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β -Cyclodextrin-Functionalized Chitosan/Alginate Compact PolyElectrolyte Complexes (CoPECs) as Functional Biomaterials with Anti-Inflammatory Properties

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ABSTRACT. Nowadays, the need for therapeutic biomaterials displaying anti-inflammatory properties to fight against inflammation-related diseases is continuously increasing. Compact PolyElectrolyte Complexes (CoPECs) represent a new class of materials obtained by

ultracentrifugation of a polyanion/polycation complexes suspension in the presence of salt. Here, a non-cytotoxic β -cyclodextrin-functionalized chitosan/alginate CoPEC was formulated, characterized and described as a promising drug carrier displaying an intrinsic anti-inflammatory property. This new material was successfully formed and due to the presence of cyclodextrins, it was able to trap and release hydrophobic drugs such as Piroxicam used as model drug. The intrinsic anti-inflammatory activity of this CoPEC was analyzed *in vitro* using murine macrophages in the presence of LipoPolySaccharide (LPS) endotoxin. In this model, it was shown that CoPEC inhibited LPS-induced TNF- α and NO release and moderated the differentiation of LPS-activated macrophages. Over time, this kind of bioactive biomaterial could constitute a new family of delivery systems and expand the list of therapeutic tools available to target inflammatory chronic diseases such as arthritis or Crohn's disease.

1. INTRODUCTION

Nowadays, the development of biomaterials such as nano and microparticles, beads, membranes or implants is of great importance in biomedical, biotechnological and pharmaceutical fields. Biomaterials features, along with bio-industrial requirements, have progressively evolved from bio-inertia to biocompatibility and biodegradability, to finally target a fully controlled bioactivity. To date the use of those therapeutic biomaterials is envisaged for the treatment of different diseases as for example inflammatory chronic diseases.¹

Inflammation is part of the innate immune response involved in the fight against pathogens and cancer. Inflammation is physiologically induced by pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP). For example,

LipoPolySaccharide (LPS), as part of Gram-negative bacteria cell wall (endotoxin), is a PAMP known to induce inflammation. Inflammation is physiologically highly regulated. However, in some cases it becomes chronic and is involved in the development of many severe diseases such as cancer, rheumatoid arthritis or Crohn's disease. Numerous anti-inflammatory biomaterials have been developed and most of them display this activity by releasing an anti-inflammatory active substance incorporated in their structure.^{2,3,4,5,6} To date, only few biomaterials showing intrinsic anti-inflammatory properties have been described, among them one can enumerate silver,^{7,8,9} cerium oxide nanoparticles^{10,11} and some hydrogels made of *N*-acryloyl glycinamide¹² or hyaluronic acid.¹³

When considering further pharmacological development, fully natural polymers or synthetic ones possessing hydrolysable bonds and/or recyclable units are generally preferred for new biomaterials.

Chitosan is a natural cationic aminopolysaccharide obtained from the *N*-deacetylation of chitin, which is a structural constituent of crustacean shells. It is biocompatible, biodegradable, nontoxic and exhibits antimicrobial activity against bacteria and fungi.¹⁴

Alginate is a natural anionic and hydrophilic polysaccharide isolated from marine brown algae species or produced by bacteria.¹⁵ It is biocompatible and has a low toxicity as well as a relatively low cost.

Due to their respective remarkable biological properties, numerous works related to the association of these two polyelectrolytes have already been conducted and have led to a large variety of biomaterials. Association of these two components find applications as membranes for soft tissue engineering,^{16,17} as hydrogels for tissue regeneration,¹⁸ as nanoparticles^{19,20,21} or micro and nanocapsules^{22,23} for drug carrying or dyeing, as polymer networks for cartilage regeneration²⁴ or as fibers for other applications.²⁵ These biomaterials show different features

depending on polymer concentrations and/or formulation conditions such as pH, ionic strength and temperature.

Recently, Schlenoff and coworkers first described so-called Compact PolyElectrolyte Complexes (CoPECs) obtained by mixing two oppositely charged synthetic polyelectrolytes, such as poly(diallyldimethylammonium) (PDADMA) and poly(styrene sulfonate) (PSS), in the presence of salt and then compacting the resulting complexes by ultracentrifugation or extrusion. These innovative materials were described as saloplastic, as they show a plastic behavior thanks to the presence of salt that plays the role of plasticizer.^{26,27} Then, as part of our research in the field of drug delivery, Reisch *et al.* worked on complexes of poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH) and showed that these materials possess remarkable self-healing and stretching properties.^{28,29} Moreover, Tirado *et al.* also demonstrated that these materials have the interesting properties to immobilize, protect and enhance the activity of enzymes incorporated inside their structure.³⁰

In a recent work, Phoeung *et al.* found that by finely tuning the experimental conditions one can process chitosan/alginate based CoPECs which are anti-adhesive against cells and non pro-inflammatory materials.³¹ As these new chitosan/alginate-based CoPECs do not have the ability to carry drugs and especially hydrophobic drugs due to their hydrophilic properties, the first aim of the present work was to modify natural chitosan and alginate to develop functionalized chitosan/alginate CoPECs to further give them the ability to carry and release drugs. For that purpose, we envisaged to functionalize these two polysaccharides with a cyclodextrin ring able to create host-guest interactions with hydrophobic drugs.

Cyclodextrins (CDs) are cyclic oligosaccharides obtained by enzymatic degradation of starch, composed of α -(1,4)-*D*-glucopyranose units. The three common natural α -, β - and γ -cyclodextrins are constituted of six, seven and eight glucose residues, respectively. Usually

presented as a 3D-truncated cone, CDs are hydrophilic on their outer surface due to the numerous hydroxyl groups but their inner cavity is essentially hydrophobic. CDs have therefore the ability to host lipophilic molecules while being soluble in water. Among the three common cyclodextrins and given the ideal size of its cavity (internal diameter of 6.0 to 6.5 Å), the β -CD (Figure 1) is particularly well-adapted to form host-guest inclusion complexes with a wide variety of molecules containing a hydrophobic moiety.^{32,33,34}

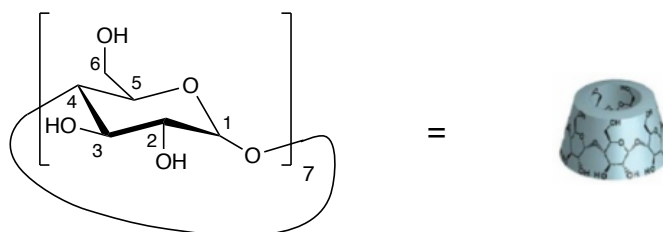


Figure 1. Structure of β -cyclodextrin.

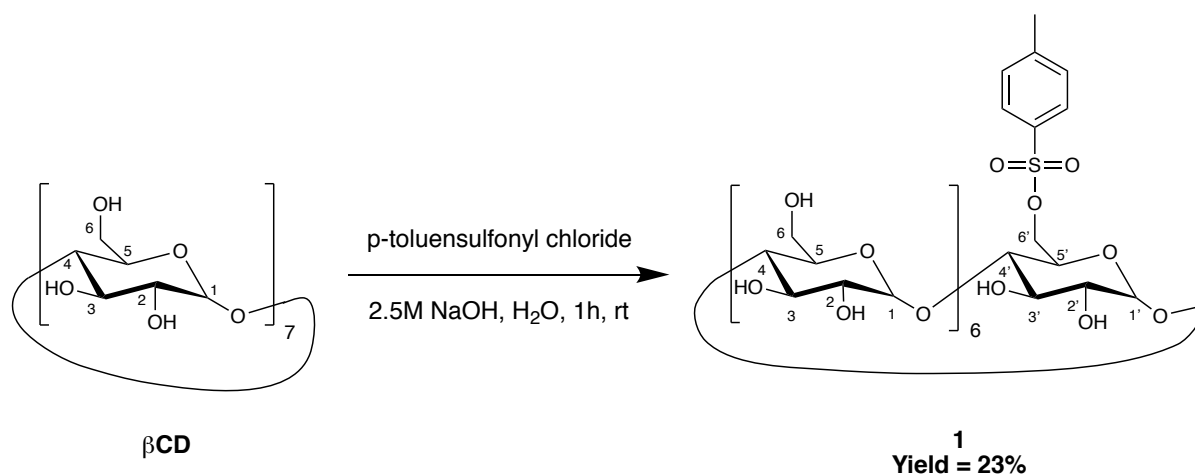
In this original work, β -cyclodextrin-functionalized chitosan/alginate CoPEC was designed to be further used as potential new carrier for drug delivery. The newly functionalized CoPEC was thoroughly characterized and its intrinsic anti-inflammatory properties were fully assessed through *in vitro* biological assays.

2. RESULTS AND DISCUSSION

2.1. Synthesis and characterization of β -cyclodextrin functionalized chitosan

Different methods to obtain functionalized β -cyclodextrins (amino, formyl, carboxy, halogeno-CDs) have already been reported in the literature.³⁵ In this work, we were interested in the functionalization of β -cyclodextrin on its primary hydroxyl group by a leaving group

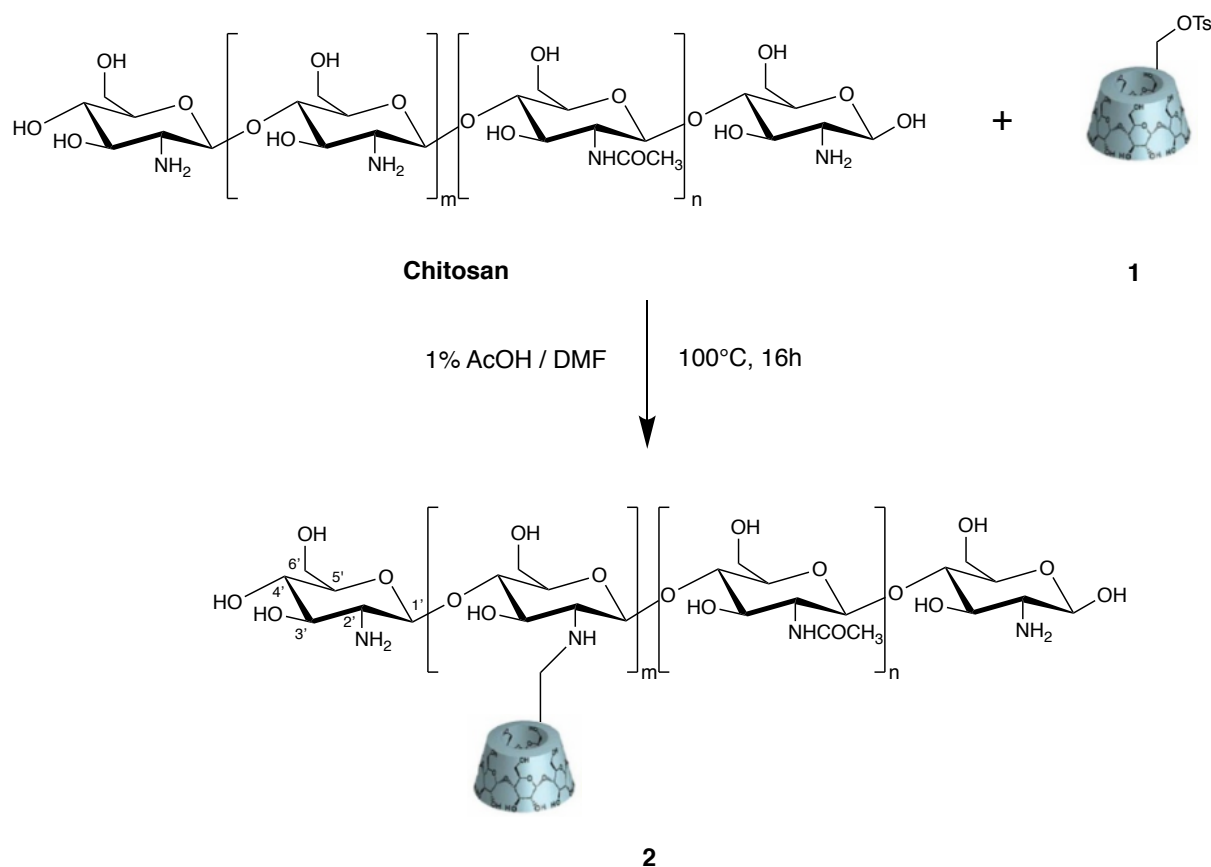
that could be further displaced by nucleophiles to synthesize useful derivatives. For that purpose, 6-OTs- β CD **1** was synthesized from commercially available β CD (Scheme 1)



Scheme 1. Synthesis of 6-OTs- β CD (**1**)

The monotosylation of β -cyclodextrin on its primary hydroxyl group has been extensively studied exploiting either aqueous or organic solvents polarity. Pyridine is a solvent reputed optimal for a selective monotosylation on the 6-OH-position of β CD. It generally results in good conversion yields, but its removal is never complete leading to sub-optimal subsequent coupling reactions with polysaccharides. In the present work the monotosylation reaction was performed accordingly to Tripodo's method,³⁶ in an aqueous NaOH solution and the purification of the crude product was further performed with a cation-exchange resin. The overall yield of this reaction (23%) and the purity of the product (99.9%) were in accordance with the literature (respectively 35% and >98%). The ¹H NMR spectrum was also in accordance with the expected product **1**, with characteristic signals of β -cyclodextrin and one tosyl group. The resulting 6-OTs- β CD **1** was further used in reactions with chitosan (Scheme 2).

Gonil *et al.* described a nucleophilic substitution between -NH₂ groups of chitosan and 6-OTs-βCD under acidic conditions (acetic acid 1% v/v in water), the tosyl being a good leaving group (Scheme 2).³⁷



Scheme 2. Synthesis of βCD-chitosan (2).

¹H NMR spectroscopy was used to assess the substitution degree (DS) of the functionalized chitosan. The integration value of the H1 anomeric proton of cyclodextrin signal at δ 4.98 ppm was compared to that of chitosan H2' protons at δ 2.89 ppm through the following equation:

$$DS (\%) = \frac{H1CD/7}{H2'Chitosan}$$

We found a DS of 25%.

2.2. Preparation of CoPECs

Recently two processing methodologies combining polysaccharides and/or synthetic polyelectrolytes showed the possibility to obtain Compact PolyElectrolyte Complexes either by ultracentrifugation/extrusion²⁷ or by evaporation¹⁷ in the presence of salt. Here we prepared CoPECs by the ultracentrifugation method. Alginate is known to form good complexes between its carboxylic groups (-COOH) and primary amino groups of the natural polysaccharide chitosan (-NH₂) through the COO⁻/NH₃⁺ ion pair. We chose this binary system as an original natural-based CoPECs formulation. In a recent work³¹ we identified suitable assembly conditions leading to handy and macroscopically homogeneous biomaterials. Besides the types of polyelectrolytes and their relative quantity, influence of pH and salt concentration on the macroscopic properties of CoPEC were studied and optimal formulation conditions were determined.

In the present work, we functionalized chitosan and alginate with β -cyclodextrin and we first formulated three new CoPECs, i. e. β CD-chitosan/alginate (β CD-Chi/Alg), chitosan/ β CD-alginate and β CD-chitosan/ β CD-alginate CoPECs. Early *in vitro* studies showed a pro-inflammatory activity of the chitosan/ β CD-alginate and β CD-chitosan/ β CD-alginate CoPECs (Supporting Information, Figures S1, S2 and S3) so that we only focus here on the β CD-chitosan/alginate CoPEC.

The functionalization of chitosan with cyclodextrins (DS = 25%) led to a decrease of the number of amino groups available for the complexation with alginate. Thus, for the formulation of β CD-chitosan/alginate CoPEC, we increased by around 25% the number of

chitosan sugar units to reach the same number of ionic groups as in the case of the CoPEC made from unmodified chitosan.

The ionic strength of the medium influences the plasticity of the material during the complexation and the ultracentrifugation processes. Indeed, low salt concentrations lead to soft biomaterials uneasy to handle, while high salt concentrations lead to more compact materials. Based on our previous results, a 1M NaBr concentration was chosen for the formulation of all the β CD functionalized chitosan/alginate CoPECs.³¹

Finally, the ultracentrifugation was performed at 308,000 g and 23°C for 12h.³¹ All these conditions led to a homogenous and easy to handle material (Figure 2a).



Figure 2. a) Physical aspect of β CD-Chi/Alg CoPEC sample obtained after ultracentrifugation: [NaBr] = 1 M; [Polysaccharides] = 1.25 mg/mL and ultracentrifugation speed = 308,000 g; b) β CD-Chi/Alg CoPEC microparticles ($\leq 300 \mu\text{m}$) obtained after crushing and sieving steps on freeze-dried CoPEC.

These materials could easily be freeze-dried and reshaped as microparticles by crushing/sieving steps, affording homogeneous CoPECs powders suitable for *in vitro* assays (Figure 2b). Here, the use of microparticles of diameter smaller than 300 μm permits to obtain a dose-response relationship during *in vitro* assays.

2.3. Surface and inner microstructures observations

Next we compared the structure of Chi/Alg and β CD-Chi/Alg CoPECs before crushing (that is to say immediately after ultracentrifugation and freeze-drying). We used Scanning Electron Microscopy for that purpose. As shown on Figure 3, the different microstructures were not porous but very homogeneous. Moreover, the general aspect of Chi/Alg (a and b) and β CD-Chi/Alg (c and d) CoPECs was similar.

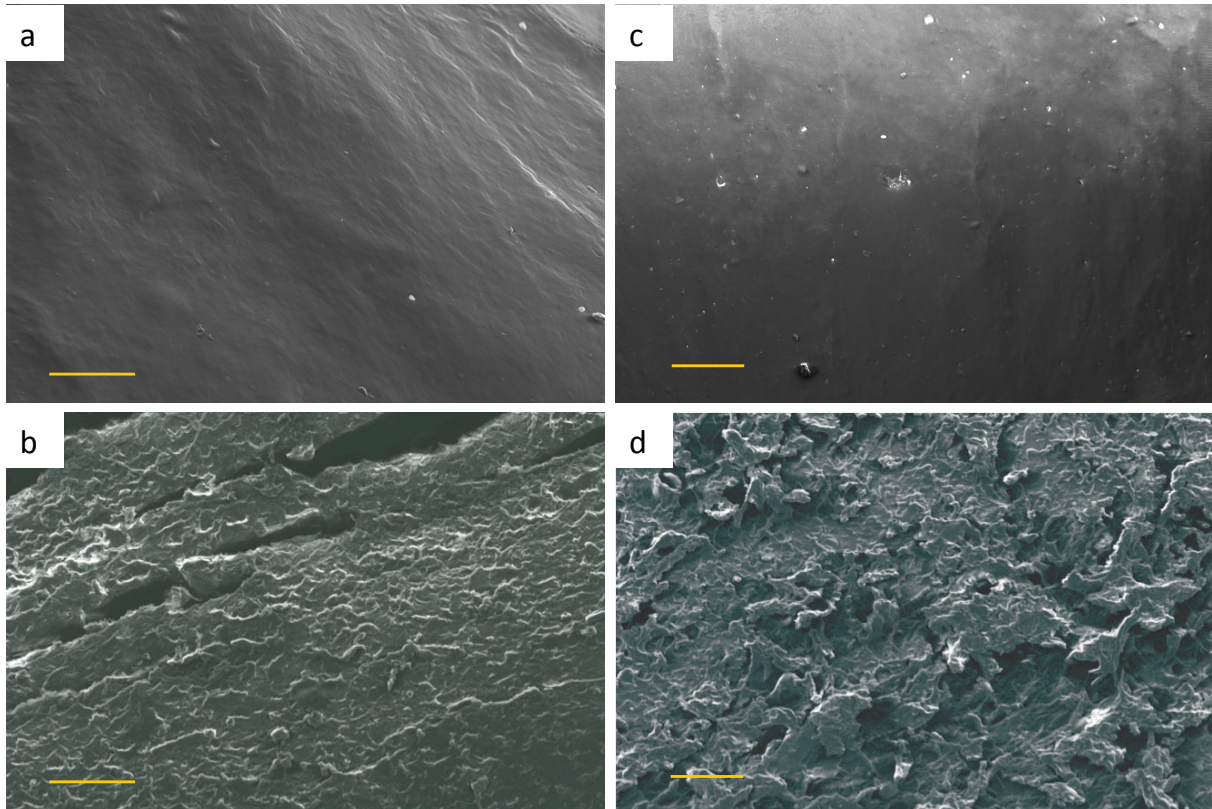


Figure 3. SEM images of the homogeneous and non porous surface and interior of Chi/Alg (a and b, respectively) and β CD-Chi/Alg (c and d, respectively) CoPECs (yellow bar = 100 μ m).

Therefore, it seems that the chemical modification of the CoPEC by β -cyclodextrin has no great impact on the microstructure of the final material.

2.4. Determination of the composition of the β CD-Chi/Alg CoPEC

During the polyelectrolytes complex formulation, all the introduced molecules are not necessarily complexed and some free molecules may remain in the supernatant. Thus, in order to evaluate the ratio between the two constituents of the material (β CD-chitosan and alginate), alginate was labeled on its carboxylate groups with rhodamine B isothiocyanate and titrated by UV-Vis spectrophotometry. The substitution degree of the labeled polymer was 0.7%, which was enough to detect the alginate and sufficiently low to keep the molar mass of the polymer and its overall charge unchanged.

According to the UV-Vis spectrophotometry measurements obtained with the supernatant before ultracentrifugation, only 0.2 mg out of 10.3 mg of alginate were found there, which means that more than 98% of alginate was complexed into the final material. That data allowed us to deduce the amount of β CD-chitosan in the final CoPEC. On a dry β CD-Chi/Alg CoPEC sample of 18.4 mg, 8.3 mg of β CD-chitosan and 10.1 mg of alginate were found corresponding to 0.051 mmol of ($\text{COO}^- + \text{COOH}$) from alginate units and 0.012 mmol of ($\text{NH}_3^+ + \text{NH}_2$) from β CD-chitosan units. The molar ratio ($\text{COO}^- + \text{COOH}$)/($\text{NH}_3^+ + \text{NH}_2$) is thus of 1:0.24. This ratio, with an excess of alginate, is probably due to the steric hindering coming from the cyclodextrins grafted onto chitosan, leading to a need for more alginate moieties to reach the different cationic groups of β CD-chitosan.

2.5. Inclusion of Piroxicam in chitosan/alginate and β CD-chitosan/alginate CoPECs

In order to evaluate the ability of our functionalized CoPEC to trap and deliver hydrophobic drugs, a model drug, Piroxicam, was included into chitosan/alginate and β CD-chitosan/alginate CoPECs. The inclusion was performed in methanol, this solvent being optimal as its modest polarity permits the solubilization of Piroxicam and forces the hydrophobic moieties to reach the cyclodextrin cavities. Included Piroxicam was then

extracted from the CoPECs using dichloromethane, a strongly hydrophobic solvent. The extracted Piroxicam was quantified by UV-Vis spectrophotometry and, from the same amount of chitosan/alginate and β CD-chitosan/alginate CoPECs (10 mg), we quantified 1.24 and 2.99 μ mol of Piroxicam, respectively. Thus, the presence of β CD in the CoPEC allowed more than a doubling of the quantity of Piroxicam included.

After performing characterizations of this new functional material and verifying its ability to trap and release a hydrophobic model drug, we conducted *in vitro* biological assays to evaluate its potential anti-inflammatory activity.

2.6. Evaluation of the anti-inflammatory activity of CoPECs

The *in vitro* evaluation of the anti-inflammatory properties of a material is usually conducted using macrophages that can be activated by chemical compounds to mimic inflammation. The anti-inflammatory activity is then estimated by assessing the amount of various inflammation mediators produced by the cells such as nitric oxide (NO) or pro-inflammatory cytokines. Moreover, inflammation induces differentiation of macrophages that results in the decrease of proliferation as well as differentiation/activation marker expression.

In our study, RAW 264.7 mouse macrophages were activated by LPS, a well-known endotoxin described as an inflammation inducer. The viability of those macrophages in the presence of CoPECs was assessed as well as the production of NO and TNF- α pro-inflammatory cytokines in cell supernatants. The expression of Ki-67 and CD40 was followed as proliferation and activation markers, respectively.

Flow cytometry analyses using a Fixable Viability Dye (FVD) demonstrated that chitosan/alginate and β CD-chitosan/alginate CoPECs were not cytotoxic, both on LPS-

activated and non-activated cells (Figure 4). Indeed the percentage of viable cells is similar when cells are not treated ($[\text{CoPEC}] = 0 \text{ mg/mL}$) and when they are treated with CoPECs.

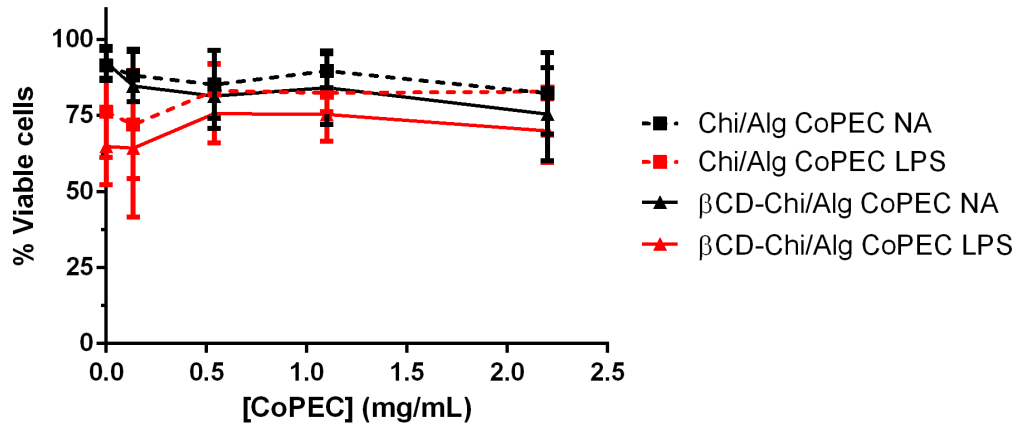


Figure 4. CoPECs functionalized or not by β -cyclodextrins are non-cytotoxic. Raw cells were preincubated 24h with CoPECs before 24h activation with 50 ng/mL LPS. Viability was assessed by cytometry with a FVD labeling ($n=3$). NA = Non-activated cells and LPS = LPS-activated cells.

To analyze the effect of CoPECs devoid of active substance on NO release and pro-inflammatory cytokine production, macrophages were incubated with CoPECs for 24 hours and then activated by LPS for 24h. As expected, LPS induced the production of NO (Figure 5a) and TNF- α (Figure 5b). The CoPECs containing or not β CD caused a decrease of NO and TNF- α detected in cell supernatants. Interestingly, although these decreased NO and TNF- α levels were observed for both CoPECs (i. e. with or without cyclodextrin), it was much more noticeable in the case of the cyclodextrin-containing one (solid red line). Moreover, one can notice that in the case of TNF- α , this cytokine is almost absent in the supernatants after treatment by the β CD-chitosan/alginate CoPEC.

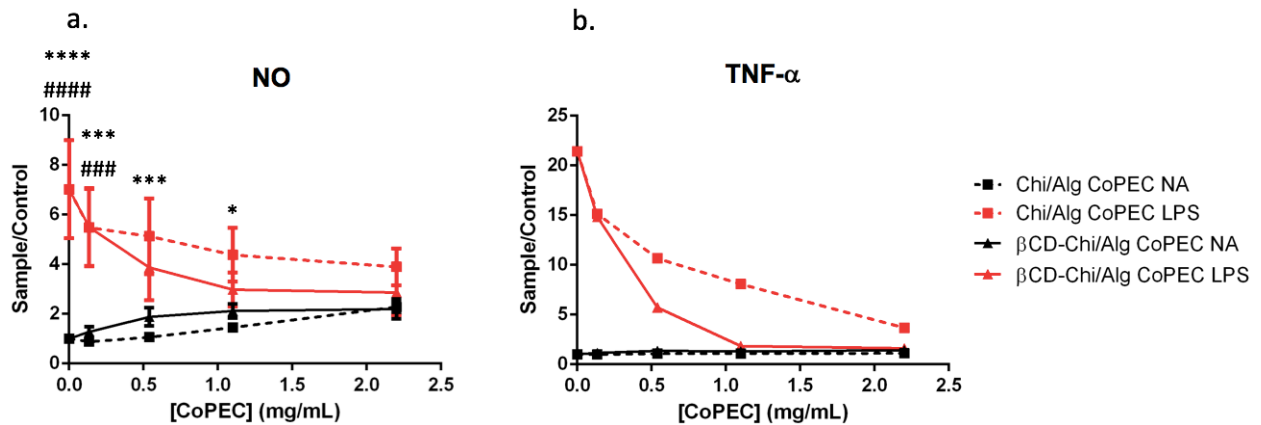


Figure 5. CoPECs functionalized or not by β -cyclodextrins decrease NO and TNF- α production by activated cells in a dose dependent manner. Raw cells were activated by 50ng/mL LPS 24h after incubation with CoPECs. Cell supernatants were collected 24h after activation. Data are presented as a ratio between the different samples and the referent untreated RAW cell supernatant (no CoPEC, no LPS). NO production (a) was assessed by Griess test (n=3) and TNF- α production (b) was estimated by ELISA test (n=1 representative of 3 experiments). NA = Non-activated cells and LPS = LPS-activated cells. ****, *** and * = p-value <0.0001, <0.001 and <0.05 respectively, difference between Chi/Alg CoPEC NA and Chi/Alg CoPEC LPS. ##### and ### = p-value <0.0001 and <0.001 respectively, difference between β CD-Chi/Alg CoPEC NA and β CD-Chi/Alg CoPEC LPS.

NO and TNF- α determinations were also conducted with the Piroxicam-containing β CD-chitosan/alginate CoPEC (Supporting Information, Figures S4 and S5). In these experiments, the different functionalized CoPEC concentrations corresponded to Piroxicam concentrations ranging from around 25 to 200 μ g/mL. From these experiments it can be noticed that the Piroxicam-containing CoPEC induces a decrease of NO and TNF- α production by activated macrophages. However, the effect of CoPEC was not significantly better than that of the CoPEC devoid of Piroxicam. This could be explained by the low quantity of Piroxicam available in this case. Indeed, in order to obtain a significant anti-inflammatory effect due to

Piroxicam alone, it is necessary to have at least 300 nmol of Piroxicam/mL as described by Chiong *et al.*³⁸ In our work, even if the cyclodextrins are saturated by Piroxicam, the concentration in Piroxicam is pretty low in the samples (62 nmol/mL for the first one) and the effect of CoPEC alone is so important that the effect of Piroxicam is completely masked by the material (Supporting Information, S8). Hereinafter and for that reason, we focused on the CoPEC without Piroxicam for further studies.

To ensure that the lowered NO and TNF- α levels detected in cell supernatants were not due to any trapping of LPS and TNF- α in the materials and in particular into the cyclodextrins, two complementary experiments were conducted. In a first experiment, LPS was incubated with β CD-chitosan/alginate CoPEC for 24h, and then supernatants were incubated with macrophages. Here, we can observe that after being in contact with CoPEC, LPS induces a NO production from macrophages comparable to that induced by fresh LPS (+ Control) (Figure 6).

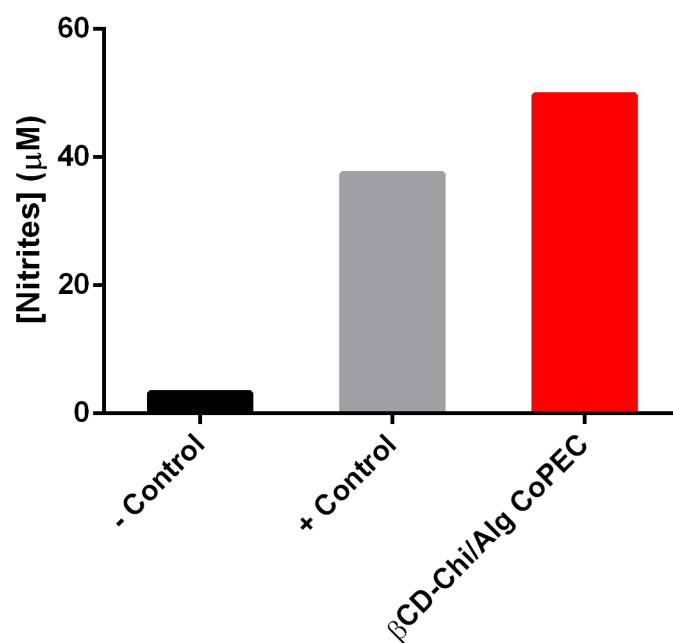


Figure 6. β CD-Chi/Alg CoPEC does not trap LPS. LPS was incubated with CoPEC during 24h. Filtered CoPEC supernatant was then placed onto macrophages. 24h later, supernatant was collected. NO production was assessed by Griess test (n = 1). – Control corresponds to the NO production of non-activated macrophages and + Control corresponds to the NO production induced by the addition of fresh LPS onto macrophages.

In a second experiment, TNF- α was incubated with β CD-chitosan/alginate CoPEC for 24h and was then quantified by ELISA. In this experiment, similar levels of TNF- α were detected both in the cases when this cytokine was incubated with CoPEC or not (Control) (Figure 7).

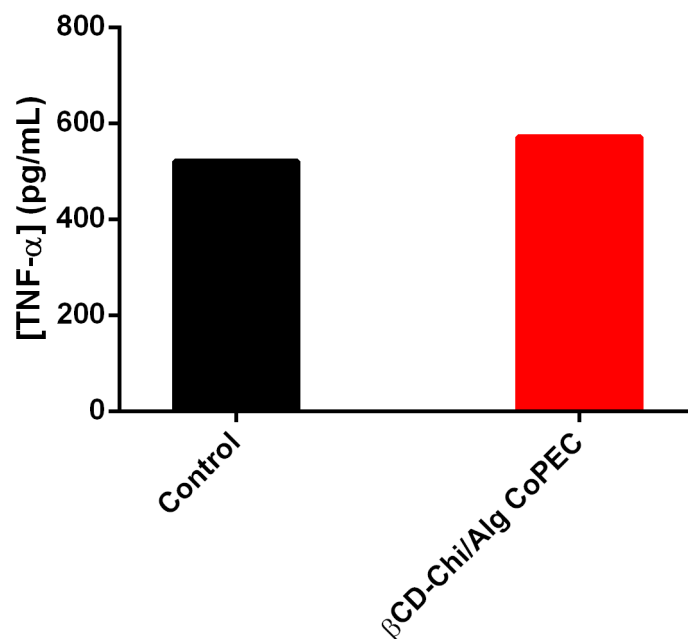


Figure 7. β CD-Chi/Alg CoPEC does not trap TNF- α . The CoPEC supernatant was collected 24 h after addition of TNF- α . TNF- α content in supernatant was quantified by ELISA test (n = 1). The control corresponds to TNF- α in culture medium without any contact with CoPEC.

From these two experiments, it can be concluded that the above mentioned inhibitions are not due to any trapping of LPS or TNF- α into the CoPECs. Thus, we can affirm that our CoPECs inhibit NO and TNF- α productions by activated macrophages, which is an evidence of the intrinsic anti-inflammatory action of our materials.

To confirm the intrinsic anti-inflammatory activity of β CD-Chi/Alg CoPECs, the differentiation state of activated macrophages was evaluated by following proliferation using Ki-67 marker and following activation using CD40 marker. As expected, Figure 8 shows that macrophages stop proliferating (Figure 8a) and express the activation CD40 marker (Figure 8b) when activated by LPS. However, in the presence of CoPECs functionalized or not, proliferation is restored and CD40 expression is decreased, showing an inhibition of macrophage differentiation, so confirming the inhibition of the inflammation.

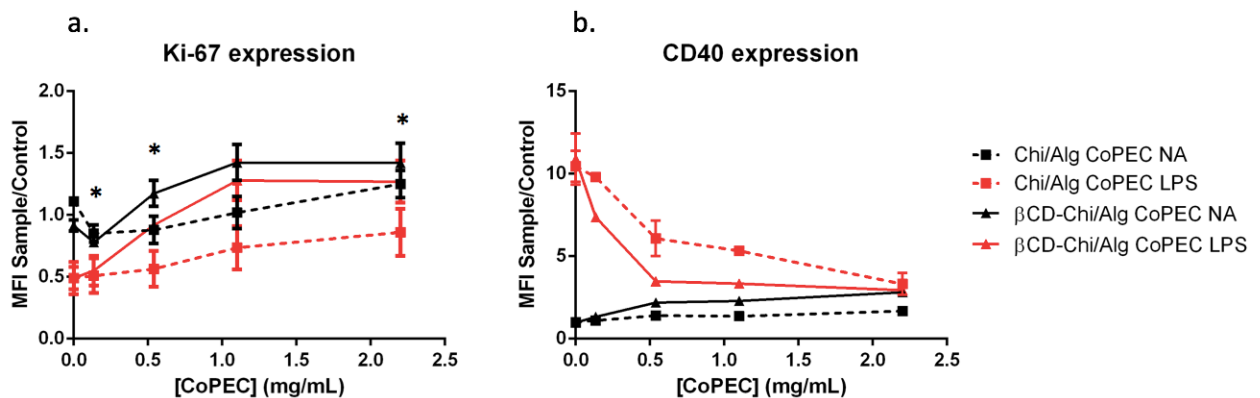


Figure 8. CoPECs functionalized or not restore cell proliferation and decrease LPS-induced cell activation. Raw cells were activated by LPS 24h after incubation with CoPECs. 24h after activation, cells were collected and cell proliferation and activation were estimated by cytometry with (a) Ki-67 (n=2) and (b) CD40 labeling (n=2), respectively. Data are represented as a Median Fluorescent Intensity (MFI) ratio between sample and untreated RAW cells (no CoPEC, no LPS). NA = Non-activated cells and LPS = LPS-activated cells. * = p-value < 0.05, difference between Chi/Alg CoPEC NA and Chi/Alg CoPEC LPS.

All together, these results show that our CoPECs have high intrinsic anti-inflammatory properties.

To better understand the biological activity of the materials, some *in vitro* evaluations have been conducted on the polyelectrolytes used for CoPEC formulation. In particular, the influence of alginate, chitosan and β CD-chitosan on the NO production by LPS-activated macrophages has been studied (Supporting Information, Figure S6). From these experiments, it can be concluded that the anti-inflammatory activity of CoPECs most probably comes from chitosan and β CD-chitosan as these two polyelectrolytes induce a lowering of NO production by activated cells. Our results seem to corroborate those obtained by Yoon *et al.*³⁹ and Zhu *et al.*⁴⁰ on chitosan oligosaccharides.

Thus, in this work, we have developed a saloplastic biomaterial made of β -cyclodextrin-functionalized chitosan and alginate that preserves the anti-inflammatory activity of chitosan and that is able to contain and release hydrophobic drugs forming an inclusion complex with β -cyclodextrin. Compared to the unmodified chitosan/alginate CoPEC, we have detected a slightly increased anti-inflammatory activity of the β -cyclodextrin-functionalized material. Two hypothesis can be formulated to justify this effect: i) the modification of the tridimensional arrangement of the polyelectrolyte chains and ii) the modification of physicochemical properties (decrease of total positive charges number and enhanced solubility for example) of chitosan due to the presence of β -cyclodextrin. Therefore, we could consider that this new functionalized CoPEC has a very high potential as bioactive material that could find applications in implantable devices.

3. CONCLUSIONS

In the present work, the recently described chitosan/alginate CoPEC was functionalized with β -cyclodextrin. This modification led to a β -cyclodextrin-chitosan/alginate CoPEC, a new material that was characterized for its composition and microstructure. The ability of this new material to carry Piroxicam drug was demonstrated. Through *in vitro* experiments with an inflammation model (activated macrophages), this new functionalized CoPEC was demonstrated to be non-cytotoxic, to inhibit NO and pro-inflammatory cytokine TNF- α production as well as to decrease LPS-induced differentiation. These are highly reliable proofs that this new biomaterial exhibits intrinsic anti-inflammatory activities. Moreover, these intrinsic anti-inflammatory activities of the CoPEC were so important that the presence of the anti-inflammatory Piroxicam could not enhanced its efficacy.

Next stage will be to include an active pharmaceutical ingredient devoid of anti-inflammatory activity (antibiotics or anticancer drug for example) into this β -cyclodextrin-chitosan/alginate CoPEC to evaluate its ability to carry and release such a molecule. Over time, this kind of biomaterial could constitute a new standard in the field of combination therapy with an intrinsic anti-inflammatory activity and an additional therapeutic effect provided by an extra active molecule included in the CoPEC.

4. EXPERIMENTAL SECTION

4.1. Materials

4.1.1. Chemical reagents and equipment

Chitosan (average molecular weight 250,000; viscosity 30-100 cP; deacetylation degree > 90%) was obtained from Glentham Life Sciences (Corsham, UK). Alginic acid sodium salt

(average molecular weight 155,000; viscosity 15-20 cP; 1% in water), rhodamine B isothiocyanate, mixed isomers and acetic acid were obtained from Sigma Aldrich (Saint-Louis, MO). β -cyclodextrin, *p*-toluenesulfonyl chloride and Piroxicam were purchased from Alfa Aesar (Ward Hill, MA). Cation exchange resin beads Amberlist® IR 120, H were obtained from Acros Organics. Purified 18 M Ω ultrapure-water (Elga, Veolia) was used in the experiments. All other reagents and anhydrous solvents were commercially available and used without further purifications.

¹H NMR, which showed a shift referenced to the residual signal of non-fully deuterated NMR solvents, was recorded on a Bruker Advance DPX400 NMR spectrometer locked to the major signal of each NMR solvent. Samples were dissolved either in D₂O or *d*₆-DMSO (Sigma Aldrich) at 25°C. Scanning Electron Microscopy (SEM) images were obtained on a Quanta™ 250 FEG from FEI Company (Eindhoven, The Netherlands).

4. 1. 2. Biological reagents and equipment

All media and supplements for cell culture were purchased from Sigma Aldrich (Saint Louis, MO). LPS from *Escherichia coli* (K12) was obtained from Invivogen (San Diego, CA).

Purified anti-mouse TNF- α antibody clone 1F3F3D4 and biotinylated anti-mouse TNF- α antibody clone XT3/XT22 for ELISA testing were purchased from eBioscience/thermoFischer Scientific (Waltham, MA). Horseradish Peroxidase Avidin (Avidin HRP) was from Jackson (West Grove, PA).

For the Flow Cytometry analysis, Fixable Viability Dye (FVD) eFluor™ 660 and Ki-67 monoclonal antibody clone SolA15 were from eBioscience and CD40 APC clone 3/23 was from BD Biosciences (San Jose, CA). Cells were analyzed on a FACScalibur from BD

(Franklin Lake, NJ) using CellQuest Software (BD) for data acquisition and FlowJo Software (FlowJo Inc) for data analysis.

4. 2. Synthesis

4. 2. 1. Synthesis of mono-6-deoxy-6-(*p*-toluensulfonyl)- β -cyclodextrin 1 (6-OTs- β CD)

6-OTs- β CD was synthesized accordingly to an organic solvent-free published methodology with minor modifications.³⁶ In a round-bottom flask, dry β -cyclodextrin (29.8 g, 26.3 mmol) was solubilized in 270 mL of milliQ-water and a 2.5M aqueous solution of NaOH (105 mL) was added. Then, 7.6 g (39.9 mmol, 1.5 eq) of *p*-toluenesulfonyl chloride were added in little portions and the suspension was stirred for 1h at room temperature. The residue was filtrated off and washed with water. To the filtrate solution (average pH=10), cation exchange resin beads (Amberlist® IR 120, H) pre-activated with a HCl 6M aqueous solution, were added under stirring at room temperature. Reaching pH value of 2-3 led to the precipitation of the reaction product. The mixture was first filtered through sieve (300 μ m) to separate the resin beads from the expected 6-OTs- β CD. Then, the precipitated white powder was filtrated on a sinter glass. The paste obtained was finally washed with water and freeze-dried to give **1** as a white solid (yield: 23%).

¹H NMR (DMSO-*d*₆) δ (ppm) 7.76 (d, 2H, Ph), 7.44 (d, 2H, Ph), 5.88-5.63 (m, 14H, OH2 and OH3 β CD), 4.90-4.75 (m, 7H, H1 β CD), 4.57-4.40 (m, 6H, OH6, β CD), 4.39-4.29 (m, 2H, H6' β CD), 4.25-4.13 (m, 1H, H5' β CD), 3.74-3.50 (m, 25H, H3, H5 et H6 β CD), 3.48-3.19 (m, H2 and H4 β CD overlap with water), 2.44 (s, 3H, Ph-CH₃).

4. 2. 2. Synthesis of chitosan grafted with β -cyclodextrin 2 (β CD-Chi)

β -cyclodextrin-grafted chitosan **2** was synthesized following Yuan's method with minor modifications.⁴¹ Chitosan (2.0 g, 12.4 mmol repeating units) was first dissolved in 1% (v/v) acetic acid (160 mL). A solution of 6-OTs- β CD **1** (7.0 g, 5.4 mmol, 0.4 eq) in DMF (80 mL) was then added to the chitosan solution and the mixture was refluxed at 100°C for 16h. After a brief evaporation by distillation to decrease the volume of solvent, the solution was dialyzed through a membrane with a Molecular Weight Cut Off (MWCO) of 3,500 Da for 2 days against 0.1M NaCl aqueous solution and then, against ultrapure water. The solution was finally freeze-dried to afford 2.7 g of **2** as a white solid (Degree of Substitution, DS=25%, determined by ¹H NMR).

¹H NMR (D₂O) δ (ppm) 7.60 (d, Ph 6-OTs- β CD), 7.28 (d, Ph 6-OTs- β CD), 4.98 (s, H1 β CD), 3.90-3.42 (m, H2, H3, H4, H5, H6, β CD and H1', H3', H4', H5', H6' chitosan), 2.89 (s, H2' chitosan), 2.32 (s, Ph-CH₃ 6-OTs- β CD).

4. 3. General method for preparation of chitosan/alginate (Chi/Alg) Compact PolyElectrolyte Complexes

Chitosan/alginate CoPECs were obtained as described in our previous work.³¹ Briefly, the alginate solution was obtained by dissolving sodium alginate in ultrapure water (1.25 mg/mL). The pH was then adjusted to 6.5 with an aqueous solution of acetic acid 0.1 M. Solid NaBr was added in little portions to reach 1M concentration.

The chitosan solution was obtained by suspending chitosan in ultrapure water (1.25 mg/mL) before adding 0.2% (v/v) of acetic acid to reach complete dissolution. Then the pH was adjusted to 4.5 using a NaOH 6M aqueous solution. Solid NaBr was also added in little portions to reach a concentration of 1M.

The CoPECs were obtained by blending alginate and chitosan solutions in equal volumes. Perfect combination was performed by simultaneously pumping both solutions using a peristaltic pump (Ismatec IPC 9329, flow rate = 100 mL/min). The resultant mixture was stirred with a magnetic stir bar at 200 rpm and the obtained suspension was then transferred into propylene centrifuge tubes and centrifuged at 9,400 g (Centrifuge Sigma 4K15C) at 20°C for 15 min. Finally, pre-compact complexes were transferred into thinwall polyallomer ultracentrifuge tubes (Beckman Coulter Inc.) with their supernatant and ultracentrifuged (Beckman Coulter Ultracentrifuge) at 308,000g using a 90Ti rotor type at 23°C for 12h to obtain CoPECs.

4. 4. Preparation of β CD-chitosan/alginate (β CD-Chi/Alg) Compact PolyElectrolyte Complexes

β CD-Chi/Alg CoPECs were obtained following the same procedure described in the previous paragraph except that the quantity of chitosan was adjusted, due to the β CD functionalization, in order to keep an equal amount of $-\text{NH}_3^+$ on chitosan and of COO^- on sodium alginate.

4. 5. Visualization of the microstructures by Scanning Electron Microscopy (SEM)

After ultracentrifugation and washing, CoPECs were cut into slices and freeze-dried. The surface and the interior of the materials were then observed by SEM.

4. 6. Determination of the composition of β CD-Chi/Alg CoPEC

4. 6. 1. Labeling of sodium alginate with rhodamine

Sodium alginate was labeled with rhodamine B isothiocyanate following the procedure described by Ziv *et al.* with little modifications.⁴² An aqueous 2% (w/w) sodium alginate solution (300 mg, 1.5 mmol) was prepared and adjusted to pH 8. Then, rhodamine B isothiocyanate, mixed isomers (20 mg, 0.04 mmol, 0.03 eq) in 8 mL DMSO was slowly added. The mixture was stirred at 40°C for 1h before addition of 5.4 g of sodium chloride to stop the reaction. The product was dialyzed through a membrane with a MWCO of 12-14,000 Da against 0.05M NaCl and finally against ultrapure water until no significant fluorescence was detected in the dialysis medium. The product was then isolated by freeze-drying leading to 264 mg of purple solid.

The substitution degree of the rhodamine-labeled alginate was determined by absorbance at 560 nm⁴³ in 0.1M pH 8 phosphate buffer using a standard curve and was found to be of 0.7%.

4. 6. 2. Preparation of the β CD-Chi/Alg-rhodamine CoPEC

As the molar mass of the sodium alginate was almost unmodified by the rhodamine labeling, the CoPEC was prepared following the same procedure and using the same polyelectrolytes proportions as for the unlabeled alginate until the ultracentrifugation step.

4. 6. 3. Estimation of the amount of alginate not included in the CoPEC

The amount of alginate present in the supernatant before the ultracentrifugation step (to say, the amount of alginate which was not included in the final material) was determined by UV-Vis spectrophotometry at 560 nm using a standard curve obtained from alginate-rhodamine solutions in 0.1M pH 8 phosphate buffer.

4. 7. Preparation of CoPECs microparticles

CoPECs (Chi/Alg and β CD-Chi/Alg) obtained by ultracentrifugation were washed during 24h twice in Dulbecco's Phosphate-Buffered Saline (PBS, Sigma Aldrich, Saint Louis, MO), i. e. a solution of 136.9 mM NaCl, 1.5 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 and 2.7 mM KCl, before the CoPECs pieces were freeze-dried. Successively they were grinded using a mortar and sieved (300 μm sieve from CISA) in order to obtain $\leq 300 \mu\text{m}$ particles.

4. 8. Inclusion, extraction and quantification of Piroxicam in chitosan/alginate and β CD-chitosan/alginate CoPECs

300 mg of CoPECs microparticles were incubated in 90 mL of a 1 mg/mL Piroxicam solution in methanol for 72h at 30°C and 90 rpm. Microparticles were collected by filtration, washed with a minimum of ultrapure water and dried at 37°C for 24h. Particles were then crushed and sieved at 300 μm before extraction of the included Piroxicam was performed by incubating microparticles in 20 mL dichloromethane for 24h at 30°C and 130 rpm. Microparticles were then separated by filtration, washed with dichloromethane and the filtrates were evaporated under reduced pressure to collect the extracted Piroxicam under its solid form. Extracted Piroxicam was dissolved in methanol, diluted in 0.1 M aqueous HCl and was then quantified by UV-Vis spectrophotometry at 333 nm and by using a standard curve built from Piroxicam solutions in 0.1 M aqueous HCl.

4. 9. Evaluation of inflammation in an in vitro model

4. 9. 1. Cell culture

RAW 264.7 mouse macrophages (ATCC® TIB-71TM) were cultured in Dulbecco's Modified Eagle Medium High glucose (DMEM) containing stabilized glutamine,

supplemented with 5% (v/v) of fetal bovine serum (FBS), Penicillin (1000 U/mL) and Streptomycin (100 μ g/mL) at 37°C in 5% CO₂, 95% humidity.

Cells were harvested at around 90% confluence using trypsin (0.5 g/L)-EDTA (0.2 g/L) for 5 min at 37°C and re-suspended in culture medium to the desired dilution.

4.9.2. Cell incubation with CoPECs

The incubation process was conducted over 3 days. The first day, 50,000 cells/well were seeded with CoPECs microparticles concentrations ranging from 0.0677 to 2.2 mg/mL to a final culture medium volume of 100 μ L in 96 well plate flat bottom during 24 hours at 37°C. On day 2, cells were stimulated with 50 ng/mL of LPS. On day 3, cells and supernatants were collected for subsequent analysis.

4.9.3. Assessment of NO production

Concentrations of nitrogen dioxide in cell supernatants (representative of the nitric oxide produced by activated cells) were evaluated by Griess test (n=3). Briefly, 60 μ L of Griess reagent (v/v mixture of 58.1 mM p-aminobenzene sulfonamide in 30% acetic acid and 3.9 mM N-(1-naphthyl)ethylenediamine dihydrochloride in 60% acetic acid) were added to 40 μ L of supernatant and the absorbance at 543 nm was measured and compared to a sodium nitrite standard curve.

4.9.4. Assessment of TNF- α production

TNF- α concentration in cell supernatants was evaluated by ELISA (n=3) using commercially available reagents and following the manufacturer instructions.

Briefly, capture antibody was diluted to 1 μ g/mL in a 0.05M pH 9.6 carbonate/bicarbonate buffer and coated 1 night at 4°C before blocking with PBS 0.05% Tween 20 1% BSA (1h,

37°C). Samples were then diluted with PBS and incubated with capture antibody (2h, 37°C) before detection antibody diluted to 0.5 µg/mL in PBS 0.05% Tween 20 1% BSA was added (1h, 37°C). Avidin HRP was then introduced (45 min, 37°C) and revelation was conducted by adding a solution of 1.25mM tetramethylbenzidine and 13.05 mM H₂O₂ in 0.1M pH 5 citrate buffer. Revelation was finally stopped by addition of 1M HCl and absorbance was measured at 450 nm.

4.9.5. Evaluation of the eventual trapping of LPS in CoPECs

On day 1, 1 mg/mL βCD-Chi/Alg CoPEC in the form of microparticles of diameter smaller than 300 µm with 10 µL of a 500 ng/mL LPS solution in culture medium were incubated in 96 well plate flat bottom during 24h at 37°C. In another plate, 50,000 cells/well were seeded and were also incubated during 24h at 37°C. On day 2, supernatants of CoPEC in contact with LPS were collected, filtrated (FLOWNI CELL STAINERS, 70 µM) and placed on cells. On day 3, supernatants were collected and the NO content was assessed through a Griess test.

4.9.6. Evaluation of the eventual trapping of TNF-α in CoPECs

On day 1, 1 mg/mL βCD-Chi/Alg CoPEC in the form of microparticles of diameter smaller than 300 µm in culture medium was placed in 96 well plate flat bottom during 24 hours at 37°C. On day 2, 10 µL of a 20 ng/mL TNF-α solution were added. On day 3, supernatants were collected and the TNF-α content in supernatants was then assessed by ELISA testing.

4.9.7. Flow Cytometry analysis

PBS supplemented with 2% FBS was used for washing and diluting the antibodies, FVD and the cells. Cells (10⁵) were stained either with 1/2000 diluted Fixable Viability Dye eFluor™ 660 during 10-15 min at 37°C (n=3) or 1 µg/mL CD40 APC during 15 min at 4°C (n=2). For Ki-67 staining, cells needed to be fixed with PFA 1% and permeabilized with cold pure acetone before addition of 0.25 µg/mL Ki-67 monoclonal antibody during 60min at room temperature (n=2). Cells were then analyzed on the cytometer.

4.9.8. Statistical analysis

The statistical significance was calculated using the GraphPad Prism 5.0 software by performing a multiple comparison RM two-way ANOVA with a Sidak's correction.

ASSOCIATED CONTENT

Supporting Information Available. Synthesis of βCD-alginate, assessment of IL-6 production from chitosan/βCD-alginate CoPEC-treated macrophages, assessment of IL-6 and TNF-α production by βCD-chitosan/βCD-alginate CoPEC-treated macrophages, assessment of NO and TNF-α production by Piroxicam-containing βCD-chitosan/alginate CoPEC-treated and activated macrophages, assessment of NO production from chitosan, βCD-chitosan and sodium alginate-treated and activated macrophages.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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ABBREVIATIONS

Avidin HRP, Horseradish Peroxidase Avidin; CD, cyclodextrin; COPEC, compact polyelectrolyte complexes; DAMP, damage-associated molecular pattern; DS, substitution degree; IL-6, interleukin 6; LPS, lipopolysaccharide; NO, nitric oxide; PAA, poly(acrylic acid); PAH, poly(allylamine hydrochloride); PAMP, pathogen-associated molecular pattern; PDADMA, poly(diallyldimethylammonium); PMMA, poly(methyl methacrylate); PSS, poly(styrene sulfonate); TNF- α , tumor necrosis factor alpha.

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