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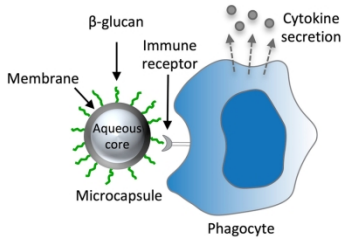
β-Glucan grafted microcapsule, a tool for studying the immunomodulatory effect of microbial cell wall polysaccharides



Useful tool in immunological study of microbial cell wall polysaccharides

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β-Glucan Grafted Microcapsule, a Tool for Studying the Immunomodulatory Effect of Microbial Cell Wall Polysaccharides

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Abstract: β-(1,3)-Glucan is one of the antigenic components of the bacterial as well as the fungal cell wall. We designed microcapsules (MCs) ligated with β-(1,3)-glucan, to study its immunomodulatory effect. The MCs were obtained by interfacial polycondensation between diacylchloride (sebacoyl chloride and terephtaloyl chloride) and diethylenetriamine in organic and aqueous phases, respectively. Planar films were first designed to optimize monomer compositions and to examine the kinetics of film formation. MCs with aqueous fluorescent core were then obtained upon controlled emulsification-polycondensation reactions using optimized monomer compositions and adding fluorescene into aqueous phase. The selected MC-formulation was grafted with curdlan, a linear β-(1,3)-glucan from *Agrobacterium species* or branched β-(1,3)-glucan isolated from the cell wall of *Aspergillus fumigatus*. These β-(1,3)-glucan grafted MCs were phagocytosed by human monocyte-derived macrophages, and stimulated cytokine secretion. Moreover, the blocking of dectin-1, a β-(1,3)-glucan recognizing receptor, did not completely inhibit the phagocytosis of these β-(1,3)-glucan grafted MCs, suggesting the involvement of other receptors in the recognition and uptake of β-(1,3)-glucan. Overall, grafted MCs are a useful tool for the study of the mechanism of phagocytosis and immunomodulatory effect of the microbial polysaccharides.

Introduction

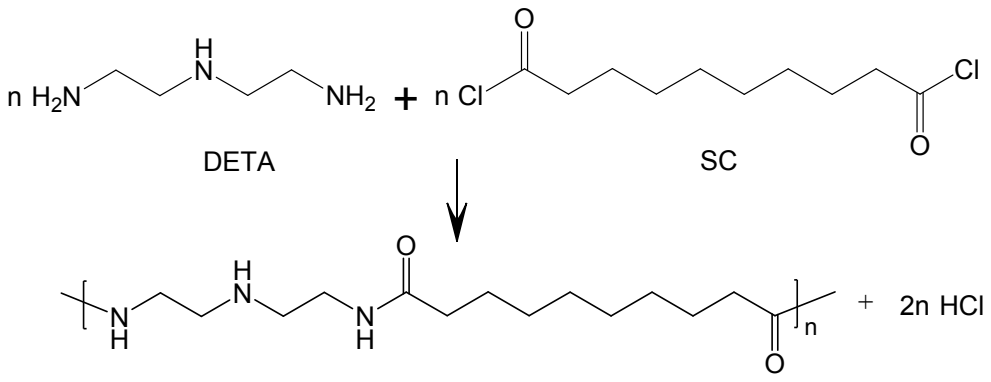
The fungal cell wall is a complex mixture of different polysaccharides, the composition of which is species-specific[1]. In pathogenic fungi, the cell wall is the first fungal component to interact with the host immune system. Therefore, these polysaccharides are potential pattern recognition-molecular patterns (PAMPs) interacting with the pattern recognition receptors (PRRs) on immune cells. The terminal immune responses of different polysaccharides are PRR-specific[2]. However, it is difficult to elucidate the role of immune response stimulation by an individual polysaccharide with the use of fungal cells, as here the cell wall is composed of multiple polysaccharides. This demands purification of individual cell wall polysaccharides for such specific study purposes.

One of the major components of the fungal cell is β-1,3-glucan[3], which is also produced by pathogenic bacteria, including *Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Alcaligenes faecalis*[4-7]. Upon purification, β-(1,3)-glucan in suspension may acquire structural alteration resulting in altered biological functions. For instance, being a part of the fungal cell wall, β-(1,3)-glucan will be internalized when fungal cells are phagocytosed by the immune cells. Whereas, isolated β-(1,3)-glucan forms particulate structures, which results in sustained activation of the immune cells[8] instead of being internalized. To overcome these drawbacks, pathogen-like particles are being conceptualized. However, in contrast to the data available on viral-like particles[9], insufficient work has been devoted to the development of a fungal-like particle. Tam et al. have designed polystyrene particles coated with β-(1,3)-glucan to understand the critical steps in early recognition of fungal polysaccharide by innate immune cells[10]; however, polystyrene particles are rigid. Our aim was to design β-(1,3)-glucan grafted microcapsules (MCs) with an aqueous core, allowing flexibility to mimic asexual spores (conidia) of *Aspergillus fumigatus*, a ubiquitous fungal pathogen. In this study, we designed β-(1,3)-glucan grafted MCs with a fluorescent aqueous core and size of *A. fumigatus* conidia. The fluorescent core of MCs facilitates tracking upon interaction with human monocyte-derived macrophages. With this advantage, the MCs were applied to the investigation of the immunomodulatory effect of β-(1,3)-glucan and the major PRRs involved in the recognition and phagocytosis of β-(1,3)-glucan.

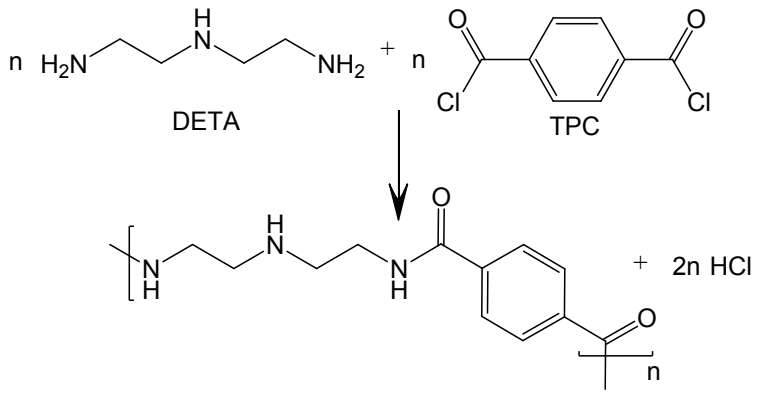
Results and Discussion

Preparation and characterization of polyamide film

Polyamide film was prepared by the interfacial polycondensation reaction[11-12] at water-cyclohexane interface using water soluble diethylene triamine (DETA) and a mixture of two lipophilic monomers, sebacoyle chloride (SC) and terephtaloyl chloride (TPC) (chemical reactions are shown in the Schemes 1 and 2) (Table 1).



Scheme 1: Interfacial polycondensation between DETA and SC



Scheme 2: Interfacial polycondensation between DETA and TPC

Table 1. Composition of aqueous and organic phases used for the preparation of the planar polyamide film. The volume ratio between organic and aqueous phases, concentrations of monomer and surfactant and temperature used in planar film preparation were kept for MC-preparation.

Composition		Product	Quantities
SOL1		Water	15 mL
		DETA	0.309 g (0.2 mol/L)
		Sodium carbonate	0.357 g (0.22 mol/L)
		Tween®80	1.5 mL (10% v/v)
SOL2		Cyclohexane	75 mL
SOL3		Cyclohexane	75 mL
	Film 1	SC 100%	1.434 g (0.8 mol/L)
	Film 2	SC/TPC (75/25%)	SC: 1.07 g (0.6 mol/L) TPC: 0.304 g (0.2 mol/L)
	Film 3	SC/TPC (50/50%)	SC: 0.717 g (0.4 mol/L) TPC: 0.605 g (0.4 mol/L)
	Film 4	SC/TPC (25/75%)	SC: 0.358 g (0.2 mol/L) TPC: 0.913 g (0.6 mol/L)
	Film 5	TPC 100%	1.121 g (0.8 mol/L)

The formation of planar films allowed their macroscopic study; the film was instantaneously formed when the two phases were in contact and within a few minutes, they became opaque and the thickness continued growing until equilibrium was reached after 12 h of reaction, which corresponded to the maximum thickness. The effect of different percentages of SC and TPC was studied (Figure 1). The film composed of 100% SC (Film 1) was non-homogeneous, fragile and composed of a plurality of layers. The base of the film (the face that is in contact with the aqueous phase) was thin and elastic, but, the polymer face in contact with the organic phase was non-homogeneous and rough. The film with 100% TPC (Film 5) was thin and homogeneous, but very fragile and less elastic than the film composed of SC. Whereas, the film composed of a low proportion of SC to TPC in the organic phase (Film 4) was the most homogeneous and resistant.

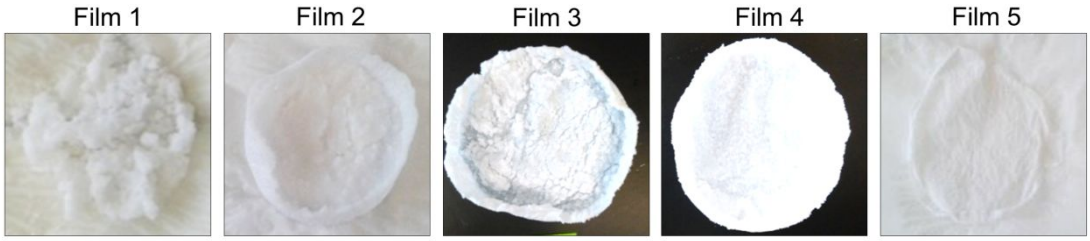


Figure 1: Macroscopic images of the planar polyamide films composed of water soluble DETA in aqueous phase and two hydrophobic monomers [SC 100%, Film 1; SC/TPC 75/25%, Film 2; SC/TPC 50/50%, Film 3; SC/TPC 25/75%, Film 4 and TPC 100%, Film 5].

The polyamide formation was followed using ATR-FTIR by the observation of amide vibration bands around 1650 and 1550 cm^{-1} (Figure 2). The amide bands are a mixed mode containing contributions from C=O/C-N stretching, C-C-N deformation vibrations, the N-H in-plane bending, the C-N stretching, and the C-N stretching vibrations. First, in each case the presence of both vibrational bands pointing the formation of amide functions was observed. Films 3 and 4 showed peaks around 700 cm^{-1} corresponding to -O-C-N groups. For all the films, the bands 2925 and 2851 cm^{-1} for $-CH_2$ asymmetric and symmetric stretching, respectively, were observed. The bands around 1400 cm^{-1} can be clearly attributed to $-CH_2$ deformation while structure at 3367 and 3289 cm^{-1} can be attributed to $C-NH_2$ in primary aliphatic amines. Indeed, we reported earlier that the polyamide film contains a small amount of DETA[12], as the amine diffuses through the polymer to interact with monomers in organic phase. Band around 1770 cm^{-1} corresponding to the presence of sebacoyl chloride was not observed.

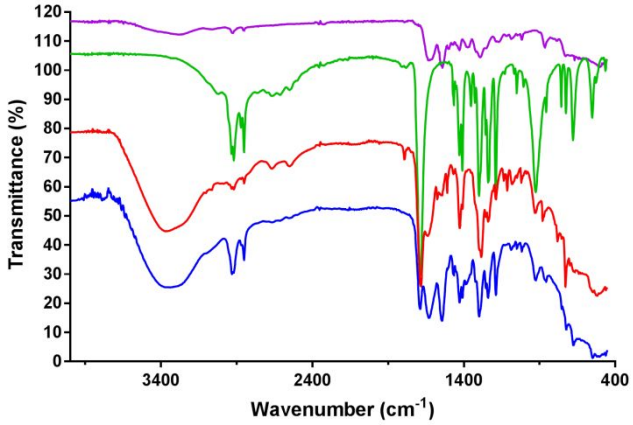


Figure 2: ATR-Infrared spectra of polyamide films obtained by the interfacial polycondensation of DETA and SC 100% (Film 1, blue), SC/TPC 50/50% (Film 3, green), SC/TPC 25/75% (Film 4, red) and TPC 100% (Film 5, purple).

Rheology measurements

Rheological experiments were carried out to characterize the kinetics of film formation for Film 3 (SC/TPC, 50/50%) and 4 (SC/TPC, 25/75%). The composition of aqueous phase (DETA, sodium carbonate and Tween® 80) was kept constant as indicated in Table 1 (SOL1). Furthermore, the effect of Tween® 80 on the interfacial rheology behavior of the film was evaluated by performing measurements without surfactant in the aqueous phase. The time-sweep was used to investigate and compare the time-course of interfacial film formation, and the evolution of the polymer formation as a function of time giving the value of G' (elastic modulus) and G" (loss modulus) over 12 h for each sample (Figure 3).

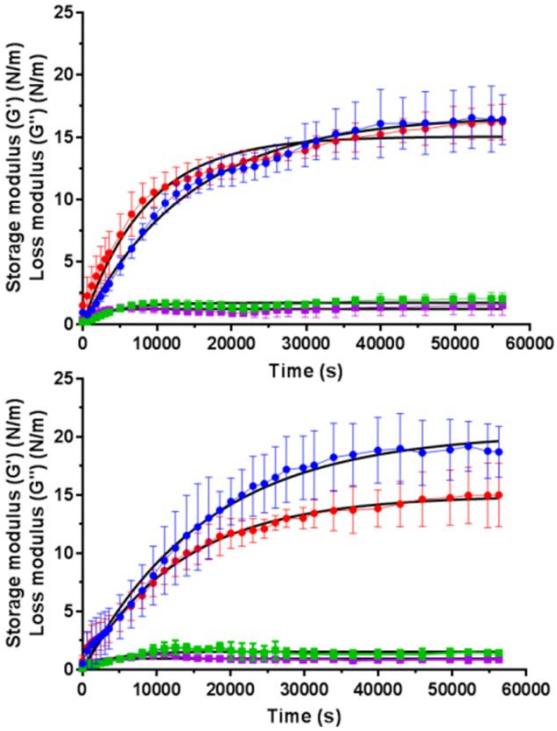


Figure 3: Elastic and loss moduli for SC/TPC film in presence of Tween® 80 (indicated by black and pink curves, respectively) and without Tween® 80 (blue and red curves, respectively) as a function of time [top, SC/TPC 50%/50% (Film 3) and bottom, SC/TPC 25%/75% (Film 4), respectively].

The results showed that interfacial elastic and viscous moduli, G' and G", respectively, reached equilibrium after about 12 h upon loading of the sample, indicating the attaining a steady state. G' was significantly higher than G", indicating that the interfacial microstructures formed are predominantly elastic. The increases in G' and G" moduli were observed whatever the composition of the phases: with/without Tween® 80. However, the resulting maximum G' value was lower in the presence of Tween®80 at SC/TPC ratio of 25/75%. Results of curves fitting for G' with the following equation: $G' = A (1 - \exp(-t/\tau))$, with A and τ constants, can be found in Table 2. Constant A is the modulus G' when t tends to infinity, and time constant τ is the time needed for G' to reach ≈ 0.63 A. Data confirmed the lower maximum G' value at a SC/TPC ratio of 25/75% with Tween® 80 and suggested that kinetics is faster at a SC/TPC ratio of 50/50% in the presence of Tween® 80 (Table 2).

Table 2. Constant A and time constant τ from curves fitting: $G' = A (1 - \exp(-t/\tau))$. Constant A is the modulus G' when t tends to infinity, and time constant τ is the time needed for G' to reach ≈ 0.63 A. Coefficient R^2 is the correlation coefficient.

Properties	Without Tween® 80		With Tween® 80	
SC/TPC ratio	25/75%	50/50%	25/75%	50/50%
A (N/m)	21 ± 2	18 ± 4	14 ± 2	15 ± 2
τ (s)	(18 ± 5) × 10^3	(15 ± 9) × 10^3	(12.3 ± 0.8) × 10^3	(9 ± 5) × 10^3
R^2	0.99	0.99	0.99	0.97

Formulation and characterization of microcapcules (MCs)

MCs composed exclusively of SC were not stable during the time of storage; microscopic observations showed that they disintegrated after 24 h of storage at room temperature. While, polyamide film composed of DETA/TPC was less elastic compared to DETA/SC film despite being more homogenous. For this reason, along with DETA, a mixture of SC and TPC was used in order to increase stability and elasticity of the polyamide film preparation and these monomers were used to obtain the MCs. Two types of polyamide shells were thus studied by modifying the SC-TPC ratio: (i) DETA-(SC/TPC, 25/75%); concentrations 0.2 mol/L/(0.2/0.6 mol/L and (ii) DETA-(SC/TPC, 50/50%); concentrations 0.2 mol/L/(0.4/0.4 mol/L). MCs obtained using these two compositions were stable after 24 h of storage at room temperature. Optical microscopy images of the two formulations showed that MCs of both formulations had a spherical shape, with sizes ranging from 1-7 µm (Figure 4).

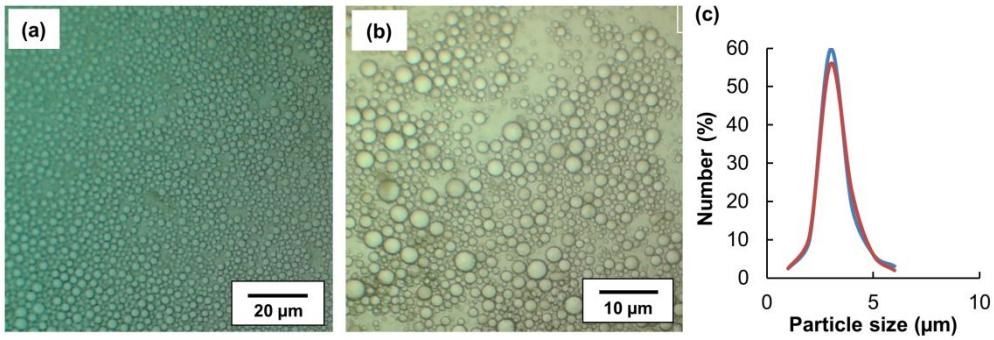


Figure 4: Optical microscopy images for MCs composed of SC/TPC at a ratio of (a) (25/75%) and (b) 50/50%. (c) Represents size distribution of MCs composed of SC/TPC (25/75%, red curve and 50/50%, blue curve).

Microcapsules (MCs) coated with polysaccharides

The morphology of the MCs observed by SEM showed spherical capsules with relatively a smooth external surface without visible pores (Figure 5). Notably, small size MCs (about 800 nm) were observed from SEM images, however, very few large MCs (3 µm) were observed, although optical microscopy (Figure 4) showed the MCs-size from 1-7 µm. This could be due to the possibility that the large MCs could not resist the drying phase of the samples, a necessary preparation process before conventional SEM analysis.

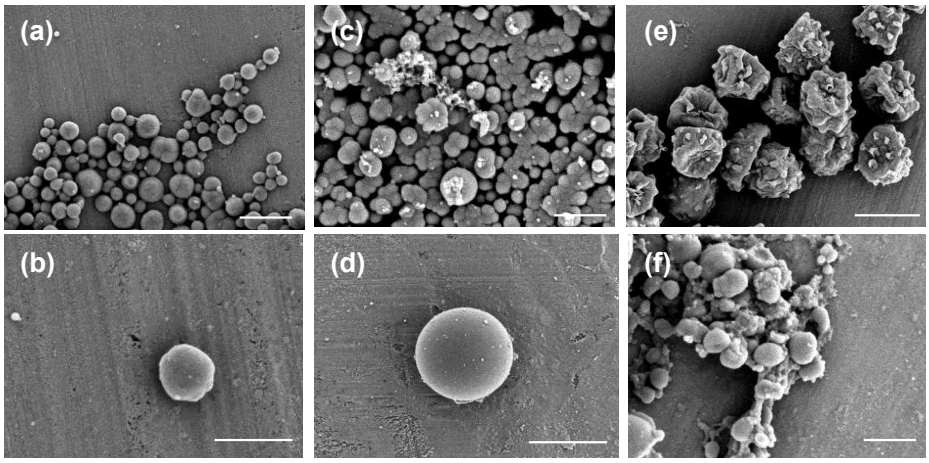


Figure 5: SEM images of MCs composed of DETA/(SC/TPC). The molar ratio between SC and TPC was 50/50% (corresponding to the concentrations of 0.4 mol/L of each monomer CS

and TPC) [(a) and (b)] or 25/75% (corresponding to the concentrations of (0.2/0.6 mol/L) [(c) and (d)]. DETA concentration was 0.2 mol/L for both formulations; (e) and (f) represent SEM images of *A. fumigatus* conidia and MCs (SC/TPC of 50/50%) coated with 17 mM curdlan, respectively; (scale bar, 3 μm).

MCs were coated with curdlan (linear β-(1,3)-glucan) at three different concentrations (10, 15 and 17 mM) as well as with 10 mM of branched β-(1,3)-glucan isolated from the *A. fumigatus* cell wall. The molecular weight of curdlan and β-(1,3)-glucan isolated from *A. fumigatus* were found to be 9.4 x 10^4 kDa and 1.42 x 10^5 kDa, respectively; the branched β-(1,3)-glucan from *A. fumigatus* showed a β-(1,6)-branching degree of 5.7%. Further, we confirmed the grafting of β-(1,3)-glucan on to MCs by immunolabeling. Grafted MCs were first treated with GNBP3[13], a receptor from drosophila that specifically binds to β-(1,3)-glucan, followed by treatment with secondary antibody conjugated to tetramethylrhodamine (TRITC); the control MCs without grafted β-(1,3)-glucan were also treated with the same procedures. A red layer was observed over β-(1,3)-glucan grafted MCs containing fluorescent core, suggesting that GNBP3 binds to the β-(1,3)-glucan ligated on the surface of these MCs (Figure 6). Control MCs did not show positive β-(1,3)-glucan labelling. The images represent MCs coated with 17 mM curdlan and 10 mM β-(1,3)-glucan isolated from the *A. fumigatus* cell wall; Figure 5(f) represents SEM image of MCs coated with 17 mM curdlan. An incomplete coating with *A. fumigatus* cell wall isolated β-(1,3)-glucan indicates that the molar concentration of the MC monomers and β-(1,3)-glucan play an essential role in the complete grafting process.

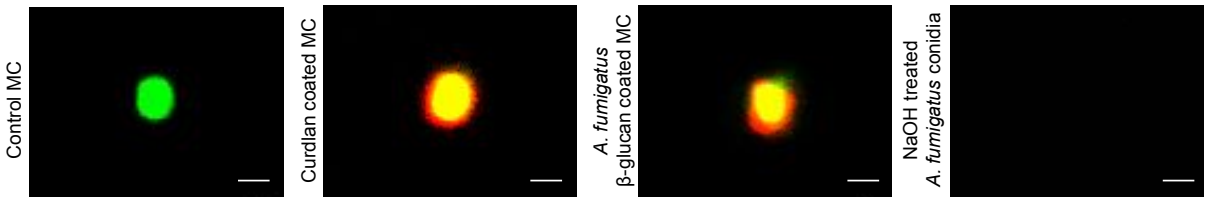


Figure 6: Fluorescent imaging of the control MCs and MCs grafted with curdlan or β-(1,3)-glucan isolated from the *A. fumigatus* cell wall, immunolabelled with GNBP3 followed by TRITC conjugated secondary antibody; *A. fumigatus* conidia were also treated with NaOH treatment to expose β-(1,3)-glucan and immunolabelled with GNBP3 followed by FITC conjugated secondary antibody (scale bar, 2 μm).

Immunological application of the grafted microcapsules

Phagocytosis is an inherent property of macrophages; therefore, even MCs without grafted β-(1,3)-glucan were phagocytosed by the human monocyte-derived macrophage (HMDM). However, only the HMDMs stimulated with β-(1,3)-glucan grafted MCs secreted cytokines (Figure 7a and 7b), confirming the immunogenic property of β-(1,3)-glucan. The amount of cytokines secreted by *A. fumigatus* cell wall β-1,3-glucan grafted MCs was less than that of curdlan grafted MCs. This could be due to the difference in concentration, 17 mM curdlan versus 10 mM of β-1,3-glucan from *A. fumigatus* used for grafting, or due to curdlan being linear β-1,3-glucan compared to branched β-1,3-glucan of *A. fumigatus*. However, when we used MCs grafted with 10 mM curdlan for HMDM stimulation assay (data not shown), the cytokine secretion was still significantly higher than the MCs grafted with β-1,3-glucan of *A. fumigatus*, suggesting that the observed differences in the stimulation of cytokine secretion between MCs grafted with curdlan and β-1,3-glucan of *A. fumigatus* is due to the branching of β-1,3-glucan from *A. fumigatus*. It was observed earlier that linear and branched β-1,3-glucan elicit differential cytokine response[14]; branched β-1,3-glucan stimulating the secretion of TNF-α, IFN-γ, GM-CSF, whereas soluble curdlan oligosaccharide could stimulate the

secretion of IFN-γ, GM-CSF but not TNF-α. Nevertheless, we did observe the secretion of TNF-α, as we used insoluble curdlan and not its oligosaccharide, suggesting a role played by the solubility/degree of polymerization of the curdlan on immunostimulation.

In addition, we also checked the cytokine response upon stimulating HMDM with linear and MC-grafted curdlan (Figure 7c). Although curdlan could stimulate cytokine secretion from HMDM compared to control, the stimulating capacity was significantly lower than HMDM stiulated with same amount of curdlan (17 mM) but grafted on MCs, suggesting that the organization and availability of β-1,3-glucan affect its immunogenic potential, as curdlan/ β-1,3-glucan are known to form particulate structure in their free form.

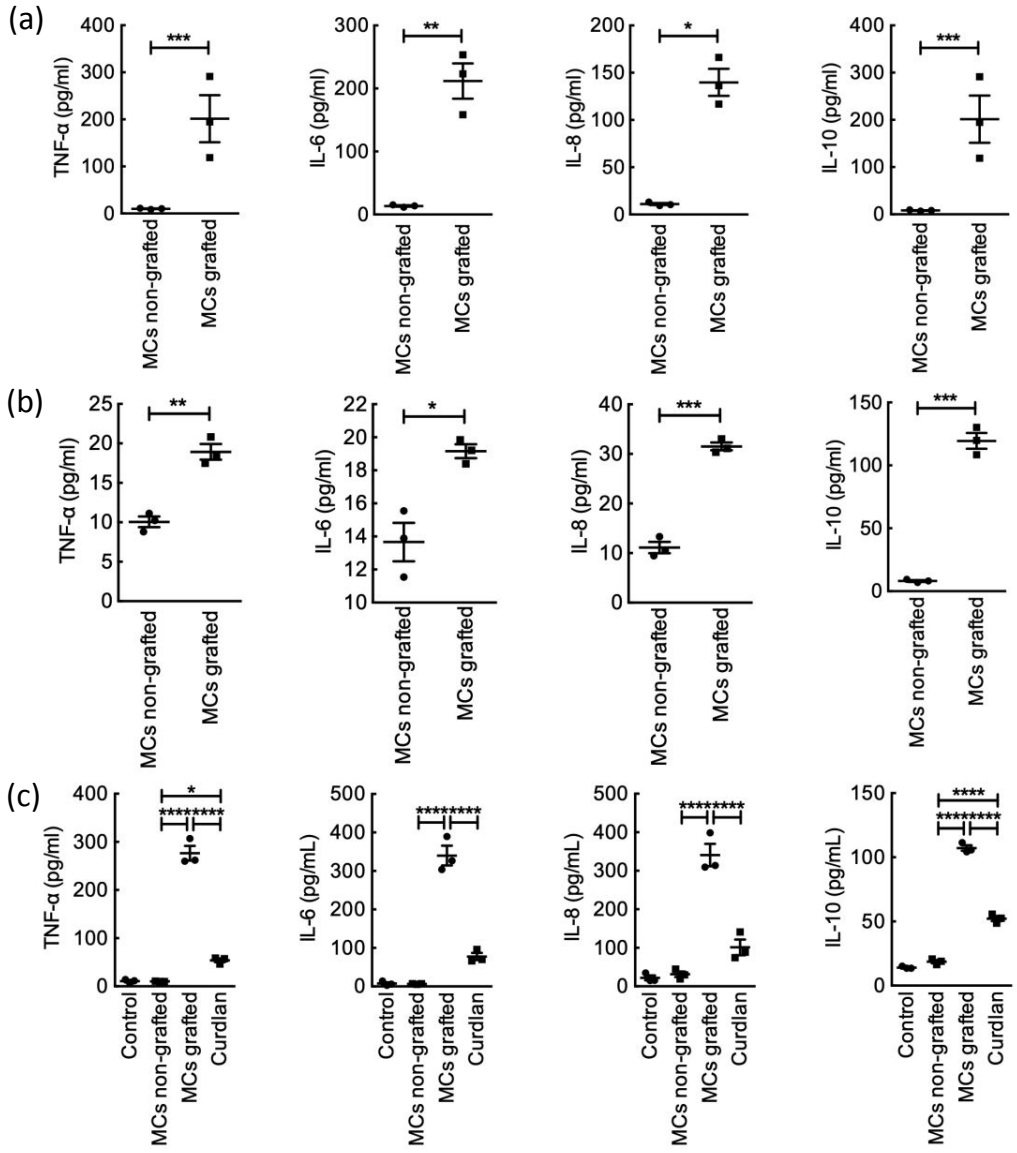


Figure 7: Evaluation of the ability of MCs composed of SC/TPC (50/50%) 0.2 mol/L/(0.4/0.4 mol/L) grafted with either **(a)** 17 mM curdlan or **(b)** 10 mM *A. fumigatus* β-1,3-glucan to stimulate TNF-α, IL-6, IL-8 and IL-10 secretion by human monocyte-derived macrophages (number of donors = 3). Control samples were the monocyte-derived macrophages stimulated with non-grafted MCs (***p<0.0005, **p<0.005 and *p<0.05). **(c)** The comparision between

MCs grafted with curdlan and curdlan alone in stimulating cytokine secretion from human monocyte-derived macrophages (number of donors = 3; *p<0.05 and ****p<0.0001).
We checked phagocytosis of curdlan grafted MCs by HMDM. Phagocytosis is an inherent characteristic of macrophages; therefore, upon incubating HMDM with MCs alone and MCs grafted with curdlan for 18 h (37°C in a CO_2 incubator) followed by washing wells with incomplete RPMI and determining fluorescence intensity, HMDM incubated with both MCs alone and MCs grafted with curdlan showed fluorescence. However, the fluorescence intensity was higher in HMDM incubated with MCs grafted with curdlan (Figure 8a). Moreover, MCs alone failed to stimulate cytokine secretion from HMDM (Figure 7). A further question we had was whether the β-(1,3)-glucan grafted MCs are phagocytosed through the classical endocytotic pathway. After 18 h of co-incubation of β-(1,3)-glucan grafted MCs with HMDM, the β-(1,3)-glucan grafted MCs were observed to be co-localized with Lamp1, which is a phagolysosomal marker (Figure 8b). The presence of these MCs in the phagolysosomes suggested that their uptake was through the conventional endocytosis, where phagolysosomes are formed following the fusion of endosomes and lysosomes.

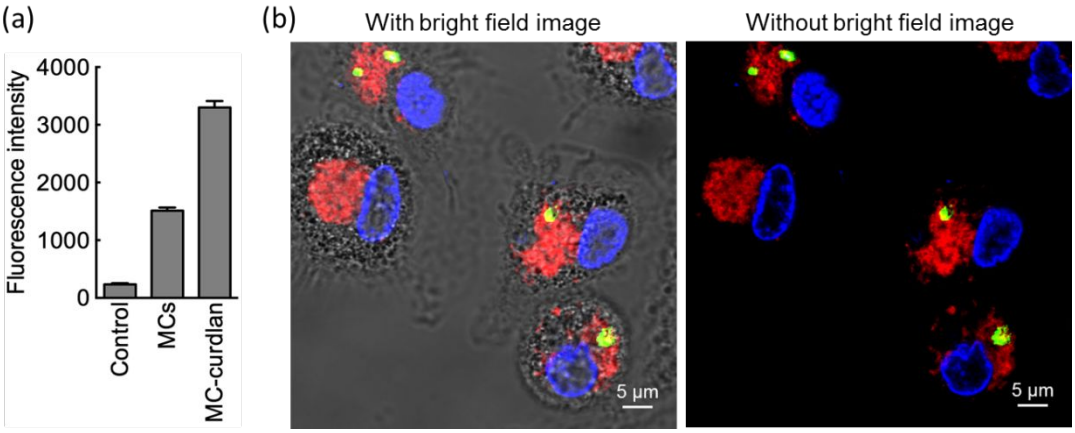


Figure 8: (a) Determination of phagocytosis by means of fluorescence intensity measurement. **(b)** co-localization of Lamp1, a phagolysosomal marker, with curdlan grafted MCs (Green, curdlan grafted MCs; blue, DAPI staining for the nuclei; red, Lamp1 staining); after 18 h or co-incubating β-(1,3)-grafted MCs and HMDM, cells were fixed, permeabilized, and labelled for Lamp1 with monoclonal anti-Lamp1 antibody conjugated with PE-Cy5.

Dectin-1 is a β-(1,3)-glucan receptor expressed by monocytes, macrophages and neutrophils[15-16]. However, blockade of dectin-1 receptors on HMDMs with anti-dectin-1 antibody did not lead to complete inhibition of the uptake of β-(1,3)-glucan grafted MCs (Figure 9), suggesting the involvement of other receptors in the recognition and uptake of β-1,3-glucan. Similarly, blocking complement receptors (CR3+CR4 with anti-CD11b, anti-CD11c and anti-CD18 antibodies) also resulted in partial inhibition of the phagocytosis of β-(1,3)-glucan grafted MCs (Figure 9), suggesting that β-1,3-glucan is not recognized exclusively by dectin-1, but also by the complement receptors, confirming earlier reports[17-20].

We also blocked both dectin-1 and complement receptors (both CR3 and CR4); however, we could still observe about 25-30% of the β-(1,3)-glucan grafted MCs being phagocytosed by HMDMs, suggesting that in addition to dectin-1 and complement receptors, there are other modes of β-(1,3)-glucan recognition by the immune cells.

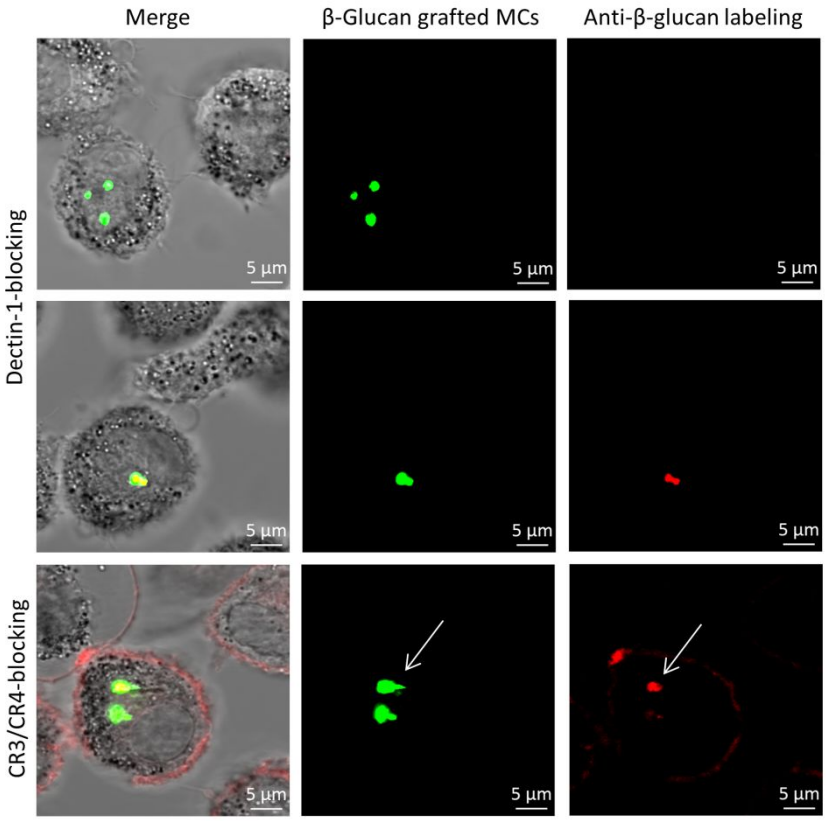


Figure 9: Phagocytosis of β-1,3-glucan grafted MCs by macrophages upon blocking dectin-1 or complement receptors (both CR3 and CR4) by respective antibodies. Human monocyte-derived macrophages were pre-incubated with either anti-dectin-1 or anti-CR3/CR4 antibodies, followed by the addition of β-(1,3)-glucan grafted MCs. Confocal images were taken after 2 h incubation at 37°C in a CO_2 incubator. Non-phagocytosed β-(1,3)-glucan grafted MCs were labelled with monoclonal anti-β-(1,3)-glucan antibodies[21] and secondary antibody (anti-mouse IgG-TRITC conjugated) (red). Note that CR4 is also labeled in red on the cell surface due to the fluorophore (PE-Cy7) of the anti-CD11c antibody. There was complete phagocytosis of β-(1,3)-glucan grafted MCs when the receptors were not blocked. While, Dectin-1 or CR3-CR4 blocking caused partial inhibition of the phagocytosis of β-(1,3)-glucan grafted MCs, evidenced by the positive labelling of some of the grafted MCs by the monoclonal β-(1,3)-glucan antibody. Note that CD11c was blocked using monoclonal antibody conjugated with PE-Cy7, could be seen on the periphery of the macrophages, which confirms the blocking of the receptor.

Conclusions

In this study, we have developed polyamide microcapsules (MCs) containing fluorescent aqueous core grafted with β-1,3-glucan, the major polysaccharide in the fungal cell wall and also produced by some bacteria. These grafted MCs overcome the drawbacks of using fungal cells or extracted polysaccharides in suspension in the study of the immunological role of individual cell wall polysaccharides. The use of fungal cell, of which the wall contains a complex mixture of different polysaccharides which obscures the effect due to individual polysaccharides, whereas isolated polysaccharides often form particulate structure thus altering the immune potential. Moreover, compared to rigid polystyrene beads, the aqueous core of these MCs allows flexibility, which mimics the structure and size of *A. fumigatus* conidia. The aqueous core is also fluorescent to allow tracking of the MCs during interaction

with and trafficking within immune cells. With these β-1,3-glucan grafted MCs, we have validated the efficiency of this tool in describing the immunological role of β-1,3-glucan as well as this polysaccharide recognition by the immune cells. In the future, other fungal cell wall polysaccharides can be grafted individually to the MCs to examine their immunological roles. MCs can also be exploited upon grafting, for example, with several polysaccharides in a proportion similar to what exists on the microbial surface, in order to study the overall effect of microbial cell wall on the host immune system.

Experimental Section

Materials: Monomers (diethylenetriamine (DETA), sebacoyl chloride(SC) and terephthaloyl chloride (TPC)), 1,1'-carbonyldiimidazole (CDI), sodium carbonate and fluorescein were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). DMSO and cyclohexane were purchased from Carlo Erba (Val de Reuil, France). Tween® 80 was purchased from Croda (Trappes, France). Glutaraldehyde and cacodylate buffer were provided by the microscopy and imaging platform of INRA of Jouy en Josas, France. Curdlan was obtained from Wako Pure Chemical Industries Ltd., Japan. β-(1,3)-glucan was isolated from alkali-insoluble fraction of the *A. fumigatus* cell wall as described before[22]. The molecular weight of curdlan/β-(1,3)-glucan isolated from *A. fumigatus* cell wall was determined by following the method described by Kumagai et al with modification, whereas the degree of branching was determined as we described earlier[3]. For molecular weight determination, the total sugar contents was determined by phenol-sulfuric acid method, whereas the reducing sugar by bicinconinic acid method[23]; average degree of polymerization (DP) was calculated as total sugar/reducing sugar in μmol of glucose, and the molecular weight as (DP x 162)+18.

Preparation of polyamide film: Achieved by adapting the method developed by Bouchemal et al[12] and Hanno et al[11]. The films were formed after very slow addition of organic phase (75 mL) to the aqueous phase (15 mL). The composition of aqueous and organic phases are detailed in Table 1. The aqueous phase was composed of DETA (0.309 g; 0.2 mol/L) in distilled water, sodium carbonate (0.357 g; 0.2 mol/L) and Tween® 80 (1.5 mL, 10% v/v). The organic phase was composed of lipophilic monomers dissolved in cyclohexane. The total concentration of lipophilic monomers was kept constant at 0.08 mol/L. DETA/lipophilic monomer molar monomers ratio was 1/2. (**SOL1**) composed of DETA, sodium carbonate, used to neutralize hydrogen chloride formed during the polymerization reaction, and Tween® 80 as a surfactant. To this phase, the organic phase was added very delicately and without sudden movement. The organic solution was composed of cyclohexane in which the monomers were dissolved at different concentrations (**SOL3**). The beaker was left at room temperature for 48 h. Thereafter the films are recovered and dried to remove excess of cyclohexane and stored at room temperature.

ATR-FTIR: Carried out for the characterization of the polyamide films using a JASCO FT-IR-4100 spectrometer. An accumulation of 60 scans per measurement was carried out in order to obtain a spectrum between 4000 and 400 cm^{-1}. The results of each spectrum were analyzed by Spectra Manager software.

Interfacial rheology: Interfacial rheology experiments were performed to characterize film formation kinetics. Interfacial measuring system was composed of a double wall ring (DWR) geometry[24] fixed to an AR-G2 stress-controlled rotational rheometer (TA Instruments, USA). Briefly, 18.8 mL of aqueous phase (aqSOL1) was poured in a circular channel, and 18.8 mL of organic phase SOL3 was added. The DWR was carefully placed at the interface between both fluids. Oscillation experiments were performed with a strain of 0.1% in the linear

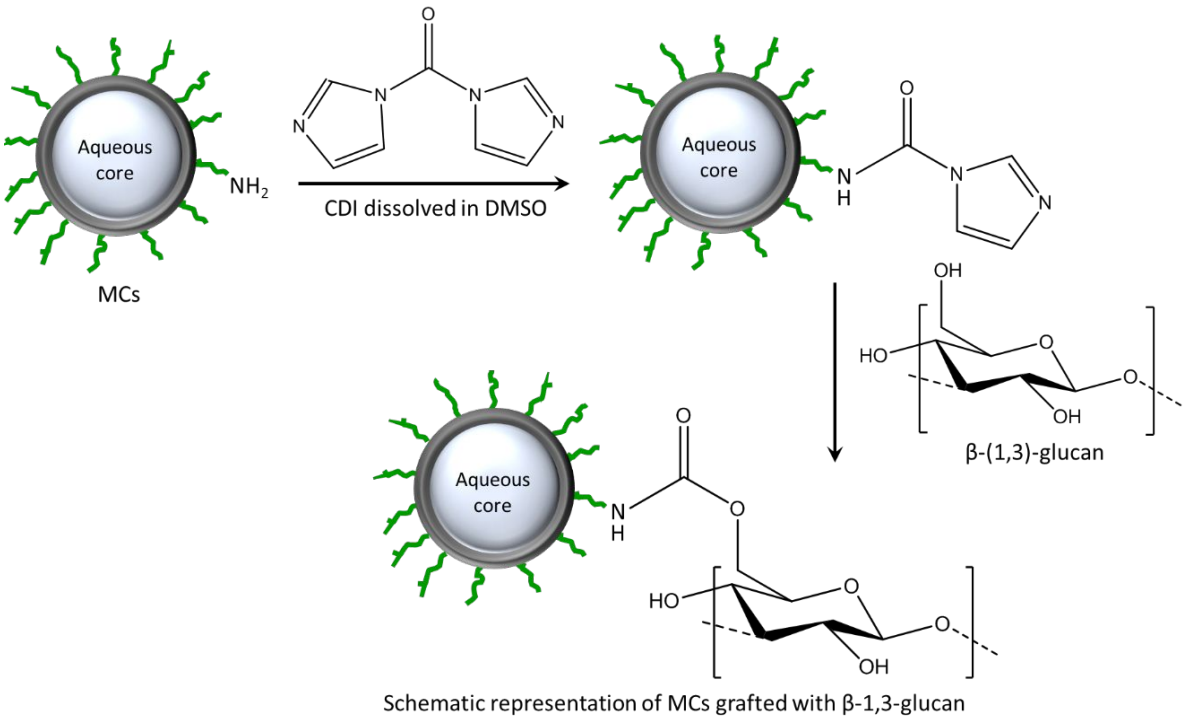
regime, with a frequency of 1 Hz and a temperature of 20°C. Interfacial elastic modulus was measured as a function of time. All experiments were performed in triplicate.

Preparation and characterization of polyamide microcapsules (MCs): The preparation of polyamide MCs was performed by the interfacial polycondensation reaction between a triamine (DETA) and a diacyl chloride (SC, TPC or their mixtures). MCs were obtained in two steps: emulsification followed by polycondensation. **Emulsification step:** W/O emulsion was obtained by adding an aqueous phase (SOL1) containing DETA, sodium carbonate and Tween® 80 to an organic solution (SOL2) composed of cyclohexane contained in a beaker of 200 mL. The mixture was then mechanically agitated for 15 min at 6000 rpm with an ultraturrax mixer (PT 3100 Polytron, Kinematica, Switzerland). **Polycondensation step:** The speed was decreased of 2000 rpm and hydrophobic monomers dissolved in cyclohexane (SOL3) were added to the emulsion, without stopping the agitation. The stirring was continued for further 15 min. The polymer, obtained by the polycondensation of monomers, precipitated at the interface and formed the membrane of the capsule. Table 1 summarizes all the products used and their quantities for the various formulations. Fluorescent MCs were obtained similarly by dispersing 3 mg of fluorescein in aqueous phase. During MC-formation, diacylchoride consumption was monitored by ATR-FTIR; very week absorption band at 1790-1815 cm^{-1}, characteristic of acylchloride group, was observed in the supernatant organic phase at the end of MC-formation, suggesting almost complete consumption of diacylchloride during the reaction. Due to the aqueous core, formed MCs sediments, which were separated from the supernatant organic phase and washed with DMSO.

MC-morphology was characterized using optical microscopy as well as scanning electron microscopy (SEM). MC suspensions were fixed in glutaraldehyde. For each sample, 300 µL of MC suspension were added to 2.7 mL of glutaraldehyde at a concentration of 2.5% in PBS, and the mixture was incubated at 4°C for 30 min. After incubation, different washes were performed with cacodylate buffer at 0.1 M as follows: a volume of 2.7 mL of the preceding mixture was removed; the same volume of cacodylate buffer at 0.1 M was added and centrifugation at 1000 rpm for 5 min was performed. The different samples were then kept at 4°C. Then, 40 µL of each sample were deposited during 2 h onto a 10 mm aluminum coupon (washed just before use by sonication in ethanol and placed into one well of a 24-well polystyrene plate). Samples were then fixed in 0.10 M cacodylate buffer containing 2.5 % (v/v) glutaraldehyde (pH 7.4) for 1 h at room temperature. Samples were then washed 3 times for 10 min with the cacodylate buffer alone. Samples were dehydrated with increasing concentrations of ethanol at room temperature (50%, 75% and 2 times with 100% for 10 min each step, and a last overnight 100% ethanol step), then 2 successive baths of 10 min of HMDS, followed by 2 h of evaporation in a fume hood for the drying. Coupons were mounted on aluminum stubs with double-sided carbon tape and were sputter coated (Polaron SC7640, Quorum Technnologies, UK) in Ar plasma with Pt (approximately 30 nm of thickness) at 10 mA and 0.8 kV over duration of 200 s. Observations were performed in FE-SEM S4500 (Hitachi, Japan) in high vacuum, with a low secondary electron detector, at 2 kV and 16 mm working distance, at the MIMA2 microscopy platform (http://www6.jouy.inra.fr/mima2). Size distribution was evaluated from optical microscopy observations. The MCs prepared were observed under a microscope (10 and 40x magnifications), and were photographed. For each image, the diameter of 500 MCs was measured to calculate the size distribution.

Preparation and characterization of MCs coated with curdlan/β-(1,3)-glucan: MCs composed of SC/CTP (50/50%) was selected for the grafting of β-(1,3)-glucan or curdlan. One milliliter of a suspension containing MCs was washed once in DMSO using centrifugal

filters containing a 0.45 µm PTFE membrane. MCs retained in the filtration membrane were resuspended in 1 mL of anhydrous DMSO containing 1,1'-carbonyldiimidazole (CDI) at a final concentration of 1 M (0.0810 g of CDI dissolved in 1 mL of DMSO). The CDI allows the activation of amine groups of the MC-shell. Then, MCs were rinsed once by anhydrous DMSO using centrifugal filters and then resuspended in DMSO containing 10 mM of β-(1,3)-glucan or curdlan at different concentrations (10 mM, 15 mM or 17 mM). The mixture with β-(1,3)-glucan or curdlan was stirred at ambient temperature for 1 h (Scheme 3). The resulting MCs were filtered with centrifugal filters to remove DMSO and resuspended in 1 mL of distilled water, washed five times with distilled water, resuspended in distilled water and stored at stored at 4°C. For washing step, centrifugations were carried out at 5000 rpm for 5 minutes and at 20°C. The grafting of β-(1,3)-glucan or curdlan on the MCs was validated by fluorescent microscopy after labelling with GNBP3, a receptor specific for β-(1,3)-glucan[13], followed by a secondary antibody (anti-mouse IgG-TRITC).



Schematic representation of MCs grafted with β-1,3-glucan

Scheme 3: Representation of the β-(1,3)-glucan grafting on microcapsules (MCs). MCs were first incubated in a solution of 1,1'-carbonyldiimidazole (CDI) in DMSO for 1 h at ambient temperature. The CDI allows the activation of the amine groups of the MC-shell. Then, MCs were rinsed once by anhydrous DMSO and resuspended in DMSO containing β-(1,3)-glucan (curdlan or isolated from *A. fumigatus* cell wall) and stirred at ambient temperature for 1 h. The resulting MCs were filtered to remove DMSO and resuspended in 1 mL of distilled water, washed with water, resuspended in water and stored at 4°C. Alternatively, CDI could also activate carboxyl groups on the MC-surface, allowing the coupling with hydroxyl groups of a polysaccharide.

Immunoassays: Fresh venous blood from three healthy donors was drawn in tubes containing EDTA and processed for PBMC isolation[25]. In brief, the blood was diluted 1:1 with phosphate buffered saline (PBS) and PBMCs were isolated using Ficoll-paque (GE Healthcare) density gradient centrifugation and washed twice in cold PBS. PBMCs (2 x 10^6/well) were seeded in 12-well culture plates in incomplete RPMI for overnight at 37°C in CO_2 incubator. The

medium was discarded and adherent cells were washed twice with incomplete RPMI. Cells were cultured with complete RPMI (RPMI containing 10% normal human serum) and granulocyte macrophage–colony-stimulating factor (10 ng/mL) (Sigma) for 5 days (37°C, CO_2 incubator) to differentiate monocyte into macrophages. The supernatant was discarded and the human monocyte-derived macrophages (HMDMs) were washed with PBS for three times. The MCs tested were of formulation with polyamide 1, DETA (SC/TPC 50/50%) 0.2 mol/L/(0.4/0.4 mol/L). The grafted MCs contained 8.2 and 5.6 µg of curdlan and β-1,3-glucan from *A. fumigatus*, respectively per 100 µL MC-suspension as determined by phenol-sulfuric acid method for total sugar estimation. Ten-microlitres of control MCs (without grafting), MCs grafted with 17 mM curdlan and MCs grafted with 10 mM *A. fumigatus* cell wall β-1,3-glucan were suspended in complete RPMI (1 mL), added to the HMDMs and incubated at 37°C in a CO_2 incubator for 18 h and 40 h, respectively, for phagocytois and cytokine measurement). After 18 h incubation, culture media were discarded, cells were washed twice with incomplete RPMI, fixed for 10 min with 1% *para*-formaldehyde, washed twice with RPMI. Washed HMDM were either taken for flurescence measurement (excitation at 495 nm and emission at 510 nm using TECAN Infinite 200 PRO microplate reader) or permeabilized with methanol for 10 min, washed twice with RPMI, labelled for Lamp1 (the phagolysosomal marker) using monoclonal anti-Lamp1 antibody conjugated to PE-Cy5 and DAPI for HMDM-nuclei, and observed under microscope (both bright-light and fluorescent). After 40 h-incubation, supernatants were collected and cytokines in the co-culture supernatant were quantified by DuoSet ELISA kits (R&D Systems).

For the immune receptors-blocking assay, HMDMs were cultured in 8-well plates (IBIDI) with similar procedures as described above. The immune receptors were blocked with corresponding antibodies for 30 min followed by the addition of grafted MCs. Dectin-1 was blocked with anti-dectin-1 antibody (GE2 1 µg/mL; 250 µL per well). Complement receptors (CR3 and CR4) were blocked with anti-CD11b antibody (Sony), anti-CD11c antibody conjugated with PE-Cy7 (Tonbo Biosciences) and anti-CD18 antibody (Calbiochem) (all were at a concentration of 1 µg/mL; 250 µL per well). Following, β-(1,3)-grafted MCs were added to receptor-blocked HMDMs and made to interact at 37°C for 2 h in a CO_2 incubator. Before microscopic observation, non-phagocytosed MCs were labelled with GNBP3[13] as described before. Experiments were also performed by using the blocking antibodies at 2 µg/ml, but the results were similar compared to blocking at 1 µg/mL concentrations.

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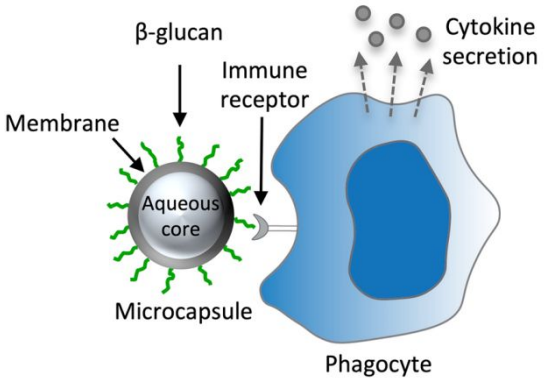
References

1. Tada, R., Latge, J. P., and Aimanianda, V. (2013) Undressing the fungal cell wall/cell membrane--the antifungal drug targets. Curr Pharm Des**.** 19, 3738-3747.
2. Bozza, S., Clavaud, C., Giovannini, G., Fontaine, T., Beauvais, A., Sarfati, J., D'Angelo, C., Perruccio, K., Bonifazi, P., Zagarella, S., et al. (2009) Immune sensing of Aspergillus fumigatus proteins, glycolipids, and polysaccharides and the impact on Th immunity and vaccination. J Immunol. 183, 2407-2414.
3. Aimanianda, V., Simenel, C., Garnaud, C., Clavaud, C., Tada, R., Barbin, L., Mouyna, I., Heddergott, C., Popolo, L., Ohya, Y., et al. (2017) The dual activity responsible for the elongation and branching of beta-(1,3)-glucan in the fungal cell wall. MBio. 8, e00619-17.
4. Honda, S., Sugino, H., Asano, T., and Kakinuma, A. (1986) Activation of the alternative pathway of complement by an antitumor (1-3)-beta-D-glucan from Alcaligenes faecalis var. myxogenes IFO 13140, and its lower molecular weight and carboxymethylated derivatives. Immunopharmacol. 11, 29-37.
5. Mennink-Kersten, M. A., Ruegebrink, D., and Verweij, P. E. (2008) Pseudomonas aeruginosa as a cause of 1,3-beta-D-glucan assay reactivity. Clin Infect Dis. 46, 1930-1931.
6. Mennink-Kersten, M. A., and Verweij, P. E., (2007) Non-culture-based diagnostics for opportunistic fungi. Infect Dis Clin North Am. 20, 711-727.
7. Stone, B. A., and Clarke, A. E. (1992) Chemistry and biology of (1–3)-beta-D-Glucan. La Trobe University Press, Melbourne, Australia.
8. Camilli, G., Eren, E., Williams, D. L., Aimanianda, V., Meunier, E., and Quintin, J. (2018) Impaired phagocytosis directs human monocyte activation in response to fungal derived beta-glucan particles. Eur J Immunol. 48, 757-770.
9. Martin, J. L., Cao, S., Maldonado, J. O., Zhang, W., and Mansky, L. M. (2016) Distinct particle morphologies revealed through comparative parallel analyses of retrovirus-like particles. J Virol. 90, 8074-8084.
10. Tam, J. M., Mansour, M. K., Khan, N. S., Yoder, N. C., and Vyas, J. M. (2012). Use of fungal derived polysaccharide-conjugated particles to probe Dectin-1 responses in innate immunity. Integr Biol (Camb). 4, 220-227.
11. Hanno, I., Anselmi, C., and Bouchemal, K. (2012) Polyamide nanocapsules and nano-emulsions containing Parsol(R) MCX and Parsol(R) 1789: in vitro release, ex vivo skin penetration and photo-stability studies. Pharm Res. 29, 559-573.
12. Bouchemal, K., Couenne, F., Briançon, S., Fessi, H., and Tayakout, M. (2006) Polyamides nanocapsules: Modeling and wall thickness estimation. AIChE. 52, 2161-2170.
13. Mishima, Y., Quintin, J., Aimanianda, V., Kellenberger, C., Coste, F., Clavaud, C., Hetru, C., Hoffmann, J. A., Latge, J. P., Ferrandon, D., et al. (2009) The N-terminal domain of Drosophila Gram-negative binding protein 3 (GNBP3) defines a novel family of fungal pattern recognition receptors. J Biol Chem. 284, 28687-28697.
14. Hida, T. H., Ishibashi, K., Miura, N. N., Adachi, Y., Shirasu, Y., and Ohno, N. (2009) Cytokine induction by a linear 1,3-glucan, curdlan-oligo, in mouse leukocytes in vitro. Inflamm Res. 58, 9-14.
15. Brown, G. D., Taylor, P. R., Reid, D. M., Willment, J. A., Williams, D. L., Martinez-Pomares, L., Wong, S. Y., and Gordon, S. (2002) Dectin-1 is a major beta-glucan receptor on macrophages. J Exp Med. 196, 407-412.
16. Taylor, P. R., Brown, G. D., Reid, D. M., Willment, J. A., Martinez-Pomares, L., Gordon, S., and Wong, S. Y. (2002) The beta-glucan receptor, dectin-1, is predominantly

expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. J Immunol. 169, 3876-82.
17. Rice, P. J., Kelley, J. L., Kogan, G., Ensley, H. E., Kalbfleisch, J. H., Browder, I. W., and Williams, D. L. (2002) Human monocyte scavenger receptors are pattern recognition receptors for (1→3)- β -D-glucans. J Leukoc Biol. 72, 140-146.
18. van Bruggen, R., Drewniak, A., Jansen, M., van Houdt, M., Roos, D., Chapel, H., Verhoeven, A. J., and Kuijpers, T. W. (2009) Complement receptor 3, not Dectin-1, is the major receptor on human neutrophils for β-glucan-bearing particles. Mol Immunol. 47, 575-581.
19. de Jong, M. A. W. P., Vriend, L. E. M., Theelen, B., Taylor, M. E., Fluitsma, D., Boekhout, T., and Geijtenbeek, T. B. H. (2010) C-type lectin Langerin is a β-glucan receptor on human Langerhans cells that recognizes opportunistic and pathogenic fungi. Mol Immunol. 47, 1216-1225.
20. Jin, Y., Li, P., and Wang, F. (2018) Beta-glucans as potential immunoadjuvants: A review on the adjuvanticity, structure-activity relationship and receptor recognition properties. Vaccine. 36, 5235-5244.
21. Matveev, A. L., Krylov, V. B., Khlusevich, Y. A., Baykov, I. K., Yashunsky, D. V., Emelyanova, L. A., Tsvetkov, Y. E., Karelin, A. A., Bardashova, A. V., Wong, S. S. W., et al. (2019) Novel mouse monoclonal antibodies specifically recognizing beta-(1-->3)-D-glucan antigen. PloS one. 14, e0215535.
22. Stephen-Victor, E., Karnam, A., Fontaine, T., Beauvais, A., Das, M., Hegde, P., Prakhar, P., Holla, S., Balaji, K. N., Kaveri, S. V., et al. (2017) Aspergillus fumigatus cell wall alpha-(1,3)-glucan stimulates regulatory T-cell polarization by inducing PD-L1 expression on human dendritic cells. J Infect Dis. 216, 1281-1294.
23. Kumagai, Y., Okuyama, M., and Kimura, A. (2016) Heat treatment of curdlan enhances the enzymatic production of biologically active beta-(1,3)-glucan oligosaccharides. Carbohydr Polym. 146, 396-401.
24. Vandebril, S., Franck, A., Fuller, G. G., Moldenaers, P., and Vermant, J. (2010) A double wall-ring geometry for interfacial shear rheometry. Rheologica Acta. 49, 131–144
25. Delsing, C. E., Becker, K. L., Simon, A., Kullberg, B. J., Bleeker-Rovers, C. P., van de Veerdonk, F. L., and Netea, M. G. (2015) Th17 cytokine deficiency in patients with Aspergillus skull base osteomyelitis. BMC Infect Dis. 15, 140-145.


β-glucan
Immune
receptor
Cytokine
secretion
Membrane
Aqueous
core
Microcapsule
Phagocyte