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Anti-fibrotic effect of ketoprofen-grafted alginate microcapsules in the transplantation of insulin producing cells

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ABSTRACT

The controlled release of small molecular modulators of the immune response from hydrogel microspheres (MS) used for cell immobilization is an attractive approach to reduce pericapsular fibrotic overgrowth (PFO) after transplantation. Ketoprofen is a well-known non-steroidal anti-inflammatory drug involved in the early stage inflammation cascade. PEGylated derivatives of ketoprofen, presenting either ester or amide linkage to the drug, were synthesized and conjugated to the hydroxyl groups of sodium alginate (Na-alg). Functionalized cell-free and MIN6 cells containing MS were produced from the resulting modified alginates. *In vitro* quantification of ketoprofen release indicated regular and sustained drug delivery over 14 days, resulting from the hydrolytic cleavage of the ester bond.

The release kinetics was enhanced over the initial 7 days by the presence of MIN6 cells, probably as a result of cell esterase activity. In the presence of amide bond, traces of ketoprofen were released over 14 days due to a much slower hydrolysis kinetics. Cell-free and MIN6 cells containing MS were transplanted in immune-competent mice, either in the peritoneal cavity or under the kidney capsule, with a follow-up period of 30 days. Comparison with non-modified Ca-alg MS transplanted in the same conditions demonstrated a clear reduction in the severity of PFO for MS functionalized with ketoprofen. Quantification of collagen deposition on MIN6 cells containing MS transplanted under the kidney capsule revealed the significant effect of ketoprofen release to decrease fibrotic tissue formation. The impact was more pronounced when the drug was covalently conjugated by an ester linkage, allowing higher concentration of the anti-inflammatory compound to be delivered at the transplantation site. The functionality of microencapsulated MIN6 cells 30 days after transplantation was confirmed by detection of insulin positive cell content.

KEYWORDS. Alginate derivatives; Antifibrotic effect; Biocompatibility; Cell microencapsulation; Controlled release; Covalent grafting; Fibrosis; Functionalized hydrogels; Ketoprofen

INTRODUCTION

The low availability of human donor material to replace dysfunctional cells, damaged tissues and end-stage diseased organs have prompted the search for alternative transplantation strategies. Initiated by Chang[1] in 1964 who proposed the concept of "artificial cells", the microencapsulation of cells and tissue appears as a promising strategy for the development of allo- or xenotransplantation therapies.[2-4] This technology has been widely studied for the immuno-isolated transplantation of endocrine cells, in particular toward the treatment of

hepatic diseases[5] and type I diabetes.[6] The main function of the encapsulation materials is to prevent the passage of immune cells and antibodies, while enabling the bidirectional diffusion of molecules essential for cell survival and metabolism such as nutrients and oxygen, and the release of metabolic products.[7,8] In addition to isolation from the host immune response, the semi-permeable immobilization matrix offers supportive structure for cells and protection against mechanical stress and deteriorating environmental effects. These combined properties have the potential to avoid or strongly reduce the use of chronic immunosuppression and to overcome the critical human donor shortage by the transplantation of encapsulated non-human cells.[9] Due to its advantageous gelling properties in contact with divalent cations such as Ba^{2+} and Ca^{2+},[10] sodium alginate (Na-alg) is by far the most widely employed polymer for cell microencapsulation. Despite the high cell compatibility of hydrogels prepared from Na-alg and derivatives,[11,12] the clinical applications of alginate-based hydrogels for cell transplantation are severely hindered by the lack of *in vivo* mechanical durability, defects in permselectivity and low host biocompatibility resulting in pericapsular fibrosis, cell necrosis and loss of graft functionality.[13-17] Several strategies have been highlighted to increase the stability of alginate microspheres (MS). The coating with polycations such as poly(L-lysine) and poly(L-ornithine) reinforced the mechanical properties of alg-based hydrogels but resulted in the adverse activation of inflammatory cytokines and growth factor *in vivo*.[18,19] The performance of alg MS, in particular their suitability for cell microencapsulation as well as their elasticity and selective permeability, could be largely improved by combination with poly(ethylene glycol) (PEG) chains equipped with complementary end reactive functionalities allowing the electrostatic network formed with divalent cations to be reinforced by covalent crosslinking from PEG derivatives.[20-23] Various approaches were reported to try to reduce pericapsular fibrotic overgrowth. Vaithilingam et al. showed that the co-encapsulation of hepato cellular carcinoma cells HuH7 with mesenchymal stem cells (MSCs), which are

known to secrete several small molecular modulators of the immune response after activation, produces a dose-dependent effect on reducing pericapsular fibrosis in a stringent xenotransplantation setting.[24] A recent study by the same group demonstrated that murine MSCs co-encapsulated with islets reduced fibrotic overgrowth and improved allograft survival in diabetic mice.[25] The beneficial effect of microencapsulated human MSCs on liver fibrosis was also observed in bile duct ligation-induced liver fibrosis in mice.[26] Different studies indicated that the co-encapsulation of pancreatic islets with anti-inflammatory and immunosuppressive drugs, enhanced the function of encapsulated cells and reduced fibrosis.[27-29] In particular, the co-encapsulation of curcumin with rat islets in alg MS improved glycemic control in diabetic mice and mitigated the formation of fibrotic tissue.[28] Finally, high purity of the encapsulating alg material[30] and coating with chitosan[31,32] had a significant positive impact on fibrosis reduction.

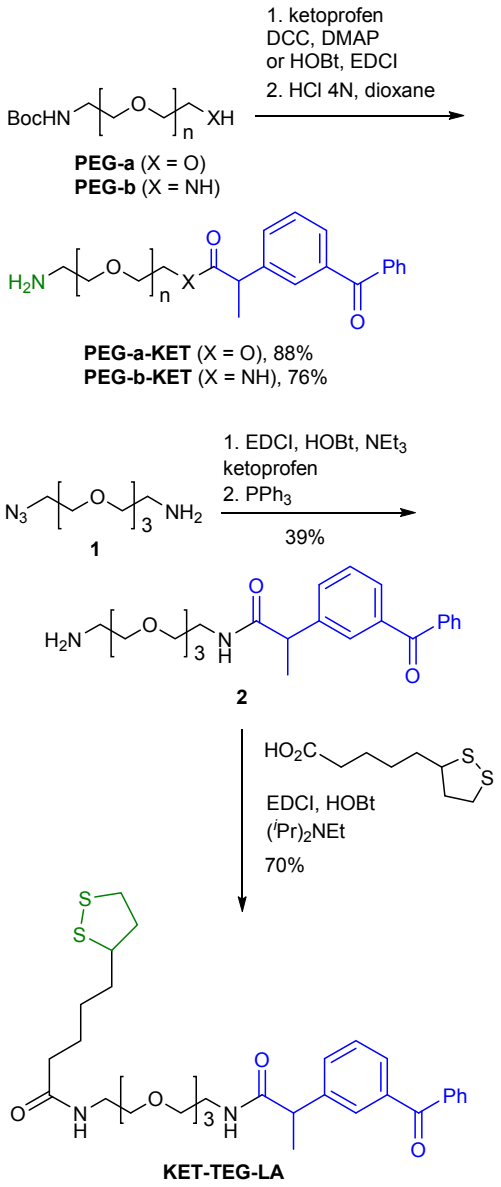
The present study intends to propose an alternative strategy to reduce fibrosis after the transplantation of microencapsulated insulin producing cells by covalent conjugation of anti-inflammatory drug on the alginate backbone allowing local controlled release from the surface of implanted microcapsules based on the hydrolytic properties of the covalent linkage *in vivo*. Ketoprofen is a non-steroidal anti-inflammatory drug exhibiting therapeutic effects which are mostly associated with the inhibition of cyclo-oxygenase I and II (COX-1 and COX-2) involved in the early stage inflammation cascade. In 2005, Ricci et al. investigated composite microcapsules prepared from alg/poly(L-ornithine)/alg system containing ketoprofen-loaded polyester MS.[33] A high burst effect was observed, associated with fast release of the active ingredient, and anti-fibrotic effect was evidenced *in vivo* for cell-free multicompartment device. However, the effect was not validated in cell-containing transplants and the preparation of MS implied the separate preparation of the drug-loaded capsules followed by assembly of the multi-component systems. First, we developed straightforward

functionalization of the encapsulation polymeric material with ketoprofen derivatives allowing the formation of MS by extrusion into a Ca^{2+} containing gelation bath. Then, the release of ketoprofen was quantified from empty and cell-loaded microcapsules followed by *in vivo* evaluation of both stability and anti-fibrotic effect with cell-free and insulin producing MIN6 cells containing capsules.

RESULTS AND DISCUSSION

Preparation of ketoprofen derivatives for covalent conjugation to Na-alg. We previously established a robust conjugation protocol to modify the hydroxyl moieties in C3 position of the mannuronic residues and C2 position of the guluronic residues of the alg backbone enabling the grafting of PEG derivatives through a carbamate linkage.[23] We envisaged the covalent functionalization of Na-alg with ketoprofen either by pegylation of the drug[34,35] followed by modification of the alg hydroxyl groups, or by derivatization of ketoprofen with a lipoyl residue allowing post-conjugation to 1,2-dithiolane containing alg derivatives (Scheme 1). Starting from semi-protected derivatives PEG-a and PEG-b (degree of polymerization DP= 44), coupling to ketoprofen was achieved either by esterification or amide bond formation, followed by treatment under acidic conditions to remove the *tert*-butyl carbamate, yielding PEG-a-KET and PEG-b-KET for conjugation to Na-alg. Alternatively, ketoprofen was coupled to tetra(ethylene glycol) derivative **1**, followed by Staudinger reduction of the terminal azide to provide intermediate **2** in 39% yield (2 steps). Conjugation to lipoic acid through amide bond formation afforded KET-TEG-LA in good yield.

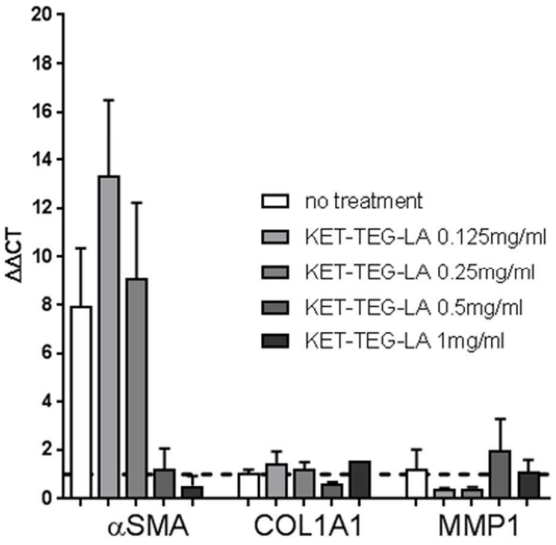
Scheme 1. Synthesis of ketoprofen derivatives for conjugation to Na-alg.



Evaluation of anti-fibrotic effect of ketoprofen derivatives. The potential anti-fibrotic effect of ketoprofen was investigated prior to conjugation to Na-alg. Primary human fibroblasts were stimulated with the pro-fibrotic transforming growth factor beta 1 (TGFβ1) to differentiate into myofibroblasts, the effector cells of fibrosis. As shown in Figure 1, 24h after TGFβ1 treatment, human fibroblasts strongly expressed α-smooth muscle actin (α-SMA), a marker for myofibroblasts, whereas the expression level of alpha-1 type I collagen (COL1A1)

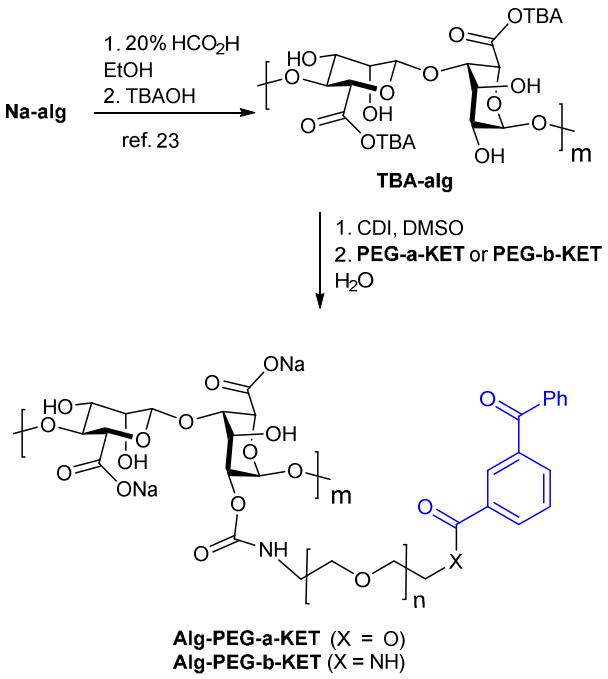
and of matrix metalloproteinase-1 (MMP-1), two components of the extracellular matrix, remained unchanged. Incubation with KET-TEG-LA at concentrations of 0.125 mg/mL and 0.25 mg/mL did not affect the expression level of the genes. However, at a concentration of 0.5 mg/mL and 1 mg/mL the expression level of α-SMA was inhibited. Furthermore, COL1A1 expression was decreased and the expression of MMP-1, an anti-fibrotic molecule, slightly increased. This result shows that KET-TEG-LA is effective in the inhibition of αSMA and COL1A1 expression, two markers of fibrotic tissue, with optimal concentration of 0.5 mg/mL. We thus hypothesized that conjugation of ketoprofen derivatives to the encapsulation polymeric material should reduce the formation of pericapsular fibrotic tissue.

Figure 1. mRNA expression of fibrotic and anti-fibrotic molecules in human fibroblasts. Quantitative RT-PCR was performed to assess the expression of α-SMA, COL1A1 and MMP1 after 24 h stimulation with TGFβ1 (10 ng/mL) (white bars) and in the presence of KET-TEG-LA at increasing concentrations (0.125, 0.25, 0.5, 1 mg/mL) (grey bars). The dashed line indicates the expression level obtained in fibroblasts cultured in medium without TGFβ1 treatment, which was set as 1. Data from 2 independent experiments are presented as means ± SEM.



Functionalization of Na-alg with PEG derivatives. First attempts focused on post-conjugation of KET-TEG-LA to alg derivative grafted with 1,2-dithiolane-terminated PEG chains,[23] aiming at the formation of disulfide clusters by mixing the two partners, with or without addition of dithiothreitol (DTT).[36] As the presence of ketoprofen on the resulting hydrogels could not be evidenced, we turned our attention to the grafting of PEGylated ketoprofen derivatives on the hydroxyl groups of the alg backbone (Scheme 2). Following a recently established protocol,[23] tetrabutyl ammonium (TBA)-alg was treated with 1,1'-carbodiimidazole (CDI) and reacted with either PEG-a-KET or PEG-b-KET (0.1 equiv.) to produce functionalized alginate derivatives which were purified by dialysis. Under those conditions, the grafting degree of pegylated KET to the alginate hydroxyl groups was evaluated to 7% (supporting information S16, Fig. S1). Using a higher amount of PEG-a-KET and PEG-b-KET (0.2 equiv.) resulted in 20% grafting degree.

Scheme 2. Functionalization of Na-alg with pegylated ketoprofen derivatives.

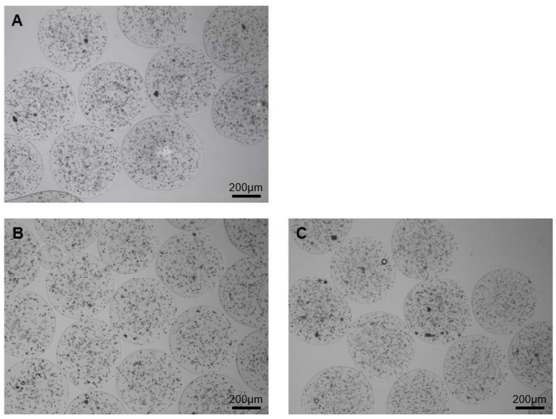


The expected chemical structures were confirmed by NMR spectroscopy. The ^{1}H-NMR spectrum of Alg-PEG-a-KET (supporting information S13) displayed characteristic signals at 7.76-7.51 (multiplet) and 1.46 (doublet) ppm for aromatic and methyl groups of ketoprofen. A large signal between 4.10 and 3.40 pm accounts for the PEG CH_2 units and the protons of the alg backbone. Similar signals were observed in the ^{1}H-NMR spectrum of Alg-PEG-b-KET (supporting information S15). Diffusion Ordered Spectroscopy (DOSY) was performed to confirm that pegylated ketoprofen derivatives were covalently conjugated to the alginate backbone (supporting information S14).

Microsphere formation and quantification of ketoprofen release. Depending on the nature of the covalent bond to ketoprofen (ester, X = O; amide, X = NH), we expected different rates of release from the polymeric materials. First, empty microspheres were produced by extruding solutions of Alg-PEG-a-KET (7% grafting degree, 3 wt %, 1 mL) or Alg-PEG-b-KET (7% grafting degree, 2.5 wt %, 1.2 mL) in 3-(*N*-morpholino) propanesulfonic acid (MOPS) (100 mM, pH = 7.4), into a gelation bath containing $CaCl_2$ as ionic crosslinker. MS were kept in MOPS solution (3 mL) containing $CaCl_2$ (100 mM) and quantification of ketoprofen release was performed over one week by LC-MS analysis of aliquots from the supernatant at regular time points (0.5, 1, 3, 6, 24, 48, 72, 96, 168 h). While MS functionalized with an ester linkage (Alg-PEG-a-KET) showed regular release of the drug over one week, MS functionalized with an amide linkage (Alg-PEG-b-KET) displayed a very slow release kinetic profile, with traces of the drug detected in the supernatant over one weeks (supporting information S17, Fig. S2). However, these MS showed irregular morphology and large size distribution, which prompted us to explore the production of MS from mixture of Alg-PEG-KET polymers (presenting 20% grafting degree) with native Na-alg. Regular morphology and narrow size distribution were achieved by extruding a mixture of solutions of

Na-alg (1.5 wt %, 2 mL) and Alg-PEG-a-KET or Alg-PEG-b-KET (20% grafting degree, 3.5 wt %, 0.86 mL) in MOPS (100 mM, pH 7.4), into a gelation bath containing $CaCl_2$ (100 mM). The resulting MS are designated as alg:Alg-PEG-a-KET and alg:Alg-PEG-b-KET, respectively. Using similar ratios, encapsulation of mouse insulinoma MIN6 cells was performed by mixing the initial solutions of polymers with cells ($3x10^6$ cells/mL of polymer solution) before the extrusion process. This one-step process delivered MS of average diameters between 500 and 600 μm, with homogeneous cells distribution within both types of functionalized alg hydrogels (Figure 2). For comparison, empty and MIN6 containing MS were produced from pure Na-alg.

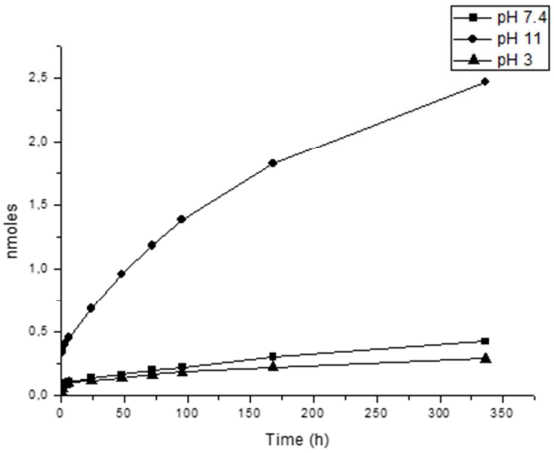
Figure 2. MIN6 cells microencapsulated in Ca-alg MS (A), alg:Alg-PEG-a-KET MS (B) and alg:Alg-PEG-b-KET MS (C).



The release of ketoprofen from empty alg:Alg-PEG-a-KET MS was studied in MOPS solution (100 mM, 2 mL) containing $CaCl_2$ (100 mM), adjusted to pH 3.0, 7.4 or 11. The release of ketoprofen was quantified by LC-MS from the supernatant at regular time points (0.5, 1, 3, 6, 24, 48, 72, 96, 168 and 336 h). At physiological pH, MS functionalized with ketoprofen through an ester linkage (alg:Alg-PEG-a-KET) showed regular release of the drug over two weeks, the covalent conjugation avoiding burst effect within the first hours

following MS formation (Fig. 3). Under the same conditions, MS functionalized with an amide linkage (alg:Alg-PEG-b-KET) released only traces of the drug over two weeks (data not shown).

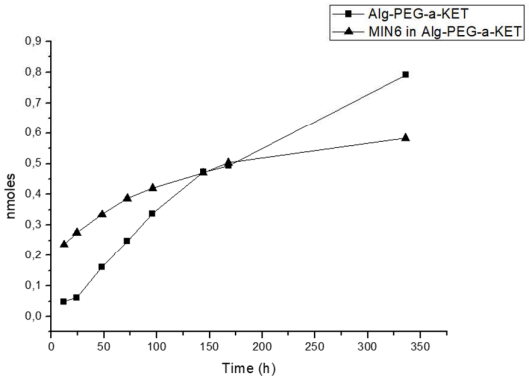
Figure 3. *In vitro* ketoprofen release from alg:Alg-PEG-a-KET and alg:Alg-PEG-b-KET MS. Empty MS (0.5 mL) were kept in MOPS solution (100 mM, 2 mL) containing $CaCl_2$ (100 mM) at the indicated pH value, under slow mechanical agitation. Aliquots of the supernatant were withdrawn after 0.5, 1, 3, 6, 24, 48, 72, 96, 168 and 336 h, and analyzed by LC-MS. At each time point, fresh supernatant was added to compensate for the withdrawn volume.



Interestingly, after 14 days, both MS types were still releasing ketoprofen so that the drug effect can be expected to have a long time impact *in vivo*. In accordance with previous reports in the literature,[37] basic conditions enhanced the rate of release from alg:Alg-PEG-a-KET MS while acidic conditions slowed down the kinetics. Empty MS remained stable over two weeks under all pH conditions (data not shown) showing the resistance of the electrostatic network toward a large range of pH.

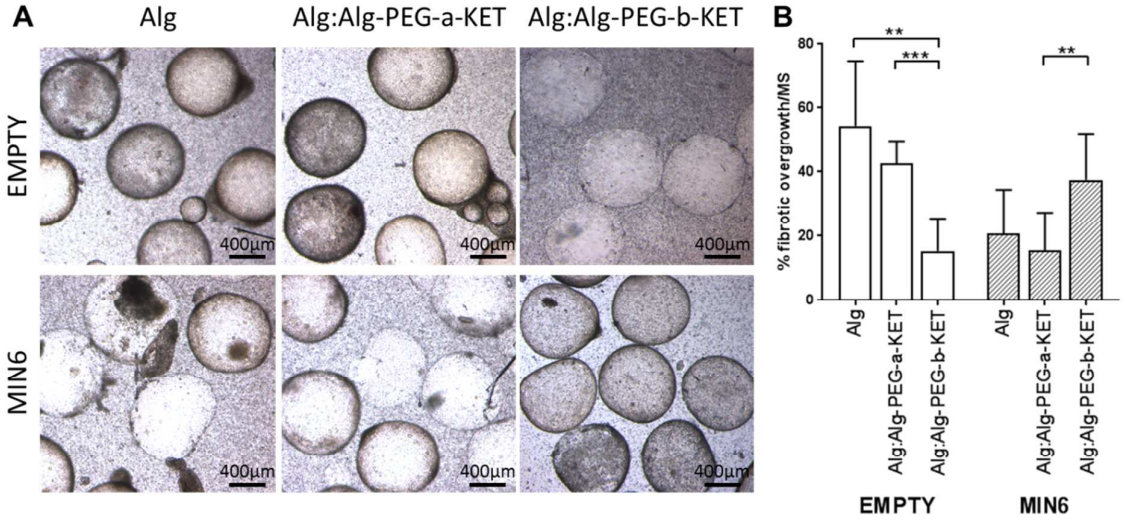
We next evaluated ketoprofen release from alg:Alg-PEG-a-KET MS containing MIN6 cells by maintaining the microcapsules in DMEM complete medium and withdrawing aliquots of the culture medium at regular time points (fresh culture medium is added at each time point to compensate for the withdrawn volume). Figure 4 presents ketoprofen release kinetic profiles from empty MS and MIN6 cells containing MS. The presence of cells induces the release of higher concentrations of ketoprofen over the first 7 days, which might be due to the presence of hydrolytic enzymes of the ester linkage in the cell content. In contrast to a co-encapsulation strategy which resulted in burst release of the drug,[33] covalent functionalization of Na-alg with ketoprofen allowed longer-term release of the drug controlled by the hydrolysis rate of the chemical linkage (ester or amide) to the PEG grafting unit.

Figure 4. *In vitro* ketoprofen release from microencapsulated MIN6 cells in alg:Alg-PEG-a-KET MS compared to empty alg:Alg-PEG-a-KET MS. Empty alg:Alg-PEG-a-KET MS and MIN6 microencapsulated in alg:Alg-PEG-a-KET MS were kept in DMEM complete medium. Aliquots of the supernatant were withdrawn after 0.5, 1, 3, 6, 24, 48, 72, 96, 168 and 336 h, and analyzed by LC-MS. At each time point, fresh culture medium was added to compensate for the withdrawn volume.



In vivo assessment of functionalized MS. The ability of ketoprofen, covently linked to the polymers, to reduce inflammation at the implantation site and prevent pericapsular fibrotic overgrowth, was evaluated by the transplantation of empty MS (Na-alg, alg:Alg-PEG-a-KET and alg:Alg-PEG-b-KET) and MIN6 cells containing MS (Na-alg, alg:Alg-PEG-a-KET and alg:Alg-PEG-b-KET) in immune-competent mice, with a follow-up period of 30 days. Pure Ca-alg MS were implanted under the same conditions. Implantation was performed either in the peritoneal cavity or under the kidney capsule. At 30 days following transplantation, the abdominal cavity of mice contained only non-adherent free-floating MS as shown in fig. S4 (supporting information S20). Therefore, we retrieved a representative amount (500 μL) of transplanted MS for analysis. These MS were examined by light microscopy and showed variable thick layers of fibrotic tissue (figure 5A). Quantification revealed that the severity of cellular overgrowth was decreased on ketoprofen-modified empty Alg:Alg-PEG-a-KET ($p<0.001$) and significantly decreased on Alg:Alg-PEG-b-KET MS ($p<0.01$) compared to pure Ca-alg empty MS (Figure 5B). Further, MS containing MIN6 cells showed similar pericapsular fibrotic overgrowth. The quantification revealed significant decrease of fibrotic overgrowth on Alg:Alg-PEG-a-KET compared with Alg:Alg-PEG-b-KET MS. This is in accordance with a higher ketoprofen concentration released from Alg:Alg-PEG-a-KET MS in the release assay (Figure 3). We also observed a non-significant decrease of fibrotic overgrowth around Alg:Alg-PEG-a-KET compared to pure Na-alg. Further, analysis of the pericapsular fibrotic overgrowth on MS recovered from the peritoneum at earlier time points (15 days) showed similar results. The reduction of fibrotic overgrowth, in the presence of MIN6 cells, was significantly decreased around Alg:Alg-PEG-a-KET and Alg:Alg-PEG-b-KET MS compared to Ca-alg MS (supporting information S21, Fig. S5).

Figure 5. Quantification of pericapsular fibrotic overgrowth on cell-free and MIN6 cells containing MS. (A) Bright-field microscopy images of MS retrieved from the peritoneum following 30 days of transplantation. Ca-alg MS (left rows), Alg:Alg-PEG-a-KET MS (middle rows) and Alg:Alg-PEG-b-KET MS (right rows); cell-free MS (upper panels) and MS with MIN6 cells (lower panels). (B) Pericapsular fibrotic overgrowth was quantified using ImageJ and expressed as a percentage of fibrotic overgrowth. For each MS, fibrotic overgrowth (grey levels) were normalized to the total area of MS measured. Fibrotic overgrowth for cell-free MS (white bars) and for MS with MIN6 cells (striped bars) are represented. All values are means ± standard deviation (SD) of at least 5 MS. **p<0.01, ***p<0.001.



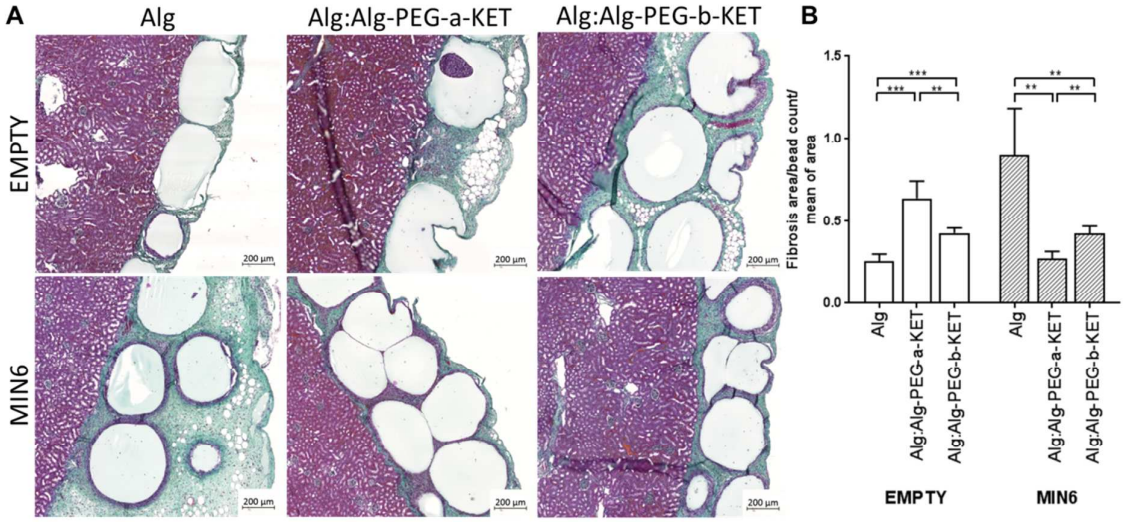
These results show that ketoprofen reduces the severity of pericapsular fibrotic overgrowth at 30 days after intraperitoneal transplantation. In the absence of MIN6 cells, the slow release of ketoprofen from Alg:Alg-PEG-b-KET might be advantageous. In contrast, the effect of ketoprofen on the fibrotic overgrowth on MS harbouring MIN6 cells is different in that MS of Alg:Alg-PEG-a-KET polymer significantly decreased pericapsular fibrotic overgrowth,

compared to Alg:Alg-PEG-b-KET, suggesting that the covalent ester linkage to ketoprofen allows a continuous release which significantly reduces the fibrotic reaction.

To evaluate the anti-fibrotic effect of ketoprofen, all three types of MS were transplanted under the kidney capsule. Histological analysis for collagen deposition using Goldner's Trichrome coloration was performed 4 weeks after transplantation. As shown, transplantation of MS under the kidney capsule induced a fibrotic reaction, revealed by fibrous collagen deposition (in green) for all types of MS (Figure 6A). Pure Ca-alg MS induced minimal collagen deposition, and collagen deposition was increased by Alg:Alg-PEG-a-KET and Alg:Alg-PEG-b-KET MS when compared to Ca-alg MS (Figure 6A, upper panels). In the presence of MIN6, collagen deposition was remarkably increased around the Ca-alg MS (Figure 6A, lower panels). Interestingly, in the presence of ketoprofen, collagen deposition appeared significantly decreased around MIN6 cells encapsulated with Alg:Alg-PEG-a-KET ($p<0.01$) and Alg:Alg-PEG-b-KET ($p<0.01$) (Figure 6B, lower panels). Furthermore, collagen deposition around Alg:Alg-PEG-a-KET MS was significantly decreased compared to collagen deposition around Alg:Alg-PEG-b-KET MS which is in line with the findings *in vitro*, that Alg:Alg-PEG-a-KET MS release higher amounts of ketoprofen compared to Alg:Alg-PEG-b-KET MS (Figure 3).

Figure 6. Quantification of collagen deposition around empty and MIN6 cells containing MS at 4 weeks after transplantation under the kidney capsule. (A) Sections of formalin-fixed and paraffin-embedded kidneys were colored with Goldner's Trichrome. Ca-alg MS (left rows), Alg:Alg-PEG-a-KET MS (middle rows) and Alg:Alg-PEG-b-KET MS (right rows); cell-free MS (upper panels) and MS with MIN6 cells (lower panels). (B) Quantification of collagen deposition was performed with Definiens software. Cell-free MS (white bars) and MS with

MIN6 cells (striped bars). All values are means ± Standard Deviation (SD) of 5 slides per condition. **p<0.01, ***p<0.001.



All together, these data suggest that transplantation of MIN6 cells in Alg:Alg-PEG-a-KET MS under the kidney capsule can mitigate fibrotic reaction (strongest reduction of collagen deposition) compared to MS of Na-alg, which correlates with highest release of ketoprofen *in vitro*. These results corroborate the observations of reduced pericapsular fibrotic overgrowth on Alg:Alg-PEG-a-KET MS in the peritoneum. The clear beneficial effect on collagen deposition around Alg:Alg-PEG-a-KET MS in the presence of MIN6 cells might be explained by the presence of MIN6-derived hydrolytic enzymes acting on ester linkage and increasing ketoprofen release as observed *in vitro* (Figure 4).

To assess the viability of transplanted encapsulated MIN6 cells, MS retrieved from IP and MS transplanted under the kidney capsules were stained for insulin after histological preparation. Immunostaining clearly detected insulin positive MIN6 cells in all three types of MS at 30 days after transplantation in mice (supporting information S22, Fig. S6), suggesting that MIN6 cells were intact and viable under such conditions.

CONCLUSION

A synthetic route was developed to prepare PEGylated conjugates of the anti-inflammatory drug ketoprofen which were grafted to the hydroxyl groups of the biopolymer Na-alg. Cell-free and MIN6 cells containing MS obtained from a mixture of Na-alg and Alg-PEG-KET derivatives were assessed *in vitro* for their ability to release the drug under physiological conditions and *in vivo* for their potential to reduce fibrotic reaction. The covalent conjugation of ketoprofen through an ester linkage resulted in regular and sustained drug release up to 14 days, the hydrolysis being accelerated in the presence of MIN6 cells, probably resulting from the action of cell esterase. In the presence of an amide linkage, trace amounts of ketoprofen were released from the MS. In comparison with non-covalent encapsulation of anti-inflammatory drugs, the functionalization strategy herein presented avoids burst release of ketoprofen within the first hours after transplantation, providing slower delivery of the active ingredient at the transplantation site. When transplanted in immune-competent mice and compared to pure Ca-alg MS, functionalized hydrogels resulted in reduced pericapsular fibrotic overgrowth 30 days after transplantation. Noteworthy, the severity of cellular deposition around pure Ca-alg MS was remarkably increased in MIN6 cells containing MS, highlighting the importance to assess fibrotic response to transplantation with cell-loaded microcapsules. Quantification of collagen deposition on MS transplanted under the kidney capsule indicated that the conjugation of ketoprofen to the hydrogel polymeric material through an ester linkage can strongly reduce pericapsular fibrotic overgrowth while maintaining the insulin secretion capacity of the microencapsulated cells. The functionalization pathway disclosed in the present study provides a new approach to mitigate fibrotic response to microencapsulated cells transplantation by controlled hydrolytic release of

anti-inflammatory drugs from the hydrogel matrix. Pericapsular fibrotic overgrowth being a major cause of graft failure, this strategy could be further applied to a variety of small molecular immune response modulators, including the possibility to combine several drugs for synergistic effects.

EXPERIMENTAL PROCEDURES

Materials and Methods. Na-alg Kelton HV (lot no. 61650A, [η] = 813 mL g^{-1} in 0.1 M NaCl, T = 25 °C, G/M = 0.6) was obtained from Kelco (San Diego, USA, CA). Linear PEG (**PEG-a** and **PEG-b**) were obtained from Jenkem Technology (Beijin, China). Other commercial reagents (Fluka, Sigma, Switzerland; TCI Europe, Zwijndrecht, Belgium) were used without further purification. Unless special mention, all reactions were performed under argon atmosphere (1 atm). Anhydrous solvents were obtained by filtration (Puresolv MD 5, Innovative Technology, Oldham, UK). Glassware was dried for 12 h in an oven (T > 100 °C) or under vacuum with a heat gun (T > 200 °C). Reactions were monitored by TLC (Merck silica gel 60F254 plates, Merck, Darmstadt, Germany). Detection was performed by UV light, $KMnO_4$, Ninhydrin or I_2. Purifications were performed by flash chromatography on silica gel (Merck N° 9385 silica gel 60, 240-400 mesh). Aqueous solutions were produced with ultrapure water obtained from Milli-Q Integral System (Merck / Millipore). NMR spectra were recorded on Bruker Avance III-400, Bruker Avance-400 or Bruker DRX-400 spectrometers at room temperature (rt) (400 MHz) (Bruker, Billerica, MA, USA). ^{1}H frequency is at 400.13 MHz, ^{13}C frequency is at 100.62 MHz. Chemical shifts are expressed in parts per million (ppm) and coupling constants (*J*) in hertz (Hz). Solvents used for NMR spectroscopy were deuterated chloroform ($CDCl_3$, Acros), deuterated methanol (CD_3OD, Acros) and deuterated water (D_2O). Mass spectra were obtained on a Nermag R-10-10C spectrometer with chemical ionization (NH_3) and mode m/z (amu) [% relative base peak

(100%)] (Nermag, Santa Clara, CA, USA). IR spectra were recorded on a Jasco FT/IR-4100 spectrometer outfitted with a PIKE technology MIRacleTM ATR accessory as neat films compressed onto a Zinc Selenide window. The spectra are reported in cm^{-1}. Mass spectra were obtained by using a Waters ACQUITY H-class UPLC/MS ACQ-SQD by electron ionization (EI positive and negative) or a Finnigan TSQ7000 by electrospray ionization (ESI+). The accurate masses were measured by ESI-TOF using a QTOF Ultima from Waters. Quantitative MS analyses were performed on and Agilent Accurate Mass Q-TOF LCMS mass spectrometer coupled to an Agilent 1290 series UHPLC system (Agilent Technologies, USA). The separation was achieved using an ACQUITY UPLC® BEH C18 1.7µm column, 2.1 mm x 50 mm (Waters) heated at 30°C.

Formation of empty microspheres. Na-alg (1.5 wt %, 2 mL) and Alg-PEG-KET polymers (3.5 wt %, 0.86 mL) were dissolved in 100 mM MOPS buffer, pH 7.4. The solutions of polymers were mixed and the resulting mixture was extruded into a gelation bath (100 mM $CaCl_2$ in 100 mM MOPS buffer, pH 7.4) containing tween 80 (diluted 1/10 000). MS were produced employing a coaxial air-flow droplet generator (Encapsulator B-395 Pro, Büchi Labortechnik AG, Flawil, Switzerland). The MS were collected by filtration, washed twice for 10 minutes with NaCl 0.9%, and finally stored in this solution at 4 °C.

Microencapsulation of MIN6 cells. MIN6 cells were cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 1 mM Na-Pyruvate, 71 µM β-mercaptoethanol, 15% decomplemented fetal calf serum (FCS), 25 mM glucose, penicillin and streptomycin (DMEM complete medium). MIN6 cells ($3x10^6$) were centrifuged at 250 g for 5 minutes and the supernatant was completely removed. The remaining cell pellet was dispersed in 1 mL of Na-alg-PEG solution using a 10 mL syringe. MS were produced using the same procedure as described above for the formation of empty MS. Encapsulated MIN6 cells were then cultured in DMEM complete medium at 37°C with 5% CO_2.

Real-time RT – PCR. mRNA expression of α-SMA, COL1A1 and MMP was analyzed by real-time reverse transcription PCR (RT-PCR), as previously described.[38] Briefly, 1.5 x 10^5 human fibroblasts were plated in DMEM supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin (P-S, Gibco-Invitrogen) and 10% FCS (Gibco-Invitrogen) at 37°C in humidified air containing 5% CO_2. After 8 hours, medium was removed and replaced by DMEM without FCS for one day. Then, 10 ng/ml of TGFβ1 diluted in DMEM with 1% FCS was added to all conditions (except negative control) and supplemented with increasing concentrations of KET-TEG-LA (0.125, 0.25, 0.5, 1 mg/ml). After 24h of incubation, total RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen, San Diego, CA, USA) according to the manufacturer's instructions. Primers listed in supporting information (S23, Table S1) were designed using Primer3 online software (http://primer3.ut.ee), tested with Primer Biosoft (http://www.premierbiosoft.com) and blasted on BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Expression of housekeeping ribosomal protein large P0 was used to normalize expression values obtained for α-SMA, COL1A1 and MMP.

Quantitative analysis of ketoprofen release by UHPLC-ESI-HRMS.

Release from empty alg:Alg-PEG-a-KET MS at different pH. Alg:Alg-PEG-a-KET MS (0.5 mL) were kept in 2 mL of gelation solution (100 mM $CaCl_2$ in 100 mM MOPS) adjusted to pH 3, 7.4 or 11, under slow mechanical agitation. From each batch, aliquots of the supernatant were withdrawn after 0.5, 1, 3, 6, 24, 48, 72, 96, 168 and 336 h, for quantitative MS analysis. At each time point, fresh supernatant was added to compensate for the withdrawn volume.

Release from encapsulated MIN6 cells in alg:Alg-PEG-a-KET MS. Alg:Alg-PEG-a-KET MS (0.5 mL) were kept in DMEM complete medium (1 mL). Aliquots of the supernatant were withdrawn after 0.5, 1, 3, 6, 24, 48, 72, 96, 168 and 336 h, for quantitative MS analysis. At each time point, fresh culture medium was added to compensate for the withdrawn volume.

Aliquots were analyzed by LC-MS and the concentration of ketoprofen released quantified according to a calibration curve obtained with ketoprofen as a reference (supporting information S19). The calibration curve was performed before and after the measurements of the samples to confirm stability of the quantification system. The cumulative release (in moles) for each time point was then calculated using the following formula:

$$n_{cumul\ x} = n_{released\ in\ x} + n_{cumul\ x-1} = \quad C_x \cdot V_x - R \cdot n_{x-1} + n_{cumul\ x-1}$$

(n, number of mole; c, concentration; V, volume; R, volume ratio left of the supernatant)

Details of the parameters for UHPLC-ESI-HRMS analysis and calibration curves are provided in supporting information (S18-S19, Fig. S3).

Transplantation of MS in mice. All animal experiments were approved by the Geneva veterinary authorities (license GE/151/16). After production, MS were kept for one day in culture. Prior to transplantation, MS were rinsed three times for 15 minutes in Hank's Balanced Salt Solution (HBSS), containing Ca^{2+}. Once MS have settled on the bottom of the tube, the supernatant was removed and remaining MS were dispersed in minimal amount of HBSS for transplantation. 1.5 mL of MS, corresponding to ~12'700 MS (supporting information S18), were introduced through a small incision into the peritoneum of Bl6 mice anesthetized by isoflurane. For kidney transplantation, kidneys were exposed through a small dorsal incision and ~ 100 µL of MS were injected under the kidney capsule using a 18G catheter. Thirty days after transplantation mice were sacrified and kidneys and MS from peritoneum were retrieved for histological analysis.

Histological analysis. MS were collected from the peritoneum using a spatulas micro spoon and added without washing to a small volume of NaCl 0.9% for immediate image acquisition using inverted bright-field microscopy (Leica DM IL) and LAS V4.5 software (Leica Microsystem, Heerbrugg, Switzerland). Kidneys were harvested, fixed in formalin for 24h

and embedded in paraffin. Histological sections of 4µm of tissue were stained for collagen using Goldner's trichrome,[26] images were acquired by Zeiss Axioscan.Z1 and processed with ZEN 2.3 lite (Carl Zeiss).

Quantification of pericapsular fibrotic overgrowth. Fibrotic overgrowth around MS retrieved from peritoneum was quantified using Image J. Briefly, each selected MS was processed with same parameters for color threshold and brightness during analysis. Total area of each MS was determined after manual selection of MS shape, which was set as 100%. The pericapsular fibrotic overgrowth (grey levels) was then evaluated in the selected area and normalized to the value of the area of each MS and therefore expressed as percentage of fibrotic overgrowth / MS. At least, 5 MS per type of polymer were analyzed to determine the percentage of fibrotic overgrowth. All values are means ± Standard Deviation (SD).

Collagen quantification around MS transplanted under the kidney capsule was performed using Definiens Software. Five kidney sections per polymer were quantified. Collagen depositions (in green) were quantified and normalized to the number of MS present in each section. Since MS size differed in each section, values were additionally normalized to the mean of the total area of MS present in a given section, therefore values are expressed as fibrosis area / MS count / mean of area. All values are means ± Standard Deviation (SD).

Synthesis protocols. Designation of the compounds refers to the chemical structures presented in Scheme 1 and Scheme 2.

Preparation of PEG-a-KET. PEG-a (1.0 equiv, 0.111 mmol, 0.234 g) was dissolved in DCM (2 mL). Ketoprofen (1.01 equiv, 0.112 mmol, 0.029 g), N,N'-dicyclohexylcarbodiimide (DCC) (1.1 equiv, 0.1224 mmol, 0.025 g) and 4-dimethylaminopyridine (DMAP) (0.1 equiv, 0.011 mmol, 0.001 g) were added to the reaction mixture. The reaction was stirred at 22°C for 12 h. After concentration under reduced pressure, the crude residue was purified by FCC on silica gel (DCM/MeOH 30:1) to afford a white solid (0.234 g, 88%). HRMS-ESI: *m/z*

2357.3321 (calcd.); 2356.599 (M+Na) (found). ^{1}H-NMR, ^{13}C-NMR and IR data (supporting information S2-S3).

This intermediate (1.0 equiv, 0.100 mmol, 0.234 g) was dissolved in a solution of HCl 4N in dioxane (3 mL) and the mixture was stirred at 22°C until completion of the reaction (monitored by TLC). The volatiles were removed under air flow and the product was dried under vacuum to afford PEG-a-KET as a beige solid (0.225 g, quant.) which was used without further purification. HRMS-ESI: *m/z* 2236.3013 (calcd.); 2236.2954 (M+H) (found). ^{1}H-NMR, ^{13}C-NMR and IR data (supporting information S3-S4).

Preparation of PEG-b-KET. PEG-b (1.0 equiv, 0.047 mmol, 0.100 g) was dissolved in DMF (5 mL). Ketoprofen (1.01 equiv, 0.047 mmol, 0.012 g), 1-Hydroxybenzotriazole hydrate (HOBt) (1.5 equiv, 0.070 mmol, 0.011 g), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (2.0 equiv, 0.094 mmol, 0.018 g) and $(^{i}Pr)_2NEt$ (3.0 equiv, 0.140 mmol, 0.023 mL) were added. The reaction mixture was stirred at 22°C for 12 h. The solvent was removed under vacuum. The residue was dissolved in DCM (10 mL) and washed with sat. NH_4Cl solution (2 x 10 mL) and brine (1 x 10 mL). The organic layer was dried with $MgSO_4$ and concentrated under vacuum. Purification by FCC on silica gel (DCM/MeOH 20:1 and then 9:1) afforded a white solid (0.084 g, 76%). HRMS-ESI: *m/z* 2357.3516 (calcd.); 2357.3511 (M+Na) (found). ^{1}H-NMR data (supporting information S5).

This intermediate (1 equiv, 0.036 mmol, 0.084 g) was dissolved in a solution of HCl 4N in dioxane (3 mL) and the mixture was stirred at 22°C until completion of the reaction (monitored by TLC). The volatiles were removed under air flow and the product was dried under vacuum to afford PEG-b-KET as a beige solid (0.083 g, quant.) which was used without further purification. ^{1}H-NMR, ^{13}C NMR and IR data (supporting information S6-S7).

Preparation of compound 2. Tetra(ethylene glycol) derivative **1**[39] (1 equiv, 0.71 mmol, 0.182 g) was dissolved in DMF (4 mL). Ketoprofen (1.01 equiv, 0.72 mmol, 0.184 g), HOBt (1.5 equiv, 1.07 mmol, 0.164 g), EDCI (2.0 equiv, 1.42 mmol, 0.274 g) and $(^{i}Pr)_2NEt$ (3.0 equiv, 2.14 mmol, 0.354 mL) were added. The mixture was stirred at 22°C for 12 h. The solvent was removed under vacuum and the residue was dissolved in DCM (10 mL). The solution was washed with sat. NH_4Cl solution (2 x 10 mL) and brine (1 X 10 mL). The organic phase was dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by FCC on silica gel (DCM/MeOH 20:1 then 9:1) to afford a yellow oil (0.223 g, 69%). HRMS-ESI: *m/z* 477.2114 (calcd); 477.2127 (found) (M+Na). ^{1}H-NMR, ^{13}C NMR and IR data (supporting information S8-S9).

This intermediate (1.0 equiv, 0.81 mmol, 0.368 g) was dissolved in THF (6 mL). Triphenylphosphine (1.4 equiv, 1.1 mmol, 0.297 g) was added and the solution was stirred at 22°C for 1 h. Water (1 mL) was added and the mixture was stirred at 22°C for 12 h. The solvents were removed under vacuum. The residue was purified by FCC on silica gel (DCM/MeOH/NEt_3 10:1:0.1) to afford **2** as a yellow oil (0.195 g, 56%). HRMS-ESI: *m/z* 429.2384 (calcd.); 429.2379 (found) (M+H). ^{1}H-NMR, ^{13}C NMR and IR data (supporting information S10-S11).

Preparation of KET-TEG-LA. To a solution of **2**[39] (1 equiv, 1.21 mmol, 0.519 g) in DMF (4 mL), were added lipoic acid (1.2 equiv, 1.45 mmol, 0.3 g), HOBt (2 equiv, 2.42 mmol, 0.371 g), EDCI (1.2 equiv, 1.45 mmol, 0.28 g) and $(^{i}Pr)_2NEt$ (3.64 mmol, 3 equiv, 0.51 ml). The reaction mixture was stirred at 22°C for 12 h. The solvent was removed under vacuum and the residue was dissolved in DCM (5 mL). The solution was washed with water (3 x 5 mL). The organic phase was dried over $MgSO_4$ and concentrated under vacuum. Purification by FCC on silica gel (DCM/MeOH, 20:1) afforded KET-TEG-LA as a yellowish solid (0.52 g, 70%).

HRMS-ESI: *m/z* 618.2559 (calcd.); 618.2563 (found) (M+H). ^{1}H-NMR, ^{13}C NMR and IR data (supporting information S11-S12).

Preparation of TBA-Alg (according to the procedure described in ref. 23). Na-alg (Kelton HV, 1.0 g) was suspended in EtOH (50 mL) and was cooled down to 0 °C. Formic acid (20 % aqueous solution, 50 mL) was added, and the mixture was stirred for 14 h at 0 °C. The resulting alginic acid powder was separated by vacuum filtration, washed with a mixture of EtOH/H_2O 1:1 (3 x 50 mL) and then acetone (3 x 50 mL). The resulting solid was further dried under vacuum at 40 °C for 20 minutes, and dispersed in water (50 mL). TBAOH (40 % in water) was added dropwise until reaching pH 7. The solution was directly freeze-dried without dialysis to afford TBA-Alg as a white solid.

Preparation of Alg-PEG-a-KET and Alg-PEG-b-KET. TBA-Alg (1.0 equiv, 0.239 mmol, 0.100 g) was dissolved in DMSO (20 mL) and the solution was stirred at 22°C for 12 h to ensure homogeneity. 1,1'-Carbonyldiimidazole (1.0 equiv, 0.239 mmol, 0.039 g) previously dissolved in a minimum volume of DMSO was added and the reaction mixture was stirred at 22°C for 30 min. To enhance precipitation of the imidazolide-alginate intermediate, acetone (40 mL) was added. The resulting precipitate was filtered (70 µm) and washed with acetone (3 × 10 mL). The solid was further dried for 15 min under vacuum at 40 °C and transferred to a round-bottom flask. Distilled water was added (10 mL), and the mixture was stirred until complete dissolution. PEG-a-KET or PEG-b-KET (0.10 equiv. 0.0239 mmol) previously dissolved in a minimum volume of water was added. The solution was stirred at 22 °C for 2 h. The reaction was quenched by addition of 0.05 M NaOH solution until reaching pH 11. The solution was directly transferred into a dialysis membrane (14 kDa) and dialyzed against distilled water. The first day, water was changed once. The dialysis was continued the next day (changing water 3 times that day). The last day, the dialysis was continued against water, with the addition of $NaHCO_3$ (180 mg in 9 L distilled water) in the dialysis bucket, to reach

pH 7. The solution was filtered (70 and 0.22 µm) and freeze-dried to obtain Alg-PEG-a-KET or Alg-PEG-b-KET as white solids. ^{1}H-NMR and DOSY spectra (supporting information S13-S15). The degree of grafting was determined by 1D ^{1}H-NMR. Due to peak overlap, separation between PEG and alginate ^{1}H resonances was performed by deconvolution (supporting information S16). Using 0.1 equiv. of PEG-a-KET or PEG-b-KET resulted in 7% grafting. Using 0.2 equiv. of PEG-a-KET resulted in 20% grafting. Before MS formation, Alg-PEG-a-KET and Alg-PEG-b-KET were subjected to UV irradiation for 20 minutes, twice.

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Author contributions

François Noverraz and Elisa Montanari contributed equally to the work. The manuscript was written through contributions of all authors. FN designed and performed the functionalization pathways, prepared and characterized the microspheres, and analyzed ketoprofen release. EM designed and performed PCR analysis, designed and evaluated microsphere fibrosis *in vivo*. JP developed and performed the quantification of fibrosis on microspheres in mice model. LS

developed the grafting procedures on alginate. DO performed the quantification of ketoprofen release. CGG, LHB and SG supervised the project, designed the experiments and prepared the manuscript.

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

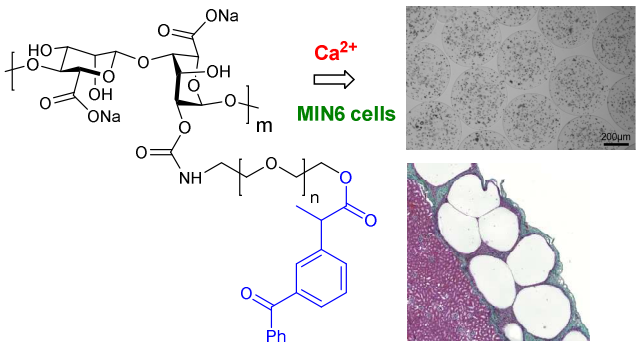
Supporting Information. The following contents are available free of charge on the ACS Publication website.

Analytical data for all synthesized compounds (^{1}H, ^{13}C and DOSY NMR data, IR data); Determination of the degree of grafting on Alg-PEG-KET polymers; Ketoprofen release from pure Alg-PEG-KET MS; Quantitative analysis by UHPLC-ESI-HRMS; Estimation of the number of MS for transplantation; Observation of free-floating MS in the peritoneum of transplanted mice; Quantification of PFO on cell-free and MIN6 cells containing MS 15 days after transplantation; Viability of encapsulated MIN6 cells 30 days after transplantation; Primers for real-time RT – PCR experiments.

ABBREVIATIONS

Alg, alginate; Boc, tert-butyloxycarbonyl; CDI, carbodiimidazole; COL1A1, alpha-1 type I collagen; DCC, *N*,*N*'-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMEM, Dulbecco Modified Eagle's Medium; DOSY, Diffusion Ordered Spectroscopy; DTT, dithiothreitol; HBSS, Hank's Balanced Salt Solution; MS, microsphere; MSCs, mesenchymal stem cells; MMP-1, matrix metalloproteinase-1; MOPS, 3-(*N*-morpholino) propanesulfonic acid; Na-alg, sodium alginate; PEG, poly(ethylene glycol); ppm, parts per million; RT-PCR, real-time reverse transcription PCR; TBA, tetrabutyl ammonium; TGFβ1, transforming growth factor; wt, weight; α-SMA, α-smooth muscle actin.

TOC GRAPHIC



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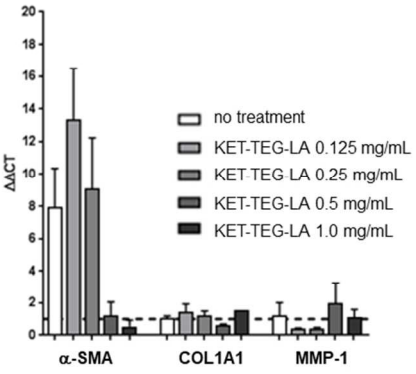

Figure 1. mRNA expression of fibrotic and anti-fibrotic molecules in human fibroblasts. Quantitative RT-PCR was performed to assess the expression of α-SMA, COL1A1 and MMP1 after 24 h stimulation with TGFβ1 (10 ng/mL) (white bars) and in the presence of KET-TEG-LA at increasing concentrations (0.125, 0.25, 0.5, 1 mg/mL) (grey bars). The dashed line indicates the expression level obtained in fibroblasts cultured in medium without TGFβ1 treatment, which was set as 1. Data from 2 independent experiments are presented as means ± SEM.

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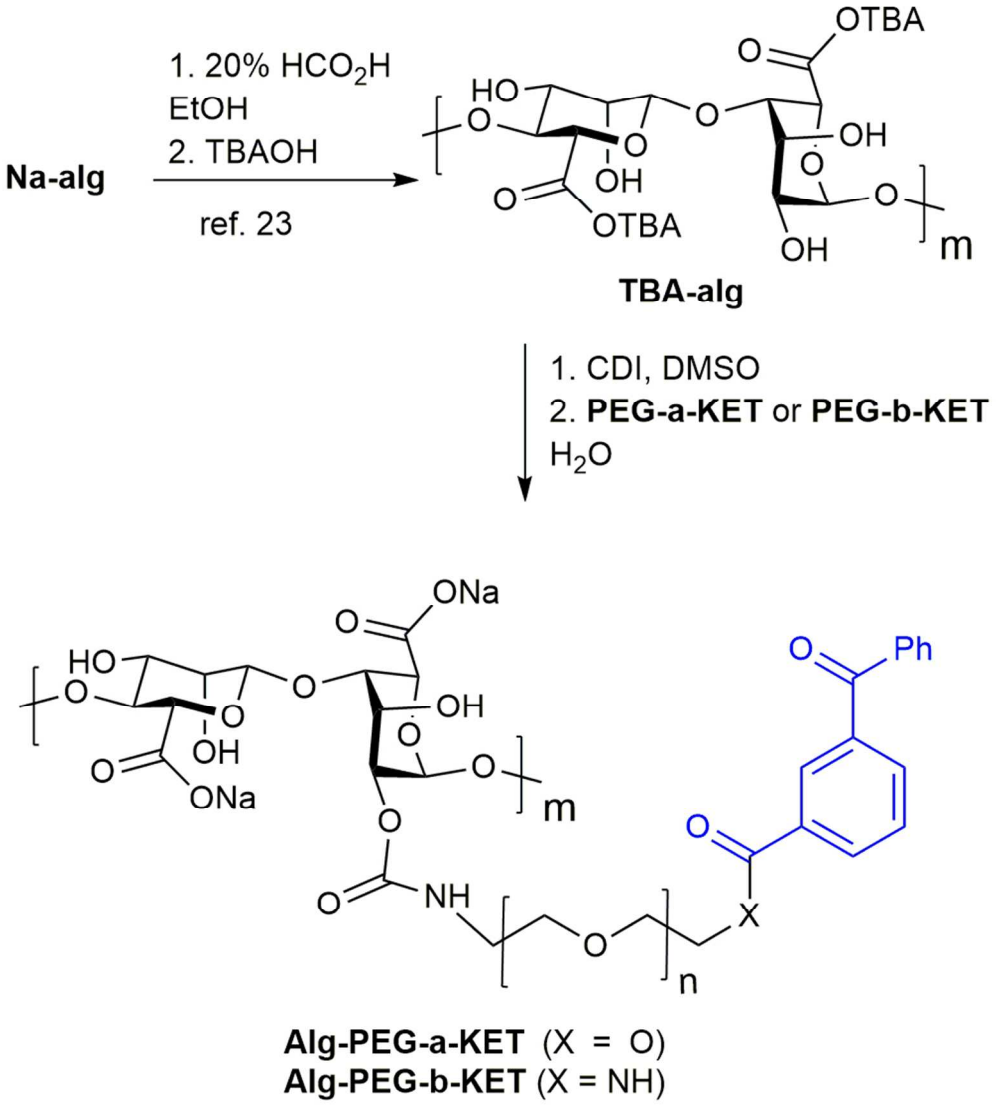

Scheme 2. Functionalization of Na-alg with pegylated ketoprofen derivatives.

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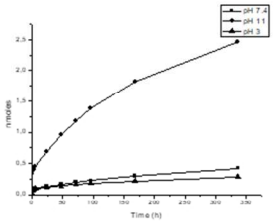

Figure 3. In vitro ketoprofen release from alg:Alg-PEG-a-KET and alg:Alg-PEG-b-KET MS. Empty MS (0.5 mL) were kept in MOPS solution (100 mM, 2 mL) containing CaCl2 (100 mM) at the indicated pH value, under slow mechanical agitation. Aliquots of the supernatant were withdrawn after 0.5, 1, 3, 6, 24, 48, 72, 96, 168 and 336 h, and analyzed by LC-MS. At each time point, fresh supernatant was added to compensate for the withdrawn volume.

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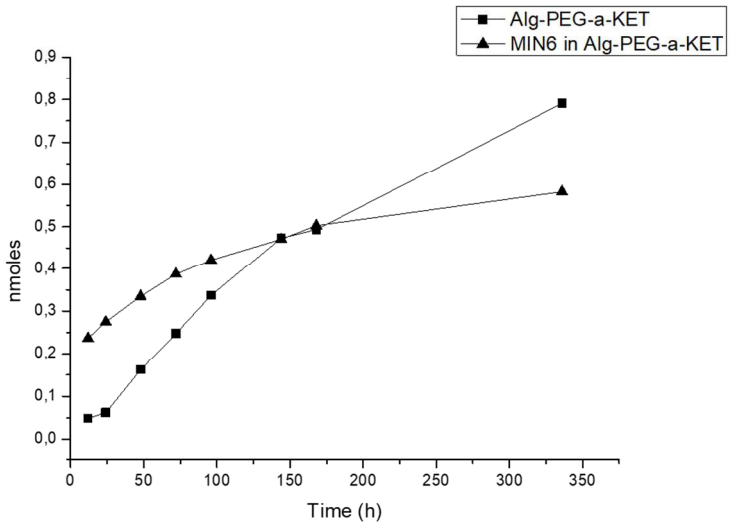

Figure 4. In vitro ketoprofen release from microencapsulated MIN6 cells in alg:Alg-PEG-a-KET MS compared to empty alg:Alg-PEG-a-KET MS. Empty alg:Alg-PEG-a-KET MS and MIN6 microencapsulated in alg:Alg-PEG-a-KET MS were kept in DMEM complete medium. Aliquots of the supernatant were withdrawn after 0.5, 1, 3, 6, 24, 48, 72, 96, 168 and 336 h, and analyzed by LC-MS. At each time point, fresh culture medium was added to compensate for the withdrawn volume.

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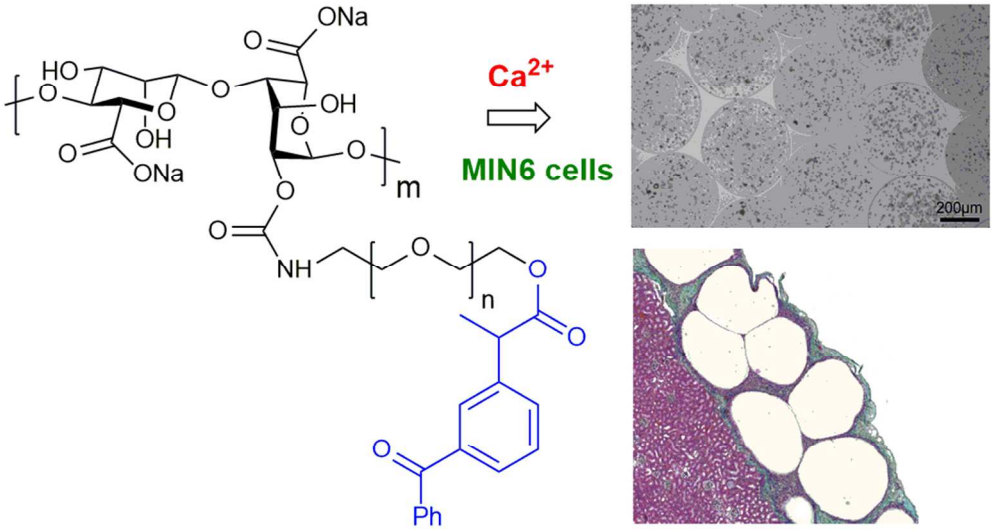

TOC graphic

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