macrophages, as determined by the release of TNFα from primary cells as well as RAW 264.7 cells (Figure 3A), which was dependent on the presence of the fatty acid side chains as all activity was lost after hydrolysis of the side chains. Initial studies with protein-contaminated crude emulsan with macrophages isolated from C3H/HeJ and C3HeB/FeJ mice indicated that TLR4 was not necessary for activation. However, further experiments, utilizing protein-free apoemulsan, demonstrated that emulsan activity was largely dependent on the TLR4 receptor (Figure 3B). While the TLR4 receptor is clearly implicated in these studies, it is important to point out that the emulsan experiments were conducted in the presence of polymyxin B and, therefore, independent of any LPS contamination in the emulsan samples. This was further confirmed by the limulus ameobocyte assay. The response is approximately 20-fold lower than that of LPS on a per weight basis. Nitric oxide was not released in response to emulsan.

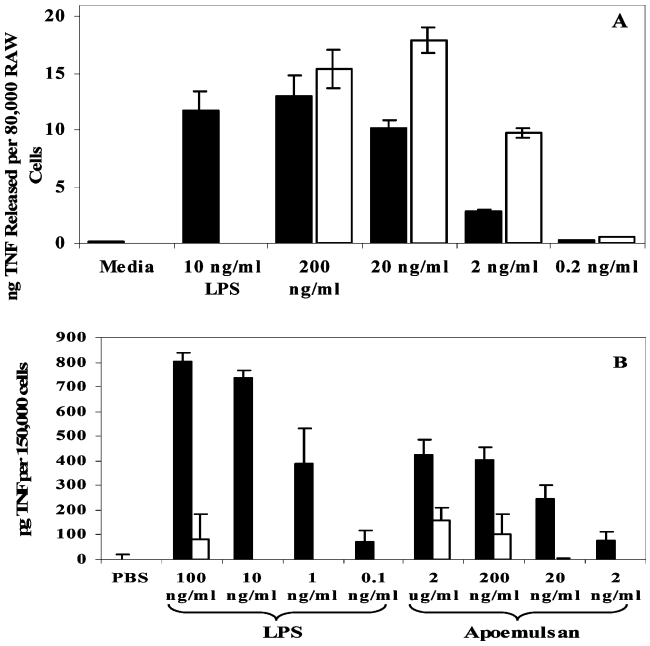

Figure 3. (A) RAW 264.7 cells were stimulated with crude (black bars) or apoemulsan (white bars) isolated from the *A. venetianus* RAG-1 parent strain fed on minimal medium and ethanol. (B) Primary macrophages from C3HeB/FeJ (black bars) and C3H/HeJ (white bars) mice were stimulated with specified doses of LPS or apoemulsan from ethanol-fed RAG-1. TNF concentrations are the average of triplicates, and the error bars represent the standard error of the mean.

Due to the ability to generate a nearly infinite number of structural variants of the emulsan polymer, it is important that screening methods be comprehensive to prevent the exclusion of valid adjuvant candidates. As a result of our focus on macrophage activation, specifically TNFα secretion, as an in vitro screening method, the nature of macrophage activation was examined in more detail utilizing an RNase

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protection assay (RPA, R&D systems). The transcript levels of cytokine (LTα, LTβ, TNFα, IL-6, IFNγ, IFNβ, and MIF) or growth factor (TGFβ1, TGFβ2, and TGFβ3) were examined (data not shown). Analysis of the RPA data indicated that the increased level of TNFα released as measured with an ELISA was found also at the message level. TGFβ1 was also induced while a modest increase in the transcript levels of IL-6 and IFNγ was also noted in response to some of the emulsan variants. MIF levels were constitutively high and unchanged with any of the treatments. The most important result of these analyses was that while other genes were induced in response to emulsan, that induction was in proportion to the amount of TNFα released as determined with an ELISA. This suggests that measurement of the amount of TNFα released is a reasonable indicator of overall macrophage activation in response to the emulsan variants.

Adjuvant Activity of Emulsan. The adjuvant activity of emulsan preparations was originally established using a classical hapten-carrier immunization protocol.[10] Total DNP-specific antibody titers revealed a significant and long-term immune response comparable to that induced by Complete Freund's Adjuvant. In addition, an examination of DNP-specific IgG2a and IgG1 titers indicated a significant increase in the T-helper type 1-associated IgG2a over antigen alone controls, with little to no increase in IgG1 titers in emulsan and Freund's adjuvant-immunized animals.

Due to several drawbacks of traditional immunizations (pain associated with injection, risk of needle sticks, spread of disease through reused and contaminated needles, and the high cost of trained personnel to administer injections), there is a clear need for alternative methods of vaccination. One of the most promising methods is intranasal delivery. Intranasal delivery of vaccine formulations has been used to examine the immune response of several antigens, including pertussis,[82] measles and rubella,[83] and influenza.[84] Aside from the advantages of the noninvasive and less technical nature of intranasal delivery, the potential for specific targeting of the mucosal immune system may induce a more effective immune response.[85] The efficacy of emulsan-based formulations for intranasal delivery was examined. Fifty 6-week-old female BALB/c mice were randomly placed in 10 groups of 5 mice each and immunized as described in Table 2. Preimmune sera were taken 3 days prior to primary immunization. Antigen and adjuvant were mixed by repeated aspiration through a 25 gauge needle. Each mouse was immunized with a total volume of 60 μL (subcutaneous) or 30 μL (intranasal) of adjuvant and/or antigen as described. Serum was collected 7 days after primary immunization, followed by a subsequent boost immunization 3 weeks after the primary immunization. Serum was collected again on days 7, 14, and 28 post-boost. Serum was analyzed for total DNP-specific antibody titers as previously described.[10] As a first indication of the relative adjuvant activity of each of the three emulsan analogues, the serum from subcutaneously immunized animals was compared. Although there was a slight decrease in the titers induced by the cerulenin−emulsan (fatty acid biosynthetic inhibitor) and the TSA−emulsan (tricosanoic acid-supplemented cultures) combinations, the overall titers 28 days post-boost were very similar and not significantly different (data not shown). Intranasal immunization with these emulsan-based formulations was highly successful. The relative titers displayed in Figure 4 indicate a significant increase in the level of the immune response to the DNP-KLH in the emulsan-based formulations when compared to the antigen alone group. These responses are slightly weaker than those observed in subcutaneously immunized animals; however, absolute titers would be needed for absolute comparisons.

Table 2. Immunization Groups for Intranasal Delivery[a]

treatment	subcutaneous	intranasal
DNP-KLH alone (50 μg)		5
DNP-KLH, 50 μg of RAG1, and EtOH	5	5
DNP-KLH, 5 μg of RAG1, and EtOH		5
DNP-KLH, 50 μg of RAG1, EtOH, and 3 mg/L cerulenin	5	5
DNP-KLH, 5 μg of RAG1, EtOH, and 3 mg/L cerulenin		5
DNP-KLH, 50 μg of RAG1, and TSA	5	5
DNP-KLH, 5 μg of RAG1, and TSA		5

[a] The emulsan analogue is denoted by the strain of *A. venetianus*, RAG-1 in all cases, the carbon source, and the supplement. EtOH represents 1% ethanol. TSA represents 0.1% tricosanoic acid.

Efficacy of Emulsan in Experimental Disease Models. Emulsan has been examined for its efficacy as an adjuvant in three separate disease models. It has proved to be either superior or equivalent to the control adjuvant, alum, in all cases. In a murine model of Lyme disease, emulsan formulations induced significantly higher antigen-specific antibody titers and protected patients from the pathology associated with challenge (arthritis) (unpublished data). In studies with a *Y. pseudotuberculosis* model of the Plague, animals were again protected from lethal challenge and exhibited high

(82) Isaka, M.; Yasuda, Y.; Taniguchi, T.; Kozuka, S.; Matano, K.; Maeyama, J.; Morokuma, K.; Ohkuma, K.; Goto, N.; Tochikubo, K. Mucosal and systemic antibody responses against an acellular pertussis vaccine in mice after intranasal co-administration with recombinant cholera toxin B subunit as an adjuvant. *Vaccine* **2003**, *21* (11−12), 1165−1173.

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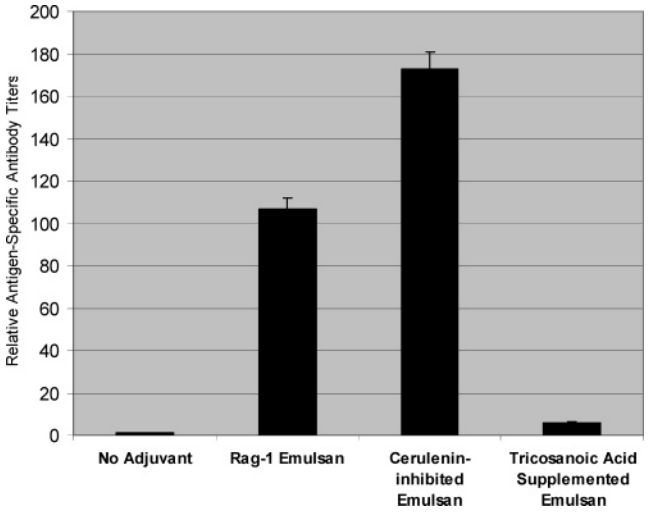

Figure 4. Relative antibody responses to DNP-KLH delivered by emulsan via the intranasal route.

antigen-specific antibody titers (unpublished data). This was again observed in a model of botulinum intoxication (unpublished data) where animals immunized twice with emulsan formulations produced titers that were higher than those of animals immunized three or more times with alum-based formulations. In addition, animals were again protected from lethal challenge.

In Vivo Toxicity of Emulsan. One important concern regarding candidate adjuvants is toxicity. No animal has died due to immunization with an emulsan-based formulation to date (more than 400 immunizations). Anecdotally, it was also noted that the mice injected with emulsan showed no more discomfort than the mice injected with antigen alone, while the mice injected with Freund's adjuvants were obviously in severe discomfort immediately following immunization. The histology results from tissue samples taken from two mice in each of the emulsan groups from the initial hapten-carrier immunization study demonstrated that exposure to 200 μg of emulsan did not increase the frequency of chronic disease over controls. Mice were euthanized and examined at approximately 42 weeks of age (36 weeks following primary immunization). Tissue samples were taken from the lungs, liver, pancreas, mesenteric lymph node, heart, and kidney. All mice, including those receiving antigen alone, exhibited mild-to-moderate lymphatic hyperplasia in the mesenteric lymph nodes. One of the six emulsan-treated animals showed evidence of a widespread lymphoma. However, one of the two control animals (no emulsan) developed a well-differentiated papillary adenocarcinoma. It is likely that these conditions are more an indication of age than treatment as the average life span of BALB\c female mice is approximately 80 weeks.[86] Taken together, the preliminary toxicity data demonstrate that the mice immunized with emulsan presented no acute toxicity or increased histopathology compared to control animals. This observation was supported in the subsequent Lyme, *Yersinia*, and *Botulinum* trials, as animals exhibited no observable effects on behavior or survival.

Summary. Emulsans make up a versatile family of complex heteropolysaccharides with capacity for immunomodulation. In addition, the polymers appear to be nontoxic and are capable of carrying payloads in the form of drugs or proteins, analogous to their natural function. Mechanisms of biological activation of emulsans appear to be similar to those of LPSs, although the ability to modulate responses in terms of both level and duration appears to be different. In addition, the ability to modulate chemistry and structure of emulsans via a combined genetic and physiological strategy provides a mode of gaining additional control of biological activation.

Chapter 3. Controlled Release of β-Glucan from Emulsan−Alginate Microspheres

Carrier Formulations for β-Glucans. Among the members of the β-glucan family, Curdlan is one of the most popular and widely used biopolymers, largely because it is considered safe by the U.S. Food and Drug Administration. However, the efficiency of Curdlan in pharmacological formulations relies on the use of other carriers as a result of poor polymer solubility in aqueous media under physiological conditions, thereby resulting in low bioavailability. Recently, low-molecular weight soluble β-glucan was accepted for clinical trials in the European Union to prevent oral mucositis.[87] However, on the basis of the low residence time in the body of soluble β-glucan, high concentrations (up to 400 mg/dose) and repetitive administrations are required to achieve the proper concentration to obtain a therapeutic dose. Nevertheless, in previous work, it has been reported that both high-molecular weight soluble and insoluble Grifolan, a β-glucan obtained from *Grifola frondosa*, are necessary for the production of TNFα by macrophages, in which cytokines IL-1 and IL-6 were stimulated.[88,89] Similarly, the presence of β-glucan-containing particles is needed to induce cytokine secretion and production of reactive oxygen species by macrophages and dendritic cells.[65,67] Particle synthesis that included a Curdlan derivative was reported earlier;[90] however, this process required chemical synthesis in a series of complex steps in organic media with the formation of toxic secondary compounds. In related work, 0.5−3.5 mm Curdlan

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crystalline-amorphous gel spheres were synthesized as drug delivery devices.[91] Besides, considering the limited solubility of Curdlan in aqueous media under physiological conditions, as well as the lack of β-hydrolases in humans, the potential use of these spheres may be limited. Thus, there remains a need to identify alternative modes of delivering β-glucan to address the current limitations described above.

Some of the most relevant drug carriers are polymeric hydrogels, because they are able to shrink or swell on the basis of environmental changes and have been extensively studied for biomedical applications, including oral delivery.[92] Hydrogels are attractive systems because they are relatively easy to manipulate and modify, and some are biocompatible. Hydrogels are hydrophilic linear or branched polymers containing chemical or physical cross-links which provide an amorphous structure. In industry, these polymers are routinely utilized for adsorption, such as in disposable diapers and napkins, or in gel actuators, drug delivery devices, and tissue engineering scaffolds.[92,93] In addition, alginates have been successfully applied in transplants with encapsulated cells and also are components in many biopharmaceutical formulations.[94] Alginates are nonbranched acid biopolymers commonly extracted from seaweed composed of β-D-mannuronic (M) and α-L-guluronic (G) acids linked by 1,4-glycosidic bonds. Alginate forms into gels in the presence of divalent ions such as calcium in aqueous systems; the encapsulation−polymerization process occurs at mild temperatures without organic solvents. Gel density and porosity can be controlled by changes in the M/G ratio and preparation conditions. Gel degradation proceeds under normal physiological conditions usually due to the displacement of the cross-linking divalent cations, releasing the resolubilized polymer into solution.[95]

Recently, emulsan−alginate microspheres were found to exhibit unique biological attributes in terms of protein adsorption and controlled release. In particular, emulsan−alginate microspheres displayed different functional features compared to those composed of alginate alone.[96,97]

Physical Characteristics of Emulsan Microspheres. Optical microscopy of alginate and emulsan−alginate microspheres showed homogeneity in spherical morphology and surface structure.[97] Emulsan−alginate microspheres are aqueous-based gels, with water content ranging from 76 to 81%. To analyze the microspheres by SEM, the absence of water using a freeze drier is a prerequisite, but changes in the microsphere morphologies induced by extensive dehydration were observed. However, more detailed analysis by SEM revealed the alginate microspheres displayed a smooth surface compared to the emulsan−alginate microspheres (Figure 5). At higher magnifications, more imperfections were apparent on the emulsan−alginate microsphere surfaces compared to the alginate ones.

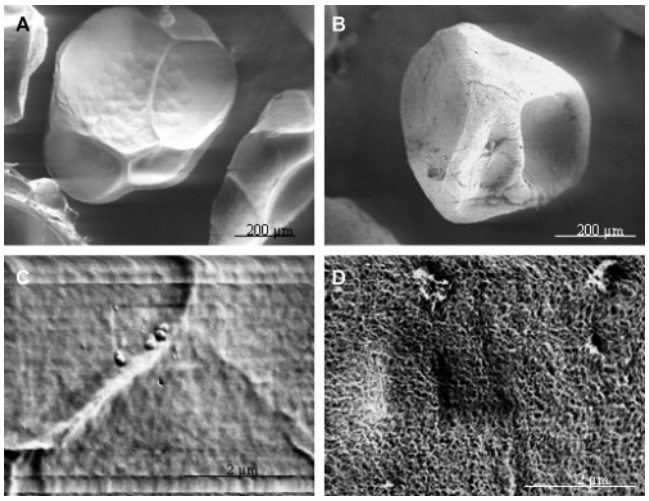

Figure 5. Scanning electron micrographs of alginate (A and C) and emulsan−alginate (B and D) microspheres.

Release of β-Glucans from Emulsan Microspheres. On the basis of previously published results in which the presence of emulsan reduced the swelling of alginate microspheres and concomitantly increased the time of release of encapsulated blue dextran used as a macromolecular drug model,[97] ANTS-β-glucan was encapsulated in the gel and the rate of release determined. Surprisingly, no differences in Curdlan release were observed when encapsulated Curdlan−alginate and emulsan−alginate microspheres were compared (Figure 6). As a result, the encapsulation technique was not considered for further studies, while adsorbed β-glucan demonstrated a slower kinetic profile. The large discrepancies between blue dextran and ANTS-Curdlan time release can be attributed to the molecular mass, where that of β-glucan is roughly 80 000 Da, which is ~25 times smaller than that of blue dextran (2×10^6 Da).

Localization of β-Glucan in Emulsan Microspheres. Considering the ability of emulsan to adsorb biomolecules, adsorption of β-glucan by the microspheres was explored.[96] No adsorption of β-glucan by the alginate microspheres was

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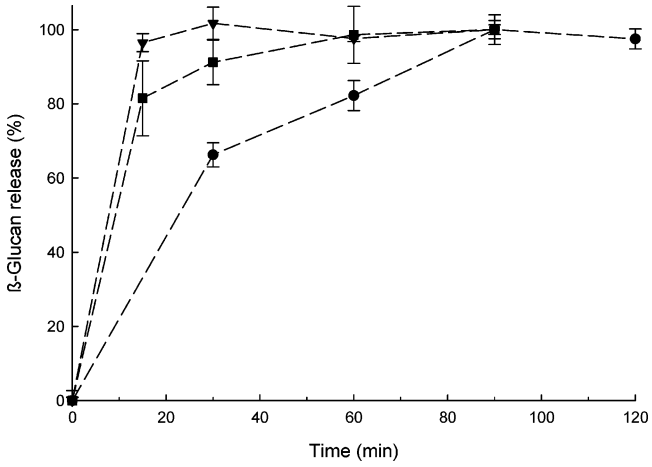

Figure 6. Release kinetics of encapsulated β-glucan from alginate microspheres (▼) and from β-glucan encapsulated (■) and adsorbed (●) in emulsan−alginate microspheres in 100 mM phosphate buffer (pH 7.5) at 37 °C.

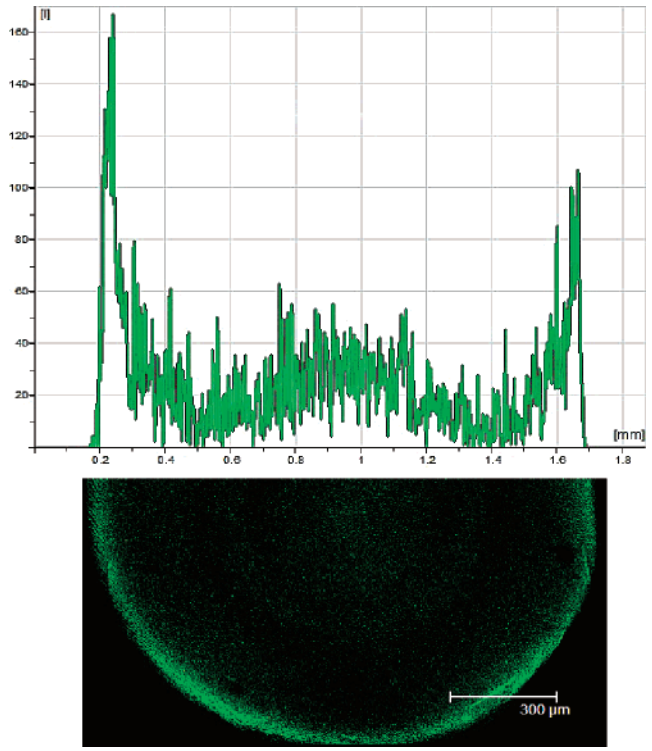

Figure 7. Distribution of β-glucan derivatized with ANTS adsorbed on emulsan−alginate microspheres.

found; meanwhile, the emulsan−alginate microspheres adsorbed 2.03 ± 0.12 μg/mg of emulsan with an efficacy of 12.3% of wet hydrogel microspheres. The adsorption of β-glucan onto a gel microsphere surface was confirmed by ANTS-labeled polymer (Figure 7). However, a weak fluorescent signal was detected in the inside of microspheres, suggesting the diffusion of low-molecular weight β-glucans to the interior of the gel.

Environmental Effects on β-Glucan Release Kinetics. The effect of temperature on release profiles of the β-glucan resulted in a linear relationship with the emulsan−alginate

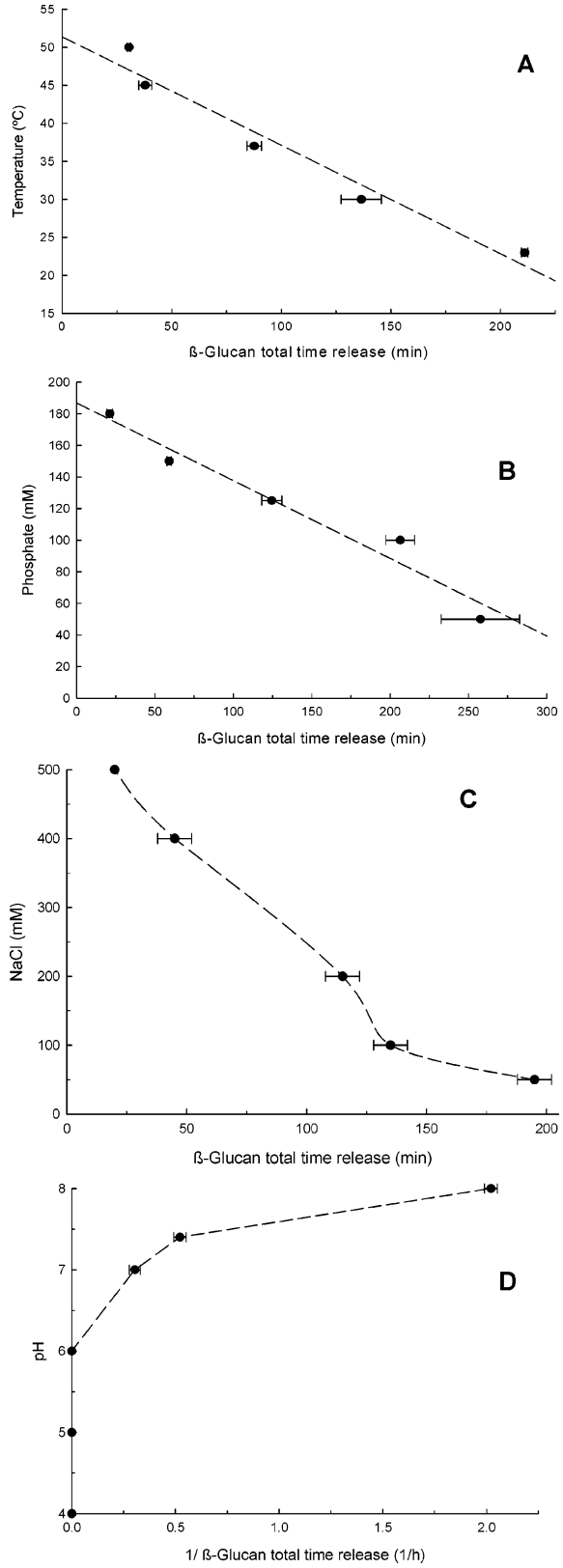

Figure 8. Effect of temperature in 100 mM phosphate buffer (pH 7.4) (A), phosphate concentration (pH 7.5) at 37 °C (B), NaCl in 50 mM phosphate buffer (pH 7.4) (C), and pH in McIlvaine or phosphate buffers (125 mM) at 37 °C (D) on the amount of β-glucan released from emulsan-coated microspheres.

Table 3. Residual Activity of β-Hydrolases in the Presence of Alginate, and Emulsan−Alginate Microspheres and Soluble Emulsan

	inhibition of β-hydrolase activity (%)		
	β-glucanases		
treatment	*Bacillus subtilis*	*Aspergillus niger*	laminarase from *Trichoderma* sp.
none	0	0	0
soluble alginate	44.4 ± 3.2	39.3 ± 2.3	42.7 ± 3.1
soluble emulsan	99.5 ± 1.8	94.6 ± 1.7	93.7 ± 3.1
alginate microspheres	32.7 ± 2.4	28.8 ± 2.4	38.9 ± 2.3
emulsan−alginate microspheres	75.3 ± 2.5	73.4 ± 3.0	72.5 ± 1.6

microspheres (Figure 8A). The fast release of β-glucan from the microspheres was at 51 °C with a total release time of 7 min. The low rate of β-glucan release in solution was approximately 6 h at 0 °C under the experimental conditions that were evaluated. Total ANTS-Curdlan release correlated with a linear regression ($r^2 = 0.96$), with a slope of 0.143 °C/min. The inverse of the slope suggested that for every increase in temperature of a degree Celsius an ∼7 min decrease in total β-glucan release time was observed (Figure 8B). Interestingly, at 36.8 °C, the total release time of β-glucan at pH 7.5 was estimated to be 102 min, sufficient time to reach the human small intestine after oral delivery to trigger biological activity.[3] Importantly, an increase in temperature which is generally associated with pathological physiological conditions, such as solid tumors, induces a reduction in the β-glucan release time, which could enhance biological response profiles.

The time required for β-glucan release displayed a linear relationship with phosphate concentration ($r^2 = 0.98$). Phosphate could play an important role in the release of the polymer from the microspheres since it is able to produce complexes by chelating calcium ions, which are the cross-linking agent for the microspheres, thus disrupting the gel structure. Phosphate is a common component of human serum involved in homeostatic processes. As expected, the β-glucan total release time increased with the reduction of phosphate concentration at 37 °C and pH 7.5 (Figure 8B). Under physiological conditions, approximately 50 mM phosphate, the β-glucan total time release estimated by linear regression was approximately 3 h.

The total release of β-glucan from the microspheres was also studied in the presence of NaCl. Total microsphere dissolution was found following hyperbolic decay between 500 and 50 mM NaCl, with release times ranging from 20 to 190 min (Figure 8C). At 154 mM NaCl, the human physiological concentration in serum, the total release time was approximately 2 h. Only at pH ≥7.0 was the release of the β-glucan observed from the microspheres at 37 °C. Faster release was found with an increase in solution alkalinity (Figure 8D). In the pH range of 7.0−8.0, the β-glucan total release time increased exponentially from 50 to 170 min, respectively. This effect is relevant in oral delivery, since the first barrier to overcome and avoid chemical hydrolysis of glucan is the stomach, where the pH ranges from 1.0 to 3.5. In the intestine, the pH is around ≥7.0 (Figure 2). Additionally, the intestine has more available adsorption surface and access to bloodstream and lymphatic systems.[4] However, one of the potential problems of delivering β-glucan is the intestine, where the presence of exogenous, microbial β-hydrolases can reduce the biological activity of β-glucan. Activities of commercial β-hydrolases were tested in the presence of soluble alginate and emulsan, and with alginate and emulsan−alginate microspheres. Alginate solutions and gel microspheres decrease β-hydrolase activity from 28 to 44%; meanwhile, in emulsan solution and microspheres, stronger β-hydrolase inhibition was detected, from 72.5 to 99.5% (Table 3).

Summary. In this study, the first in vitro release studies of β-glucans adsorbed on stimuli-sensitive emulsan−alginate microspheres were conducted. The effects of temperature, pH, and ionic strength on the release of Curdlan from these carriers were analyzed. The activity of β-glucanases in the presence of alginate and alginate−emulsan microspheres was also analyzed. The findings support the potential utility of these new carriers for the delivery of Curdlan due to the facile fabrication and loading requirements, the release profiles, and the synergy anticipated due to the biological activation features of the carrier (emulsan) as well as the Curdlan.

Conclusion

Given the established potential of β-glucan and the emulsan family of polymers in a wide range of biomedical applications, the results presented in Chapter 3 lend support to the hypothesis that these molecules can function in combinatorial delivery formulations. The synthesis of microspheres containing β-glucan for drug delivery is performed in a simple procedure and biologically compatible aqueous media using biopolymers without requiring further purification. The release of β-glucan from emulsan−alginate microspheres showed stimulus sensitivity to factors such as pH and temperature, suggesting possibilities for tuning β-glucan release profiles under specified physiological conditions by tailoring emulsan fatty acid composition. In addition, the decrease in microbial β-hydrolase activities in the presence of emulsan is another beneficial property that preserves the biological activity of β-glucan for longer times with a reduced concentration inside the body. It should be noted that Curdlan did not adsorb significantly to the alginate microspheres; thus, the interaction between the glucan and the beads was dependent on the emulsan chemistry which is dominated by the fatty acid side chains. Since we have

previously reported methods for altering the composition of these fatty acids,[73] new options for matching chemistry with release profiles would be an interesting area to explore. A change in the hydrophobicity of emulsan is one strategy for increasing or reducing the adsorption capability of the carrier biopolymer.[73] While individual pharmaceuticals will need to be examined for compatibility within this system, the efficacy of emulsan as a biological adjuvant, combined with the properties of β-glucan as a biological response modifier, suggests synergy in delivery of β-glucans for the treatment of human diseases where the immune system is compromised, such as HIV and cancer.

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