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Research article

Preparation and investigation of P28GST-loaded PLGA microparticles for immunomodulation of experimental colitis

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ABSTRACT

The aim of this work was to prepare and characterize (in vitro and in vivo) PLGA-based microparticles loaded with an enzymatic protein derived from the helminth parasite *Schistosoma haematobium*: glutathione S-transferase P28GST (P28GST). This protein is not only a promising candidate vaccine against schistosomiasis, it also exhibits interesting immunomodulating effects, which can be helpful for the regulation of inflammatory diseases. Helminths express a regulatory role on intestinal inflammation, and immunization by P28GST has recently been shown to be as efficient as infection to reduce inflammation in a murine colitis model. As an alternative to the combination with a classical adjuvant, long acting P28GST microparticles were prepared in order to induce colitis prevention. PLGA was used as biodegradable and biocompatible matrix former, and a W/O/W emulsion/solvent extraction technique applied to prepare different types of microparticles. The effects of key formulation and processing parameters (e.g., the polymer molecular weight, drug loading, W/O/W phase volumes and stirring rates of the primary/secondary emulsions) on the systems' performance were studied. Microparticles providing about constant P28GST release during several weeks were selected and their effects in an experimental model of colitis evaluated. Mice received P28GST-loaded or P28GST-free PLGA microparticles (s.c.) on Day 0, and optionally also on Days 14 and 28. Colitis was induced on Day 35, the animals were sacrificed on Day 37. Interestingly, the Wallace score (being a measure of the severity of the inflammation) was significantly lower in mice treated with P28GST microparticles compared to placebo after 1 or 3 injections. As immunogenicity markers, increased anti-P28GST IgG levels were detected after three P28GST PLGA microparticle injections, but not in the control groups. Thus, the proposed microparticles offer an interesting potential for the preventive treatment of experimental colitis, while the underlying mechanism of action is still to be investigated.

1. Introduction

Inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, represent a major health burden in many countries. The exact pathogenesis of IBD has not yet been elucidated, but it is commonly believed that an inappropriate activation of the mucosal immune system plays a major role, leading to chronic inflammation (Strober et al., 2012).

Interestingly, epidemiological evidence suggests that the importance of IBD increases in regions which underwent intensive deworming treatments (Wammes et al., 2014). Thus, parasitic infections might help keeping the incidence of chronic inflammatory bowel diseases limited. It has for instance been hypothesized that helminthic infections can have positive immune modulatory effects, which can modulate autoimmune inflammatory diseases (Weinstock and Elliott, 2009; Smallwood et al., 2017). However, an infection with a parasite is not a viable treatment option or prevention technique. For this reason, well characterized helminth molecules have been proposed instead. In this respect, P28GSTs (schistosome enzymes with glutathione S-transferase activity) have been identified as potential vaccine candidates against schistosomiasis, and produced under recombinant form (Capron et al., 2005). Besides 28 kDa glutathione S-transferase of *Schistosoma mansoni* (Sm28GST), *Schistosoma haematobium* (Sh) 28GST has been further characterized, from molecular cloning to crystallization (Johnson et al., 2003). Sm28GST has been developed as a schistosome vaccine (Riveau et al., 2012), and is considered as the most advanced schistosome vaccine candidate (Tebeje et al., 2016). Recently, the potent pro-Th2 immunogenicity and anti-inflammatory potential of

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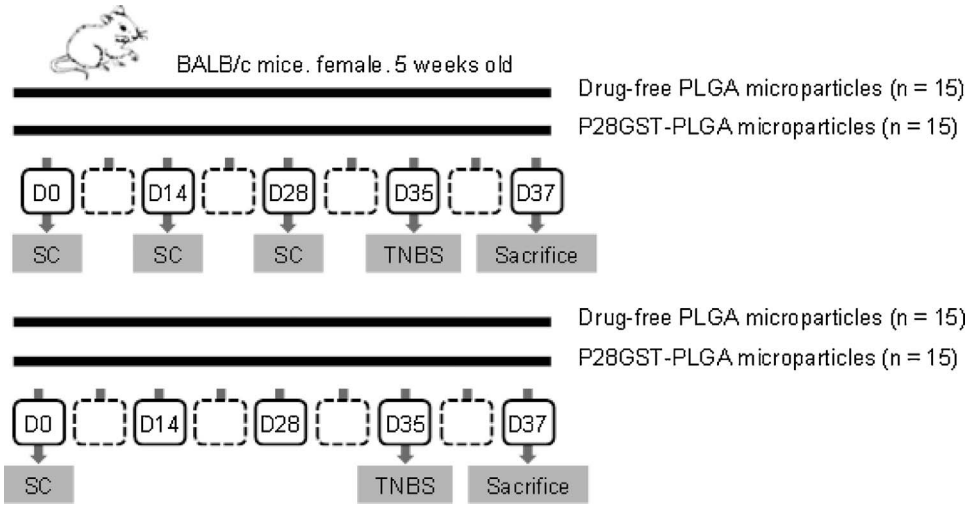

Fig. 1. Design of the in vivo study: The mice were divided into 4 groups (n = 15 in each case): (1) One group received 3 injections of drug-free PLGA microparticles: on Day 0, 14 and 28. (2) One group received 3 injections of P28GST-PLGA microparticles (5 μg/kg, 100 μL): on Day 0, 14 and 28. (3) One group received 1 injection of drug-free PLGA microparticles on Day 0. (4) One group received 1 injection of P28GST-PLGA microparticles (5 μg/kg, 100 μL) on Day 0. In all groups, colitis was induced on Day 35 using TNBS. All animals were sacrificed on Day 37.

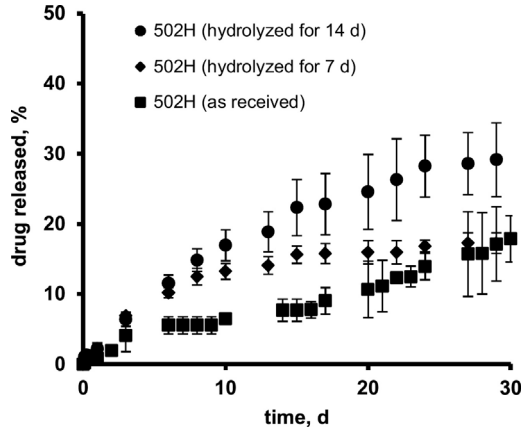

Fig. 2. Impact of the polymer molecular weight on P28GST release from PLGA microparticles prepared with a *solution of P28GST in ammonium bicarbonate buffer* (6.3% theoretical P28GST loading; volumes of the W/O/W phases: 0.846/2.82/200 mL; stirring speeds of the primary and secondary emulsion: 1500/500 rpm). The PLGA used for microparticle preparation was 502H as received, or 502H after 7 or 14 d hydrolysis in 1 mL water at 37 °C, as indicated in the diagram.

Sh28GST have been exploited to prevent intestinal inflammation in an experimental colitis model (Driss et al., 2016). Upon s.c. injection of rSh28GST in the presence of aluminum hydroxide to rats or mice, TNBS-induced colitis could be prevented, as efficiently as with infection with living parasites. This led us to the initiation of a phase 2 clinical trial in Crohn's disease patients, currently in progress (NCT 02281916), as recently reviewed (Smallwood et al., 2017).

However, aluminum compounds used as adjuvants are known for their inability to induce cell-mediated immunity (Gupta et al., 1995; Jiang et al., 2005). Furthermore, the possibility to control the rate at which P28GST is released into the living organism might allow optimizing its immunomodulating effects (Tamber et al., 2005). For these reasons, P28GST-loaded, poly (lactic-co-glycolic acid) (PLGA)-based microparticles were prepared and characterized in this study. PLGA microparticles have been reported to elicit cytotoxic T lymphocytes responses and to offer an interesting potential for mucosal immunization (e.g., Gupta and Siber, 1995; O'Hagan et al., 1998). Previous studies have shown that Sm28GST can be encapsulated in different types of biodegradable microparticles, aiming at controlled antigen release (Baras et al., 2000). A single i.p. injection was able to induce specific antibody responses during several months. Sm28GST-loaded PLGA and polycaprolactone (PCL) microparticles were also administered intranasally and orally to allow for mucosal vaccination against schistosomiasis (Baras et al., 1999). Furthermore, a liposomal formulation containing Sm28GST was proposed for oral vaccination, inducing mucosal and systemic immune response in mice (Ivanoff et al., 1996).

Importantly, PLGA-based microparticles are frequently used to control the release of active agents, and several drug products are available on the market. But despite their great practical importance, the underlying mass transport phenomena are not yet always fully understood (Siepmann and Goepferich, 2001; Siepmann and Siepmann, 2008). This can be attributed to the potential complexity of the involved physico-chemical processes, such as water penetration into the systems, drug dissolution (Siepmann and Siepmann, 2013), PLGA ester

Table 1
Processing parameters and key properties of PLGA microparticles prepared with *P28GST dissolved in ammonium bicarbonate buffer pH 8.0*.

PLGA type	Theoretical drug loading	Phase volume, mL			Stirring speed, rpm		Practical drug loading	Encapsulation efficiency	d min, μm	d mean, μm	d max, μm
		W	O	W	W/O	W/O/W					
502H	6.3%	0.846	2.82	200	1500	500	3.8%	59.6%	4.2	11.8	29.1
502H hydrolyzed for 7d[a]	6.3%	0.846	2.82	200	1500	500	3.9%	62.4%	7.35	14.3	40.0
502H hydrolyzed for 14d[a]	6.3%	0.846	2.82	200	1500	500	3.0%	48.3%	2.33	9.77	79.8
502H	6.3%	0.846	2.82	200	2000	500	2.7%	43.0%	4.7	10.2	26.5
502H	8.3%	0.846	2.82	200	2000	500	3.0%	36.5%	3.4	10.0	28.6
502H	10%	2.1	7	100	5000	1500	5.7%	57.6%	< 1	< 1	2.1
502H	10%	2.1	7	100	7500	1500	5.7%	57.3%	< 1	1.1	2.8
502H	10%	2.1	7	100	10000	1500	5.5%	55.0%	< 1	< 1	< 1
502H	20%	2.1	7	100	5000	1500	11.0%	55.0%	< 1	< 1	1.5
502H	20%	2.1	7	100	7500	1500	10.7%	53.6%	< 1	< 1	< 1
502H	20%	2.1	7	100	10000	1500	11.2%	56.2%	< 1	< 1	< 1

[a] As described in the *Materials Section*.

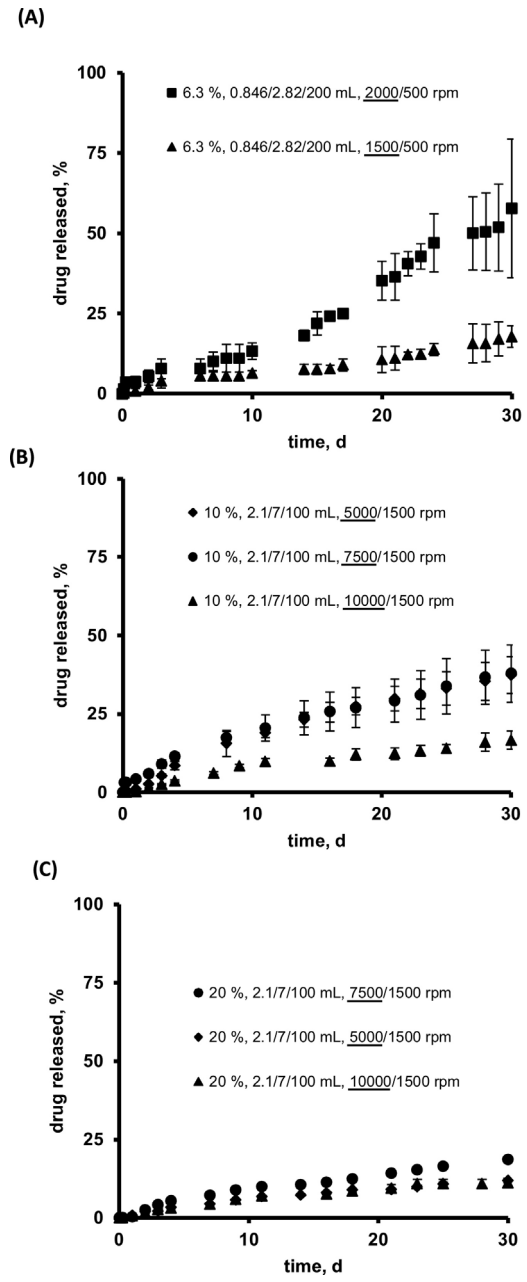

Fig. 3. Effects of the stirring speed of the primary W/O emulsion on P28GST release from PLGA microparticles prepared with a *solution of P28GST in ammonium bicarbonate buffer*: (A) 6.3% theoretical drug loading; volumes of the W/O/W phases: 0.846/2.82/200 mL; stirring speed of the secondary emulsion: 500 rpm; (B) 10% theoretical drug loading; volumes of the W/O/W phases: 2.1/7/100 mL; stirring speed of the secondary emulsion: 1500 rpm; (C) 20% theoretical drug loading; volumes of the W/O/W phases: 2.1/7/100 mL; stirring speed of the secondary emulsion: 1500 rpm. In all cases, 502H PLGA was used as received.

bond cleavage, the generation of shorter chain acids within the systems, microparticle swelling (Gasmi et al., 2015a,b, 2016; Gu et al., 2016), eventual plasticizing effects of drugs (Albertini et al., 2015), drug acylation (Shirangi et al., 2016), pore closures (Kang and Schwendeman, 2007; Huang et al., 2015), as well as the potential occurrence of autocatalytic effects (Siepmann et al., 2005; Klose et al., 2006; Fredenberg et al., 2011). The latter can result from the fact that water penetration into PLGA microparticles is much faster than the subsequent ester hydrolysis. Consequently, the systems undergo so called "bulk erosion": Polymer degradation occurs throughout the microparticles. If the generated acids are sufficiently short, they become water-soluble and diffuse out, being neutralized in the surrounding bulk fluid. In addition, bases from the surrounding environment can diffuse into the microparticles and neutralize the generated acids. However, diffusional mass transport in PLGA microparticles is relatively slow and the rate at which the acids are generated can be higher than the rate at which they are neutralized. Hence, the pH within the microparticles might significantly drop (Brunner et al., 1999; Li and Schwendeman, 2005). This is particularly true for the center of the systems, since the diffusion pathways to be overcome are the longest. As ester bond hydrolysis is catalyzed by protons, such local drops in micro-pH can lead to accelerated PLGA degradation. The importance of this type of autocatalytic effects (and their consequences for drug release) strongly depends on the systems' properties, especially the microparticle size and porosity (Siepmann et al., 2005; Klose et al., 2006).

The aim of this study was to prepare different types of PLGA-based microparticles loaded with the ShP28GST in order to control inflammatory bowel diseases. The systems were prepared using a solvent extraction/evaporation technique (W/O/W) and the impact of key formulation and processing parameters on drug release was studied. Microparticles providing about constant P28GST release during several weeks were selected for an in vivo study in mice and the therapeutic efficacy and immunomodulating effects were monitored.

2. Materials and methods

2.1. Materials

Batches of P28GST were produced and purified from recombinant *Saccharomyces cerevisiae* culture under Good Manufacturing Practice conditions by Eurogentec S.A (Seraing, Belgium). P28GST was received in the form of: (a) a solution in 10 mM ammonium bicarbonate buffer pH 8.0, and (b) a freeze-dried powder, co-processed with lactose as cryo-protectant. Poly (D,L-lactic-co-glycolic acid) (50:50) (PLGA; Resomer RG 502H) was obtained from Evonik (Darmstadt, Germany). Partially hydrolyzed PLGA 502H was prepared by placing 1 g polymer (as received) in a 2 mL Eppendorf tube, filled with 1 mL distilled water. The tube was kept at room temperature (no stirring) for 7 or 14 d (as indicated). Afterwards, the samples were freeze-dried. Poly (vinyl alcohol) (Mowiol 4–88), sodium hydroxide, bicinchoninic acid disodium, anti-mouse IgG peroxidase Ab and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma-Aldrich (Lyon, France). Dichloromethane, sodium carbonate anhydrous, disodium tartrate dihydrate, copper (II) sulfate pentahydrate and potassium phosphate dibasic were obtained from Fisher (Illkirch, France), gelatin from Merck (Darmstadt, Germany), and ammonium bicarbonate as well as potassium dihydrogen phosphate from Acros Organics (Geel, Belgium).

2.2. Microparticle preparation

PLGA-based microparticles were prepared using a water-in-oil-in-water (W/O/W) double emulsion – solvent extraction/evaporation technique. The relative amounts of raw materials and processing parameters were varied as indicated. The inner aqueous phase was either a P28GST solution in 10 mM ammonium bicarbonate buffer pH 8.0, or a solution of the lactose-containing freeze-dried enzyme powder in distilled water. The organic phase was a solution of PLGA in dichloromethane. The inner aqueous phase was emulsified into the organic phase by sonication (30 s, Sonopuls HD 2070, fitted with a Sonotrode MS72; Bandelin, Berlin, Germany). The resulting primary W/O emulsion was injected through a 21G-needle into an outer aqueous poly(vinyl alcohol) solution (5% w/w) under stirring using a 3-bladed Eurostar Power-b stirrer (Ika, Staufen, Germany) or a T25 UltraTurrax disperser equipped with a S25N-25G dispersing tool (Ika) (as indicated), to obtain a W/O/W double emulsion. After 1 min, 300 mL distilled water was added to the outer aqueous phase, and the system was further stirred during 30 min to allow for the extraction and

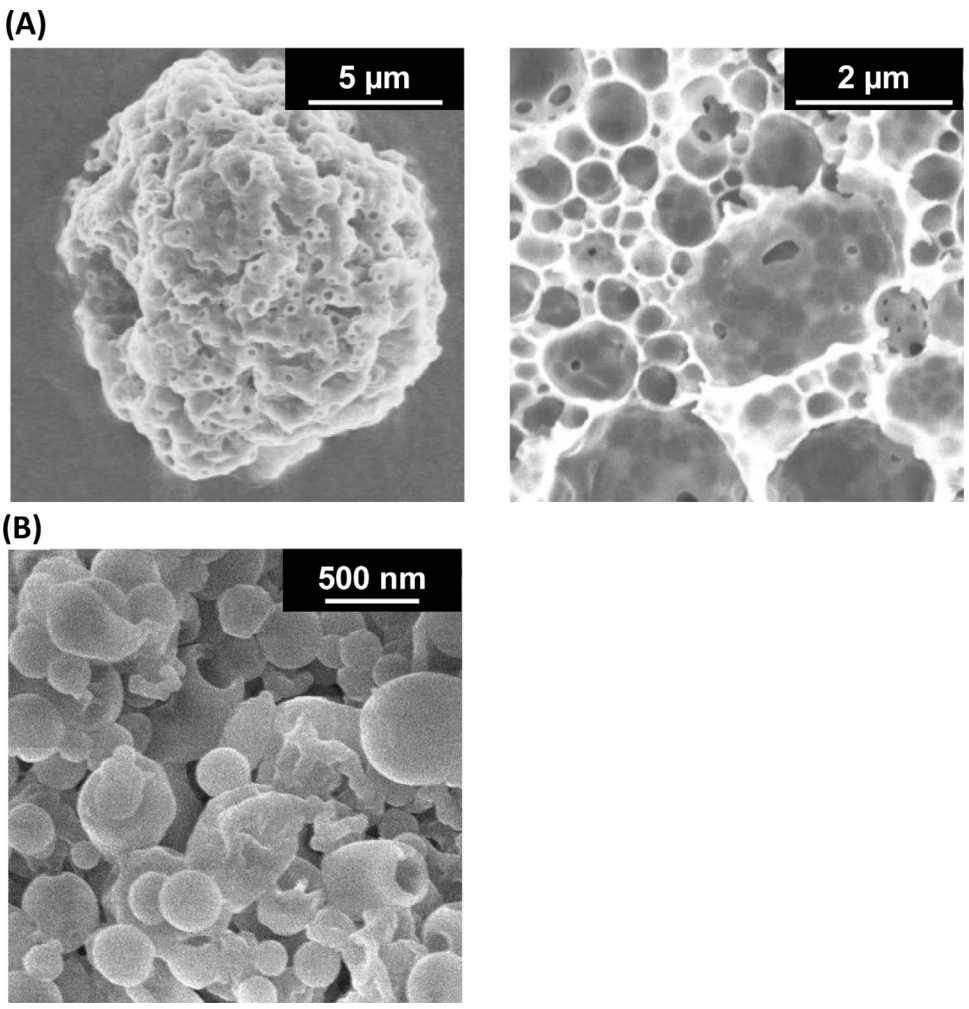

Fig. 4. SEM pictures of microparticles prepared with a *solution of P28GST in ammonium bicarbonate buffer*: (A) 8.3% theoretical drug loading; volumes of the W/O/W phases: 0.846/2.82/200 mL, stirring speeds of the primary and secondary emulsion: 2000/500 rpm (left hand side: surface, right hand side: cross-section). (B) 10% theoretical drug loading; volumes of the W/O/W phases: 2.1/7/100 mL, stirring speeds of the primary and secondary emulsion: 10000/1500 rpm (surfaces). In all cases, 502H PLGA was used as received.

evaporation of the organic solvent. The obtained microparticles were separated by filtration through a 0.2 µm-nitrocellulose membrane (Merck Millipore, Fontenay-sous-bois, France), and washed with distilled water. They were subsequently freeze-dried (Epsilon 2–4 LSC; Christ, Osterode, Germany) and stored at 4 °C. For reasons of comparison, drug-free microparticles were prepared in the same manner, but without P28GST.

2.3. Particle size measurements

Particle sizes were determined using a microscope Axioscope equipped with an AxioCam ICc1 camera and an AxioVision LE imaging system (Carl Zeiss, Jena, Germany). Each measurement included 300 particles. Minimal, maximal and mean diameters are reported.

2.4. Determination of the protein loading

Accurately measured amounts of microparticles (approximately 1 mg) were dissolved in 0.3 mL 0.2 M NaOH. PLGA hydrolysis took place at room temperature under occasional and gentle shaking during 24 h, until the samples were completely dissolved. The total protein content was determined using a bicinchoninic acid (BCA) assay as follows: Differently concentrated standard solutions of P28GST (in the range of 0.5–30 µg/mL) were prepared by diluting the protein solution, which contained 0.8 mg/mL P28GST with 0.2 M NaOH. In addition, a working solution was freshly prepared, consisting of 25 parts (v/v) of a sodium carbonate/tartrate buffer pH 11.25 (0.65 M sodium carbonate anhydrous, 0.08 M disodium tartrate dihydrate, pH adjusted using 0.2 M NaOH), 25 parts (v/v) of an aqueous solution of the disodium salt of bicinchoninic acid (4%, w/w) and 1 part (v/v) of an aqueous copper (II) sulfate pentahydrate solution (4%, w/w). One hundred µL samples were blended with 100 µL standard solution and 100 µL of the working solution in a 96 well microtiter plate. The plate was placed in an oven at 60 °C for 1 h. Upon 15 min cooling to room temperature, the absorption was measured spectrophotometrically at 562 nm (EL 405 microtiter plate reader, Bio-Tek; Cysoing, France). The limits of detection and quantification were equal to 0.63 and 1.91 µg/mL, respectively. Each experiment was conducted in triplicate. As a negative control, drug-free PLGA microparticles were treated in the same way.

2.5. P28GST release measurements

Drug release was measured as total protein release in phosphate buffer pH 7.4 (USP 35) at 37 °C. Approximately 2 mg samples were placed in 1.5 mL Eppendorf vials, filled with 0.3 mL release medium and shaken at 80 rpm (GFL 3033, Gesellschaft für Labortechnik, Burgwedel, Germany). At predetermined time points, samples were withdrawn and replaced with fresh release medium. The total protein concentration in the withdrawn samples was determined using the BCA assay described above [using phosphate buffer pH 7.4 (USP 35) instead of 0.2 M NaOH for the dilution of the standard solutions; the limits of detection and quantification were equal to 0.40 and 1.22 µg/mL, respectively].

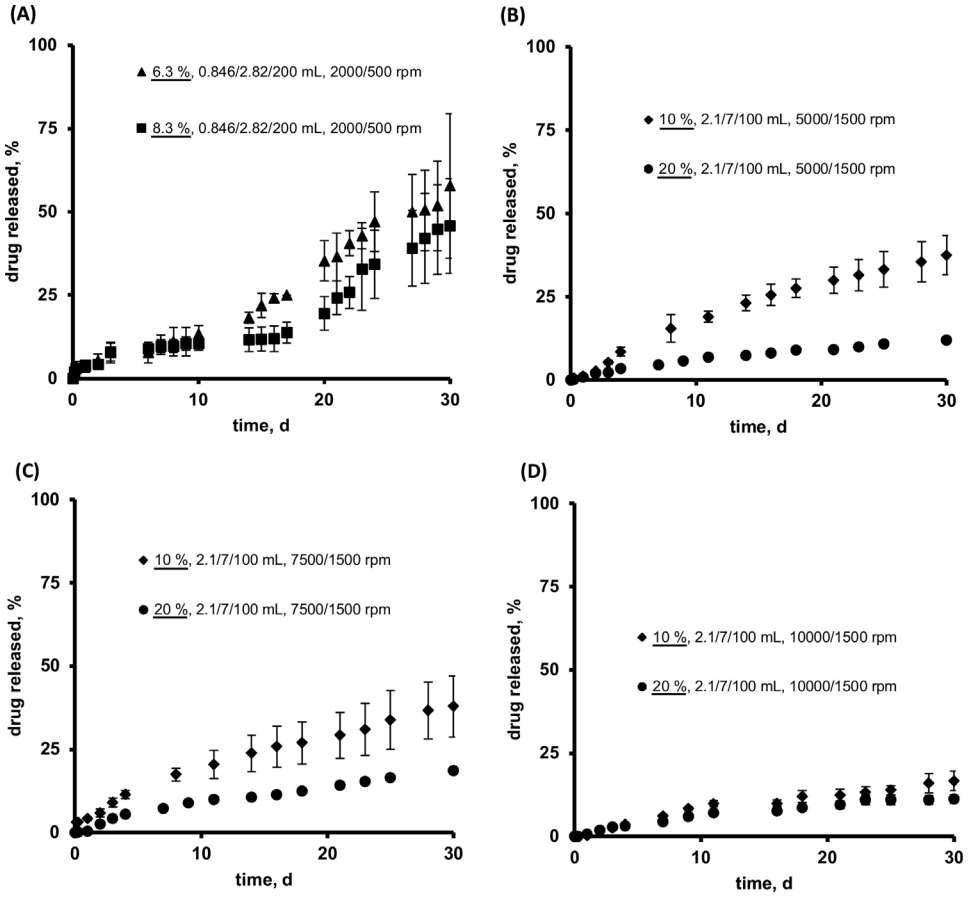

Fig. 5. Impact of the theoretical drug loading (indicated in the diagrams) on P28GST release from PLGA microparticles prepared with a *solution of P28GST in ammonium bicarbonate buffer*: (A) 6.3% versus 8.3% (volumes of the W/O/W phases: 0.846/2.82/200 mL; stirring speeds of the primary and secondary emulsion: 2000/500 rpm). (B) 10% versus 20% (volumes of the W/O/W phases: 2.1/7/100 mL; stirring speeds of the primary and secondary emulsion: 5000/1500 rpm). (C) 10% versus 20% (volumes of the W/O/W phases: 2.1/7/100 mL; stirring speeds of the primary and secondary emulsion: 7500/1500 rpm). (D) 10% versus 20% (volumes of the W/O/W phases: 2.1/7/100 mL; stirring speeds of the primary and secondary emulsion: 10000/1500 rpm). In all cases, 502H PLGA was used as received.

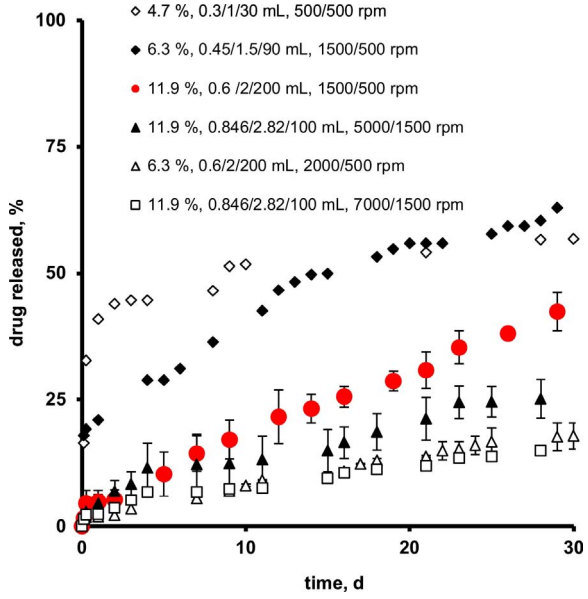

Fig. 6. Impact of formulation and processing parameters on P28GST release from PLGA microparticles prepared with an *aqueous solution of P28GST and lactose*: The theoretical drug loading, volumes of the W/O/W phases and stirring speeds of the primary and secondary emulsions are indicated in the diagram. In all cases, 502H PLGA was used as received.

2.6. Scanning electron microscopy

The morphology of the microparticles was studied using a Hitachi S4700 scanning electron microscope (Tokyo, Japan) operating at an accelerated voltage of 5 kV. Samples were placed onto a double-sided carbon adhesive tape, which was attached to an aluminum stub and sputter coated with carbon.

2.7. Animal study

An optimized P28GST-PLGA microparticle formulation was selected for in vivo evaluation (the composition and preparation procedure of these microparticles are described in the text). Female, 5 weeks old BALB/c mice (20 g, Charles Rives, Saint-Germain-Nuelles, France) were used for this study, which was conducted in the animal facilities of the Pasteur Institute in Lille (France), respecting all governmental guidelines (including n°2010/63/UE; Décret 2013-118) and ethical rules (local Animal Care Ethical Committee CEEA Agreement no. 352012).

Five animals were housed per cage and had free access to standard chow and tap water. The mice were divided into 4 groups and immunized by subcutaneous injections (in the neck region) of microparticles dispersed in an aqueous 0.9% NaCl solution, as follows (Fig. 1): (1) One group (n = 15) received 3 injections of drug-free PLGA microparticles: on Day 0, 14 and 28. (2) One group (n = 15) received 3 injections of P28GST-PLGA microparticles (5 μg/kg, 100 μL): on Day 0, 14 and 28. (3) One group (n = 15) received 1 injection of drug-free PLGA microparticles on Day 0. (4) One group (n = 15) received 1 injection of P28GST-PLGA microparticles (5 μg/kg, 100 μL) on Day 0. On Day 35, colitis was induced in all animals as follows: mice were anesthetized by a subcutaneous injection of xylazine (50 mg/kg) and ketamine (50 mg/kg) and received an intrarectal administration of 2,4,6-trinirobenzenesulfonic acid (TNBS) (150 mg/kg, 40 μL) in 50% ethanol. All animals were sacrificed on Day 37.

Macroscopic lesions of colitis were evaluated blindly by two investigators. The opened colonic segment of each mouse was examined and scored according to the Wallace criteria (Wallace et al., 1989). Briefly, the Wallace score rates macroscopic lesions on a scale from 0 to

Table 2
Processing parameters and key properties of PLGA microparticles prepared with an *aqueous solution of P28GST and lactose*. In all cases PLGA 502H was used as received.

Theoretical drug loading	Phase volume, mL			Stirring speed, rpm		Practical drug loading	Encapsulation efficiency	d min, μm	d mean, μm	d max, μm
	W	O	W	W/O	W/O/W					
4.7%	0.3	1	30	500	500	2.2%	46.7%	17.2	50.0	106.5
6.3%	0.45	1.5	90	1500	500	2.0%	31.4%	5.6	12.6	46.3
6.3%	0.6	2	200	2000	500	1.7%	27.4%	5.2	8.9	17.4
11.9%	0.6	2	200	1500	500	6.2%	52.3%	6.2	11.4	31.2
11.9%	0.846	2.82	100	5000	1500	6.6%	57.0%	3.9	15.0	22.1
11.9%	0.846	2.82	100	7000	1500	6.2%	52.3%	2.3	6.2	21.8

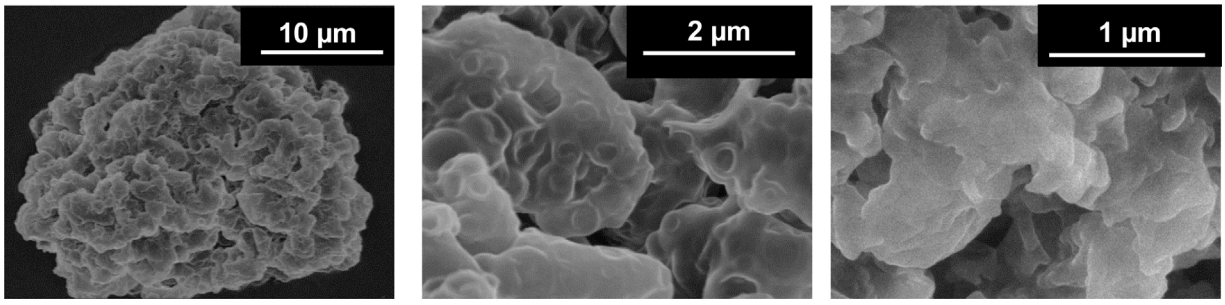

Fig. 7. SEM pictures of microparticles administered in mice prepared with an *aqueous solution of P28GST and lactose* (11.9% theoretical drug loading; volumes of the W/O/W phases: 0.6/2/200 mL; stirring speeds of the primary and secondary emulsion: 1500/500 rpm).

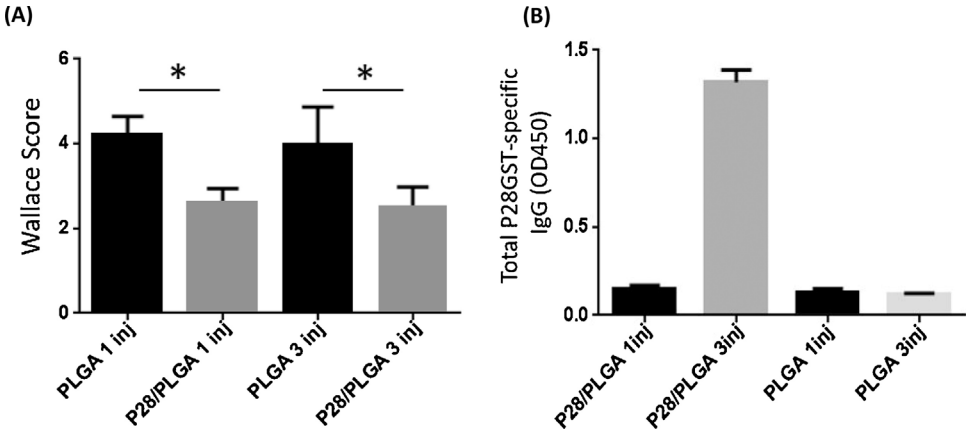

Fig. 8. In vivo performance of the P28GST-loaded PLGA microparticles prepared with an *aqueous solution of P28GST and lactose* (11.9% theoretical drug loading; volumes of the W/O/W phases: 0.6/2/200 mL; stirring speeds of the primary and secondary emulsion: 1500/500 rpm): (A) Wallace score, and (B) Anti-P28GST total IgG in the different mice groups (determined on Day 37, study design see Fig. 1; *: $p < 0.05$).

10, based on features reflecting inflammation such as hyperemia, thickening of the bowel, and the extent of ulceration. The higher the score, the more pronounced is the disease state.

Immunogenicity was assessed via specific antibody levels in individual sera, which were determined by ELISA as follows: P28GST (5 μg/mL) was coated on 96-well plates (Nunc, Roskilde, Denmark) for 2.5 h at 37 °C. The plates were blocked with phosphate-buffered saline containing 0.5% gelatin. Then, serial dilutions of individual sera (dilution for IgG 1/1; 1/10; 1/100) were added and incubated for 4 h at 37 °C. Anti-mouse IgG peroxidase Ab was incubated (2 h at 37 °C) at a 1/10000 dilution for IgG detection. Colorimetric development was performed with substrate solution (50 μL at room temperature, 20 min) and the reaction was stopped with 50 μL of Stop solution (R & D systems, Lille, France). The absorbance (OD) was measured at 450 nm. Results were defined as the highest dilution yielding an absorbance value three times above the background (wells containing buffer instead of serum in the same plate).

2.8. Statistics

The graphs and statistical analyses were generated using GraphPad Prism 5 (Graph Pad Software). Statistical significance was calculated using unpaired Mann-Whitney *U* test. All data are expressed as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results and discussion

The aim of this study was to prepare P28GST-loaded, PLGA-based microparticles in order to evaluate their potential for the prevention of experimental colitis. Triple emulsion/solvent evaporation methods were applied for the manufacturing of the microparticles, using either a P28GST solution in ammonium bicarbonate buffer pH 8.0 or a P28GST/lactose solution in water as inner aqueous phases. In each case, the impact of different formulation and processing parameters on the systems' key properties were studied. Based on the observed in vitro drug release kinetics, one type of microparticles (described in Section 3.3.) was selected for in vivo studies.

3.1. Microparticles prepared with a solution of P28GST in ammonium bicarbonate buffer

Fig. 2 shows the impact of the polymer molecular weight of the PLGA on the resulting drug release kinetics from microparticles prepared with 0.846/2.82/200 mL inner aqueous/organic/outer aqueous

phase. The stirring speeds of the primary and secondary emulsions were 1500 and 500 rpm, respectively. The theoretical P28GST loading was 6.3%. The PLGA was Resomer RG 502H as received, or Resomer RG 502H, which was partially hydrolyzed during 7 or 14 d, respectively (details are given in the *Materials Section*). As it can be seen, drug release was sustained in all cases over several weeks. The release rate increased with decreasing PLGA polymer molecular weight. This can probably be attributed to one or more of the following phenomena: (i) With decreasing chain length, the hydrophilicity of the polymer increases, driving more water into the system. The higher the water content, the higher the mobility of the water soluble drug P28GST, and the more drug can be dissolved in case of limited drug solubility effects (please note that such effects can be of importance even in the case of freely water-soluble drugs, e.g., Siepmann et al., 2017). (ii) The degree of macromolecular entanglement decreases with decreasing chain length, resulting in increased PLGA chain mobility and, thus, increased drug mobility within the polymeric matrix. (iii) The critical molecular threshold value below which the PLGA degradation products become water-soluble and leach out of the system is more rapidly reached, resulting in accelerated matrix erosion and increased matrix porosity and, hence, increased drug mobility. In contrast, the observed differences in the P28GST release rates shown in Fig. 2 are unlikely to be due to differences in the practical drug loadings (which were similar in all cases, Table 1), or differences in the microparticle size (Table 1).

The impact of the stirring speed used for the preparation of the primary O/W emulsion during microparticle manufacturing on P28GST release is illustrated in Fig. 3. Three different systems were studied. The theoretical drug loadings, volumes of the W/O/W phases and stirring speeds of the primary and secondary emulsions were varied as indicated. In all cases the matrix forming polymer was 502H PLGA (used as received). The practical drug loadings and sizes of the corresponding microparticles are given in Table 1. As it can be seen, the variation of the stirring speed of the primary emulsion partially affected the resulting P28GST release kinetics, but to different extents and in different directions. Whereas an increase in the stirring speed of the primary emulsion resulted in an increase in the drug release rate in Fig. 3A, the opposite effect was observed in Fig. 3B, and almost no impact was seen in Fig. 3C. Differences in the practical drug loadings or microparticle sizes are unlikely to fully explain these observations (Table 1). Eventually, the inner structure of the PLGA microparticles was altered by the stirring speed of the primary emulsion, but it remains unclear how this affected the drug release kinetics. It was beyond the scope of this study to investigate this aspect in more detail.

Overall, it can be seen that with increasing theoretical (and practical) drug loading and decreasing particle size, the relative drug release rate decreased (Fig. 3 and Table 1): Increasing the stirring speed of the secondary emulsion from 500 to 1500 rpm (Fig. 3A vs. C,D) resulted in a clear decrease in particle size: down to the nanometer range (Table 1). Fig. 4 shows some examples of SEM pictures of larger and smaller P28GST-loaded PLGA micro/nanoparticles. Importantly, the larger microparticles were highly porous. The observed decrease in the relative release rate with decreasing microparticle size might be attributable to a decrease in the importance of autocatalytic effects (Brunner et al., 1999; Li and Schwendeman, 2005; Siepmann et al., 2005; Klose et al., 2006). The smaller the particles, the shorter are the diffusion pathways to be overcome by acids generated within the systems in order to be released into the surrounding bulk fluid. Also, bases penetrating from the release medium into the particles (neutralizing the acids generated upon ester bond cleavage) have to overcome shorter pathways. Thus, substantial drops in the micro-pH become unlikely in very small PLGA particles and polymer degradation is not accelerated in these cases.

Furthermore, increasing the practical drug loading from roughly 3 to 11% might lead to limited drug solubility effects, even in the case of freely water-soluble compounds (Siepmann et al., 2017). It has to be pointed out that the amounts of water penetrating into these PLGA-based particles are limited, and this water is partially bound to the polymer. Thus, the entire P28GST loading might not immediately be dissolved. In these cases, dissolved and non-dissolved drug co-exist. Importantly, only dissolved drug is available for diffusion (Siepmann and Siepmann, 2012). Looking at Fig. 5 it becomes obvious that this relationship "theoretical/practical P28GST loading – drug release rate" is generally valid. The impact of varying the theoretical (and hence practical) protein content in four "systems" is illustrated. In each diagram, the process parameters (volumes of the inner aqueous/organic/outer aqueous phases and stirring speeds of the primary and secondary emulsions) were kept constant. Clearly, the overall tendency was "the higher the theoretical/practical drug loading, the slower drug release" (for the reasons discussed above).

3.2. Microparticles prepared with an aqueous solution of P28GST and lactose

In these cases, freeze-dried P28GST/lactose blends were dissolved in distilled water, and the aqueous solutions were used as inner phases in the triple emulsions for microparticle preparation. Fig. 6 shows the resulting drug release kinetics from different types of microparticles, which were prepared varying the W/O/W volumes, stirring speeds of the primary and secondary emulsions and theoretical drug loading. Table 2 shows the respective practical P28GST loadings and sizes of these microparticles. Please note that in these cases no nanoparticles were obtained. At similar practical drug loadings (1.7–2.2% on the one hand side, and 6.2–6.6% on the other hand side), a rather clear effect of the microparticle size on drug release was observed: With decreasing particle size the relative release rate decreased ("open diamonds vs. filled diamonds vs. open triangle" and "red circles vs. filled triangles vs. open squares" in Fig. 6). This can probably at least partially be explained to a decreasing importance of autocatalytic effects in these PLGA-based microparticles, as discussed above. The differences in the microparticle sizes can be explained by the differences in the stirring speeds and W/O/W phase volumes.

3.3. In vivo performance

Based on the above described key properties of the investigated P28GST-loaded PLGA microparticles (in particular the linearity of the observed release profile during several weeks), the following system was selected for in vivo studies in mice: microparticles prepared with a P28GST/lactose solution as inner aqueous phase, a theoretical protein loading of 11.9%, PLGA 502H, W/O/W phase volumes equal to 0.6/2/200 mL and stirring speeds of 1500 and 500 rpm for the primary and secondary emulsions, respectively. The red circles in Fig. 6 illustrate the in vitro P28GST release kinetics from these microparticles, and Fig. 7 shows SEM pictures of these systems (at different magnifications). The primary reason for choosing these microparticles was the about constant P28GST release rate of about 10%/week. Please note that in vivo, in many cases, drug release from PLGA-based microparticles has been reported to be faster than in vitro (e.g., Hirota et al., 2016; Doty et al., 2017).

The in vivo study design is shown in Fig. 1 (representative of 2 independent experiments). Briefly, P28GST-loaded microparticles were injected subcutaneously into mice: one single administration was compared to 3 administrations (separated by 2 weeks). On Day 35, colitis was induced using TNBS. On Day 37, all animals were sacrificed and their Wallace scores as well as anti-P28GST IgG antibody levels were determined. The Wallace score is a measure for the severity of the colitis: The macroscopic lesions are evaluated on a scale from 0 to 10 (10 indicating the worst case). For purposes of comparison, also P28GST-*free* PLGA microparticles were administered (1 and 3 times, like the drug-loaded systems, Fig. 1). As shown in Fig. 8A, the Wallace scores determined on Day 37 were significantly lower in mice treated with the P28GST-loaded microparticles compared to the control groups,

suggesting that the injection of P28GST PLGA microparticles induced a reduction of colitis severity. Interestingly, this significant effect was obtained as soon as after 1 single injection of P28GST microparticles, with no obvious difference when 3 injections were performed (it has to be said that the protocol is based on an immunization procedure, which usually is not strictly dose-dependent). As immunogenicity markers, increased anti-P28GST IgG levels were detected in mice after three P28GST PLGA microparticle injections, but not in drug free control groups (Fig. 8B). The presence of anti-P28GST IgG antibodies in serum of mice immunized with P28GST PLGA microparticles but not in control sera, indicated that P28GST encapsulated into PLGA microparticles kept its immunogenic properties. However, 3 injections were needed to obtain elevated IgG levels, suggesting that specific anti-P28GST IgG antibodies are not directly linked to the anti-inflammatory effect. Further studies are now needed in order to investigate the mode of action of P28GST PLGA microparticles, and in particular their effects on the regulation of inflammatory cytokines. In any case, P28GST-loaded PLGA microparticles offer an interesting potential for the prevention of colitis.

4. Conclusion

This study showed the promising potential of using P28GST-loaded PLGA-based microparticles for a new preventive treatment to control inflammation in colitis. This type of advanced drug delivery systems might help re-regulating the inappropriate activation of the mucosal immune system in chronic inflammatory bowel diseases using the “benefits” of appropriate helminth derived molecules.

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