In vivo evaluation of post-operative pain reduction on rat model after implantation of intraperitoneal PET meshes functionalised with cyclodextrins and loaded with ropivacaine

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Abstract (200 words)

The avoidance of post-herniorrhaphy pain can be challenging for hernia repair and has the greatest impact on patient's quality of life, health care utilisation and cost to society. Visceral meshes, functionalised with an efficient drug carrier system – hydroxypropyl beta-cyclodextrin polymer (polyHPβCD) coating, were developed to give a prolonged intraperitoneal analgesic drug release. We attempted to evaluate the *in vivo* pain-relief efficacy of ropivacaine loaded polyHPβCD functionalised polyester meshes in a rat model of visceral pain induced by colorectal distension (CRD). *In vivo* safety, pharmacokinetic profile and biodegradation were measured via histological analysis and high-performance liquid chromatography, *etc*. The results confirmed that the polyHPβCD on the functionalised meshes has a high adsorption capacity of ropivacaine and resulted in a sustained drug release in rats after mesh implantation. This was further reaffirmed by an elevated pain threshold (30%) up to 4 days after implantation in the rat CRD model, compared to 1-2 days for non-adapted meshes. Neither polyHPβCD nor the loaded ropivacaine had a major impact on the inflammatory response. This evidence strongly suggests that polyHPβCD functionalised visceral mesh could be a promising approach for post-operative pain control by improving the intraperitoneal drug delivery and bioavailability.

Keywords:

Post-operative pain; Visceral mesh; Cyclodextrin polymer; ropivacaine ; Colorectal distension, drug delivery

1 Introduction

Hernia repair is one of the most commonly performed surgical procedures in the world, with around 800,000 operations/year in USA and 160,000 operations/year in France [1-3]. Modern advances in hernia repair are credited with reduced recurrence rate; thus, surgeons' attention is shifted from preventing recurrence to the new topic of post-operative pain. It is reported that around 12.3% of patients feel persistent pain 3 months after surgery (post-herniorrhaphy pain syndrome) [4]. Though several pre- or per-operative risk factors [5-7] have been identified that generate pain after hernia repair, the exact cause is unclear, and a lack of an evidence-based treatment path presents a significant issue in the effective management of this surgical complication, representing a genuine socio-economic challenge in industrialised countries considering its secondary complications such as depression or sexual dysfunction [5,8].

Even if the effectiveness of the surgical technique (use of laparoscopic surgery instead of open surgery) for post-operative pain control has long been proven, the clinical reality is, unfortunately, still far from satisfactory [9]. The use of medication [10] is also promoted to relieve the pain. Besides non-steroidal anti-inflammatory drugs (NSAIDs), paracetamol and anti-epileptics molecules, anaesthetic techniques also have a major influence on the quality of pain control. Local anaesthesia injection in combination with parenteral administration of NSAIDs has been reported to improve postoperative analgesia [11], and thus, is very commonly used. Ropivacaine is the most often used local anaesthetic agent in hernia surgery. It has a higher toxic threshold than other long-acting local anaesthetics and its half-life of elimination after intravenous injection is 1.8 hours with an active duration from 2.5 to 3.5 hours. Different means of local anaesthetic drug (bupivacaine, ropivacaine or ketoprofen) delivery around the surgical site were reported, such as infiltration techniques (an elastomeric pump or a multi-perforated catheter) [12-14] and polymeric materials (polycaprolactone / polylactic-co-glycolic acid [15] or polysebacic-co-ricinoleic acid material [16]) based drug release systems, and the best among them showed a significant effect on managing post-operative pain with *in vitro* drug release of up to 14 days.

Cyclodextrins (CDs) are torus-shaped cyclic oligosaccharides derived from the enzymatic degradation of starch. The CD family is composed of three main types of "native" cyclodextrins made of six (α-cyclodextrin), seven (β-cyclodextrin) or eight (γ-cyclodextrin) anhydroglucose units (AGU). Furthermore, CD's derivatives can be created by substituting the hydroxyl groups of AGU of native cyclodextrins with a wide range of chemical groups. On the internal face of the molecular torus, the inner cavity presents a hydrophobic side and gives CDs the ability to form inclusion complexes with a wide range of lipophilic molecules whose water solubility can significantly increase. The inclusion of a guest molecule is not only determined by the cavity dimensions, but also by the nature of the substituents in CD's derivatives. For example, 2-

Hydroxypropyl-β-cyclodextrin (HPβCD) has higher solubility in water than pristine β-CD and displays enhanced solubilising power toward ropivacaine [17].

In the past 15 years, our team has been working on the use of cyclodextrins polymers (polyCDs) crosslinked with citric acid as efficient drug release systems (DDS) [18, 19]. With proved *in vitro* and *in vivo* safety [20, 21], we reported that these polyCDs could be firmly coated on diverse biomaterials, e.g. woven/knitted polyester vascular prostheses [22], nonwoven polypropylene and polyester meshes [23, 24], cellulose membranes [25], polyamide structures [26], porous silicon wafers [27], metallic stents [28] and bio-ceramics [29, 30], for further loading / delivering different drugs (such as antibiotics, antiseptics, anticancer and antimitotic molecules). More recently, different visceral meshes based on resorbable polylactic acid on the one hand and on polyethylene terephthalate on the other hand have been modified with polyHPβCD and displayed high adsorption capacities and *in vitro* prolonged release properties toward ciprofloxacin and ropivacaine respectively [24, 31].

The development of devices with drug-delivery properties mostly relies on *in vitro* evaluation of release performance. It is important to highlight that even though several *in vitro* tests have been performed, there is no *in vitro* standard technique available for simulating *in vivo* conditions and avoiding non-physiological turbulence. However, when *in vivo* tests were performed, the choice of the experimental model was still an important but difficult task in a well-designed experiment. Various models including rats and mice have been reported for evaluating the analgesic activity [24]. Among these commonly used methods, the Randall-Selitto test [32], intended to serve as a tool to assess the effect of analgesic agents on the response thresholds to mechanical pressure stimulation, has been used by a number of investigators to evaluate painful inflammatory responses [33-36]. Based on the Randall-Selitto principle, an electronic device has been further developed for quantitatively testing pain by pressuring different areas of the animal body. Subsequently, a specific test for visceral pain, inherent in hernia repair, was developed by Ness *et al* [37], in which a rectal balloon in rat was inflated progressively with air and a contraction of the abdomen, indicating a pain reaction, appeared when reaching the pain threshold. More recently, Rousseaux et *al* [38, 39] applied this colorectal distension (CRD) test on rats to evaluate the efficacy of abdominal pain treatment in healthy rats and in rat models of irritable bowel syndrome.

In such a context, we attempted to evaluate the *in vivo* pain-relief efficacy of our anaesthetic (ropivacaine) loaded polyHPβCD functionalised polyester visceral meshes in the above-mentioned rat CRD model. At the same time, *in vivo* tissue response, pharmacokinetics profile and biodegradation behaviour were investigated using histological analysis, high-performance liquid chromatography (HPLC) and toluidine blue staining, respectively.

2 Materials and methods

2.1 Materials and reagents

The visceral meshes used in this study are knitted multi-filament polyethylene terephthalate (PET) meshes (Biomesh®), "A1L" (PCTA1LIGHT, 38g/m²), manufactured by Cousin Biotech (Wervicq-Sud, France). Hydroxypropyl-β-cyclodextrin (HPβCD, Kleptose HP, MS = 0.62) was purchased from Roquette Frères (Lestrem, France). Citric acid, sodium dihydrogen hypophosphite (NaH_2PO_4), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH) and ropivacaine hydrochloride monohydrate powder were purchased from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France). All solutions used throughout the experiments were prepared freshly in ultrapure water obtained from a MilliQ - Ultrapure water system (Millipore®, France).

Ropivacaine hydrochloride 2mg/mL (Naropan®) and 10mg/mL (Kabi®) solutions for infusion were purchased from AstraZeneca (London, UK) and Kabi (Bad Homburg, Germany) respectively.

2.2 Functionalisation of visceral meshes with polyHPβCD

The functionalisation of A1L mesh with polyHPβCD was based on a pad/dry/cure textile finishing process as previously reported [19, 24]. First, A1L meshes (15 * 5cm) were thoroughly washed by soxhlet extraction with diethyl ether (24 cycles, 1 hour per cycle), according to the process approved by the manufacturer (Cousin Biotech), in order to eliminate lubricants and other residual compounds from the manufacturing process. The textile samples were dried at 104°C for 1 hr and desiccated for 30 min. The A1L meshes were impregnated and roll-squeezed (2 m/min, 2 bars, Laboratory Padder LDP, LAB-PRO, Wikon, Switzerland) in an aqueous solution containing three compounds (ratio denoted as X/Y/Z): citric acid (CTR, X g/100 mL) as a crosslinking agent, sodium dihydrogen hypophosphite (NaH_2PO_2, Y g/100 mL) as a catalyst, and hydroxypropyl-β-cyclodextrin (HPβCD, Z g/L). In order to set up a series of meshes, A1L meshes were impregnated in reactant's solutions with ratios of 80/10/100, 120/15/150 and 160/20/200. The resulting functionalised meshes were named as "A1L-CD180", "A1L-CD270" and "A1L-CD360" respectively, with the number characters representing the weights of citric acid plus HPβCD dissolved in one litre of impregnating solutions.

Impregnated meshes in CTR/NaH_2PO_2/HPβCD solutions mentioned above were dried in an oven (Model FD, BINDER, Tuttlingen, Germany) at 90°C for 5 min and then cured at 140°C for 120 min, in order to firmly coat the PET fibres by a HPβCD polymer crosslinked with citric acid (polyHPβCD). After a rapid wash (twice 3 min in ultrapure water), the samples were soaked in a sodium carbonate solution (4g/L) in order to neutralise the residual acidity from polyHPβCD. They were then rinsed again twice rapidly in water as above, the meshes were

further thoroughly washed in ultrapure water at 80°C (FISONS C1, Thermo Scientific Haake, Karlsruhe, Germany) for 20 min. Finally, the functionalised meshes were dried at 104°C for 1 hr and cooled in a desiccator for 30 min into finished material. The degree of functionalisation (DF%) of treated meshed was reported as their weight gain according to:

$$DF = \frac{m_f - m_i}{m_i} \times 100$$

where m_i and m_f correspond respectively to the sample weight before and after treatment, measured with a precision balance (± $4 \cdot 10^{-4}$ g, Kern).

The degree of functionalisation of the treated meshed were 13.7 +/- 3.6%wt; 17.4 +/-1.0 %wt and 27.7 +/- 3.0 %wt for AL1-CD180, AL1-CD270 and AL1-CD360 respectively. The virgin and functionalised meshes were then cut into rectangular (1.5 × 1.5cm) or disk (∅ 11 mm) samples according to the requirement of different experiments. Ethylene oxide (EtO) sterilisation of samples was conducted according to the production process of Cousin Biotech. The characterisation of functionalised meshes was reported in our previous paper [24].

2.3 Ropivacaine loading on meshes

EtO sterilised disk samples (∅ 11mm, n=3) of functionalised meshes (A1L-180, A1L-270 and A1L-CD360) were soaked in the ropivacaine hydrochloride solutions (2mg/mL or 10 mg/mL) in the ratio of “6 disks / 20mL ropivacaine solution” at different times (from 5 to 60 min), then dipped in 10mL ultrapure water for 1 min to remove the non-specific adsorbed drug. The specific adsorbed drug was "desorbed" from the meshes by soaking the mesh in 5mL of 0.1 M sodium hydroxide solution (4 hr, 37°C), which leads to a complete hydrolysis of the polyHPβCD coating, thereby ensuring complete liberation of ropivacaine from the samples in the supernatant. The amount of desorbed ropivacaine in 10mL water and 5mL of 0.1 N NaOH was determined using an HPLC method. Ropivacaine was analysed by high-performance liquid chromatography (LC2010A-HT, Shimadzu, Noisiel, France) equipped with ultraviolet-visible detector and PC Dell integrator/recorder with LabSolutions software. The chromatograph was fitted with a Gemini-NX C18 column (5µm, 250 × 4.6mm, Phenomenex, Le Pecq, France). The mobile phase consisted of a mixture of 72% phosphate buffer (pH 2.5) and 28% acetonitrile. The chromatographic separation was performed at 25°C with a flow rate of 1mL/min and monitored at 201nm [40, 41]. Approximate retention time for ropivacaine at 210 nm was 5.5 min under these conditions.

To assess the homogeneity and reproducibility of the drug loading process, disk samples (∅ 11 mm) were cut from four corners and the centre of a rectangular band (15 × 3 cm) of functionalised mesh (A1L-CD360, n=3). Disk samples were soaked in a ropivacaine 10mg/mL

solution for 5 min and successively soaked in ultrapure water and in a 0.1 N NaOH solution as described above. The extracted amount of ropivacaine was analysed by HPLC as described above.

2.4 Rat model for visceral pain assessment

The visceral pain was monitored in the rats, who received the controlled isobaric colorectal distension (CRD) with a balloon introduced and inflated in the colon. The animal nociception was estimated by measuring the intra-colonic pressure required to induce a certain behavioural response.

All animal experiments were performed by the laboratory of Intestinal Biotech Development (IBD biotech, Lille) in an accredited facility at the Institute Pasteur of Lille, respecting the European directive (2010/63/UE), governmental guidelines (articles R214-87 to R214-137 rural code update 13 February 2013) and those of the regional Ethical Committee for Animal Use (N° B59-35009).

2.4.1 Animals

Male Sprague Dawley rats weighing around 200g (Charles River Laboratories, L'Abresle, France) were housed 3 per cage under optimum light, temperature and humidity conditions (12-hr light/12-hr dark cycle, 22 ± 2°C, under 45 ± 15% humidity). The rats were allowed to acclimatise for at least 7 days before starting the experiments and received water and food *ad libitum*. Great care was taken with regard to living conditions in order to avoid or minimise discomfort of the animals. All studies were performed in accordance with the proposal of the committee for Research and Ethical Issues of the International Association for the Study of Pain [42].

2.4.1 Pharmacokinetic study

Two groups of 10 rats which were implanted with virgin meshes (1.5 × 1.5 cm) with an intra-peritoneal (i.p.) injection of 1mg ropivacaine (A1L+ropi) and functionalised meshes (1.5 × 1.5 cm) loaded with 1mg ropivacaine (A1L-CD360+ropi) respectively, were examined for pharmacokinetics of ropivacaine in rat blood within 24-hr implantation. In short, blood samples were drawn under anaesthesia from the femoral artery after mesh implantation: every 10 min during the first half hour and at hour intervals during 6 hr. The blood samples were immediately centrifuged to collect plasma samples, which were then stored at -80°C until analysis. Ropivacaine was extracted from plasma samples (140 µL) with a 90:10 v/v heptane-ethyl acetate admixture (300 µL) after adding a bupivacaine 20 µg/mL solution (50 µL) as internal standard and 0.5 N NaOH (5 µL). After evaporation (Stuart SBHCONC/1, Roissy, France) with liquid nitrogen (Nitrogen 4.5, Linde, Porcheville, France) at 40°C, the extracted ropivacaine

was reconstituted in 140 µL of methanol/water (50:50 v/v) and analysed by HPLC as described in the above drug sorption study.

2.4.3 Colorectal distension experiments

Animal nociception was assessed by measuring the intracolonic pressure required to induce a behavioural response during colorectal distension (CRD) due to the inflation of a balloon introduced in the colon. This response is characterised by an elevation of the hind part of the animal body and clearly visible abdominal contraction corresponding to the severe contractions [43, 44]. The rats were anesthetised for a short period with volatile anaesthesia (2% isoflurane), the balloon (prepared as previously described [44]) was inserted intrarectally in a minimally invasive manner to 7cm from the anus, and the catheter was taped to the base of the tail. After 5 minutes, the rats were placed in the middle of a 40x40cm Plexiglas box and the catheter was connected to an electronic barostat apparatus (Distender Series IIR™, G& J Electronics). Increasing pressure was continuously applied until pain behaviour was displayed or a cut-off pressure of 80 mm Hg was reached. “pain reflex” responses were characterised by specific behaviours of the rats such as an elevation of the back part of the animal and a clearly visible abdominal contraction corresponding to severe contractions (as described by Bourdu *et al* [45])

2.4.4 Measurement of the analgesic effect of ropivacaine administered by injection

A proof-of-concept study of the efficacy of ropivacaine and determination of the optimal dosage on visceral hypersensitivity was performed in the above-mentioned CRD model by intraperitoneal (i.p). injection of ropivacaine.

Ropivacaine was i.p. injected into 10 rats with declining doses (1.5; 1.0; 0.5 and 0.2mg / rat / day) by bolus with a wash-out period of five days between each dose. Another group of 10 rats received the vehicle injection under the same regime as control. 30 min after drug injection, the analgesic effect on visceral pain was evaluated by CRD method as described above. The change in body weight was also monitored as well as their consumption of food and water.

2.4.5 Measurement of analgesic effect of ropivacaine loaded visceral meshes

The analgesic effect of ropivacaine loaded virgin or functionalised visceral meshes (1.5 × 1.5cm) was further investigated by CRD method. EtO sterilised meshes were soaked in a 10mg/mL ropivacaine solution for 5 min before implantation. The shape and size of mesh implants for the rat were chosen according to the ratio of meshes / abdomen surface in a human patient. Four groups of 10 rats including: virgin meshes (A1L), virgin meshes plus i.p. injection of 1mg ropivacaine (A1L+ropi), maximum functionalised meshes (A1L-CD360), maximum functionalised meshes loaded with ropivacaine by soaking process (A1L-CD360+ropi), were studied in order to determine their pain threshold under the experimental

regime. To avoid excessive discomfort to the rat, only half of each group of rats received the CRD each day and were allowed 24-hr recovery before receiving the next CRD intervention.

2.4.6 Histological analysis

The animals (4 groups of 10 rats) from the above CRD study were sacrificed 8 days after the implantation. All mesh implants were carefully explanted for the biodegradation study, and different tissue specimens surrounding the implant were explanted, fixed in 10% buffered formalin and embedded in paraffin. Tissue pieces were sectioned into 4 μm thick sections and stained with haematoxylin & eosin (H&E). Qualitative and semi-quantitative analysis of various parameters, concerning fibroplasias/fibrosis, neovascularisation, oedema, presence of neutrophils, lymphocytes, plasma cells, macrophages and giant cells, were carried out applying a 0 - 5 scoring system (0 for absence and 5 for very severe). The presence of textile fibres has also been reported during analysis.

2.4.7 *In vivo* degradation of polyHPβCD coating

The animals from the above pharmacokinetic study and visceral pain study were sacrificed after 24 hours and 8 days respectively and the visceral mesh implants were explanted. The explant's mesh was soaked in cold 0.25% trypsin/EDTA solution at 4°C for 6 hr in order to digest the tissue adherent to the mesh. The amount of polyHPβCD remaining on the functionalised meshes was then semi-quantitatively assessed by toluidine blue ortho (TBO) staining method, which was measured at 634nm with a spectrophotometer as described by Taha *et al* [30]. As the cationic TBO dye can interact with both COOH groups (present in the CTR crosslinker) and HPβCD (inclusion complex), the amount of adsorbed TBO was found strictly proportional to that of polyHPβCD [30].

2.5 Statistics

GraphPad Prism 5.03.0001 was used for statistical analysis. Results are reported as mean ± SD for each experimental group. Since the data of CRD assays were not normally distributed (D'Agostino and Pearson omnibus normality test), nonparametric analysis using Kruskal–Wallis ANOVA for pairwise comparisons was undertaken followed by Bonferroni correction for assessment of differences between time points for the various meshes. Pharmacokinetic parameters (C_{max}, T_{max}, and AUC) and homogeneity evaluation were compared with a two-side non-parametric Mann-Whitney U test. The $p<0.05$ was considered statistically significant.

3 Results

3.1 Drug sorption

3.1.1 Kinetics of ropivacaine sorption on meshes

Figure 1 clearly shows the effect of the concentration of soaking solution (2mg/mL or 10mg/mL ropivacaine) and soaking time (from 5 to 60 min) on the amount of ropivacaine sorption to different functionalised visceral meshes (A1L-CD180, A1L-CD270 and A1L-CD360) by successive extraction in ultrapure water and in 0.1 N NaOH. The water-extracted fraction of ropivacaine corresponds to the non-specific ropivacaine sorption to textile by capillary forces, while the NaOH-extracted ropivacaine fraction corresponds to specific ropivacaine sorption through i) ionic interactions between protonated amino groups of ropivacaine and carboxylate groups of polyHPβCD, ii) inclusion complexation in HPβCD cavities and iii) hydrogen bonding with the hydroxyl groups of HPβCD polymer network. It was found that, regardless of the concentration of soaking solution, the amount of non-specific adsorbed ropivacaine (released in water bath) was much higher than that of specific absorption (released in NaOH bath). Such phenomenon has already been observed for other drugs (antibiotics) loaded on other cyclodextrin coated biomaterials [46]. It was also noticed that standard deviation of water-extraction data is much higher than that of NaOH extraction, which is due to the non-controllable variance of ropivacaine solution absorption volume on textile when the sample is taken off from the soaking solution. Consequently, the degree of functionalisation shows no significant effect ($p>0.05$) on the water-extraction amount of drug. However, the influence of the soaking solution concentration was indeed found on the amount of water-extracted ropivacaine ($p<0.05$), which grew with the increased soaking solution concentration, *i.e.* 9.0 ± 2.5 mg/g for soaking in a ropivacaine 2mg/mL solution and 53.3 ± 12.4 mg/g for that in 10mg/mL solution.

Regarding the amount of extracted drug in NaOH, it was found to be affected not only by the concentration of ropivacaine soaking solution, but also by the DF% of the meshes. The amount of specific drug adsorption, very logically, increased with the increasing amount of polyHPβCD immobilised on the meshes per surface unit ($p<0.05$): in the case of the 60-min soak in a ropivacaine 2mg/mL solution, *i.e.* 5.1 ($\pm$ 0.1); 7.2 ($\pm$ 0.5) and 9.4 ($\pm$ 0.8) mg/g drug for functionalised meshes A1L-CD180, A1L-CD270 and A1L-CD360 respectively. This is also consistent with our previous studies of polyHPβCD functionalisation of other medical devices [47]. Similarly, in the case of the 60-min soak in a ropivacaine 10mg/mL solution, the specific drug adsorption amount also increased in similar way ($p<0.05$), *i.e.* 18.8 ± 0.1; 23.1 ± 1.1 and 33.9 ± 1.4mg/g ropivacaine for functionalised meshes A1L-CD180, A1L-CD270 and A1L-CD360 respectively.

Concerning the sorption kinetics, whatever the soaking solution concentration, the specific adsorption isotherms very quickly (generally 5 to 15min soaking) reached a plateau (saturation of the adsorption sites).

Thus, the A1L-CD360 meshes (1.5 × 1.5 cm, 38 g/m²), which were coated with the maximum amount of polyHPβCD and had a total amount of 1mg ropivacaine sorption capacity (by 5-min soak in a ropivacaine 10mg/mL solution), will be the chosen functionalised meshes in the following *in vivo* investigation. This amount of ropivacaine (1mg) loaded on A1L-CD360 mesh implant will be taken into account for defining the control group (A1L).

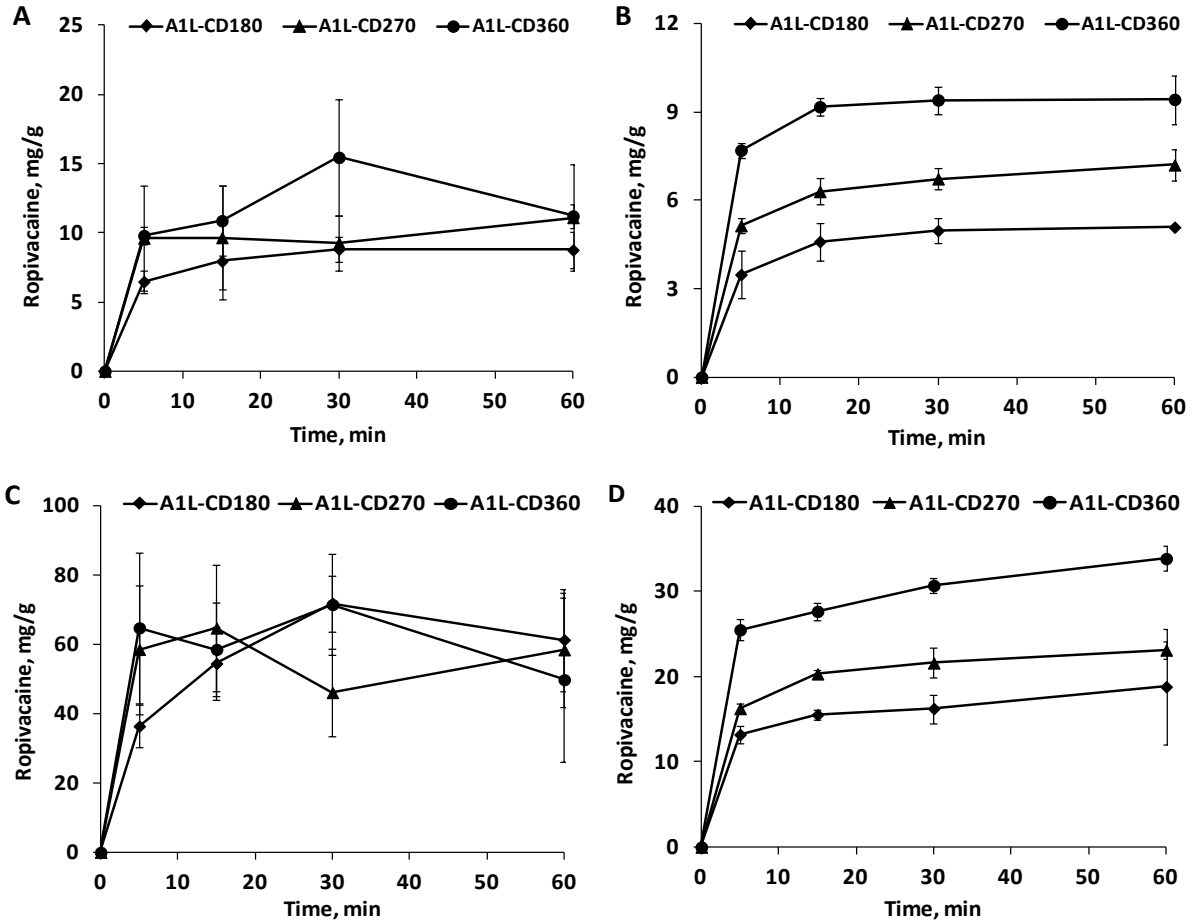

Figure 1: Determination of effect of the concentration of soaking solution (2mg/mL or 10 mg/mL ropivacaine) and soaking time (from 5 to 60 min) on the amount of ropivacaine adsorption to different functionalised visceral meshes (A1L-CD180, A1L-CD270 and A1L-CD360, n=3) by successive extraction in ultrapure water and in 0.1 N NaOH. A: ropivacaine 2mg/mL soak / water extraction; B: ropivacaine 2mg/mL soak / NaOH extraction; C: ropivacaine 10mg/mL soak / water extraction; D: ropivacaine 10mg/mL soak / NaOH extraction.

3.1.2 Homogeneity of the ropivacaine sorption on meshes

The quantification of the adsorbed drug on the cut disk pieces from different places on the rectangular functionalised meshes A1L-CD360 after 5-min soak in 10mg/L ropivacaine solution was carried out in order to determine the homogeneity of DF% at the scale of the whole sample.

As reported in Figure 2, no significant difference could be observed, neither for water extracted fraction (p<0.05) nor for NaOH extracted fraction (p<0.001) in terms of ropivacaine sorption among sample aliquots cut off at different places on the functionalised meshes. This result is also consistent with the results shown above (Figure 1C, D) under the same conditions. Thus, 5-min soaking in 10mg/L ropivacaine solution is a reproducible drug loading condition for A1L-CD360 mesh to have homogeneous drug adsorption.

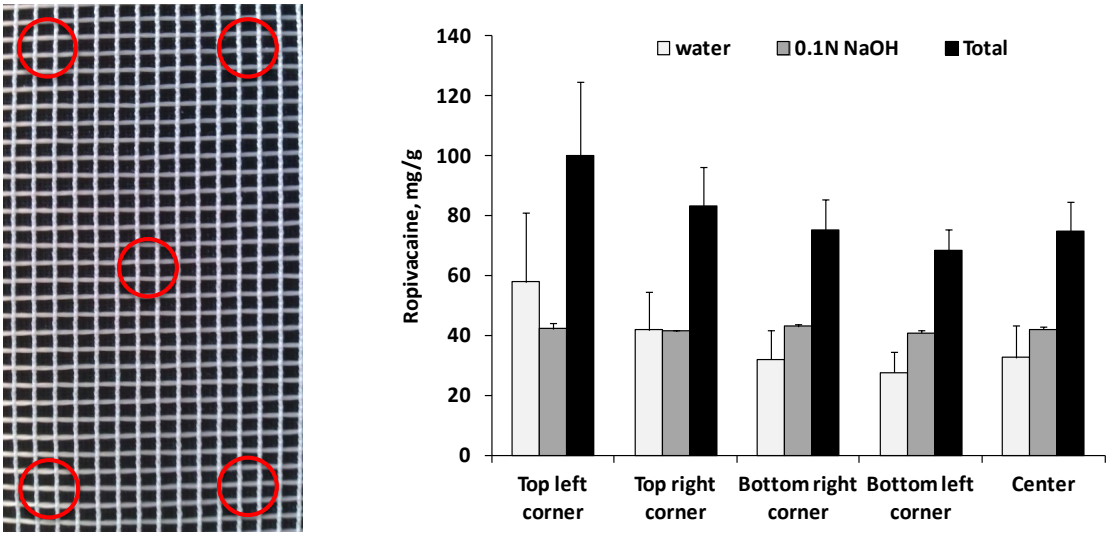

Figure 2: Amount of extracted ropivacaine fraction in ultrapure water and in 0.1 N NaOH from cut sample pieces (left) of each rectangular A1L-CD360 meshes (n=3) after 5-min soak in a ropivacaine 10mg/mL solution. Statistical analysis with Mann-Whitney U test

3.2 *In vivo* study of analgesic effect

3.2.1 Pharmacokinetics

The plasma concentration time profiles of ropivacaine after implantation of the drug-loaded functionalised meshes (A1L360+ropi) or virgin meshes (A1L+ropi) in rats are shown in Figure 3. The average maximum plasma concentration (C_{max}) of ropivacaine for A1L-360+ropi (0.60 ± 0.29mg/L) was 4-fold (p<0.05) that for A1L+ropi (0.16 ± 0.05mg/L). In addition, the time (T_{max}), at which the C_{max} is observed, was also significantly different (p<0.05): 57 ± 29 min for A1L+ropi *vs.* 93 ± 21 min for A1L-CD360+ropi. Compared with the drug-loaded raw material, the relatively slower T_{max} of drug-loaded functionalised meshes pro ved a significant slow-release behaviour in the plasma, which is consistent with the results of *in vitro* release study previously described by Vermet *et al* [24]. Finally, a significant difference is observed on area under curve (AUC) between both groups: 24.4 ± 11.1 *vs.* 86.9 ± 32.9mg·min/L, for A1L+ropi and A1L-CD360+ropi, respectively (p<0.05). Under the context of the same amount of ropivacaine loading, the drug release of A1L-CD360+ropi showed a distinct rapid strong release during the first 2 hours and then, also decreased rapidly with time;

whereas the drug release of A1L+ropi appeared generally slower and weaker throughout the whole experiment duration. It indicates that the polyHPβCD, as a drug carrier on the functionalised meshes, provides a sustained drug release profile in the rat and may also further improve the intraperitoneal bioavailability of ropivacaine.

The diffusion of local anaesthetics in tissue and their distribution were also analysed from the 1-day sacrificed animals by means of drug extraction of explanted implant and the surrounding tissue, but all samples showed no detectable ropivacaine (results not shown here).

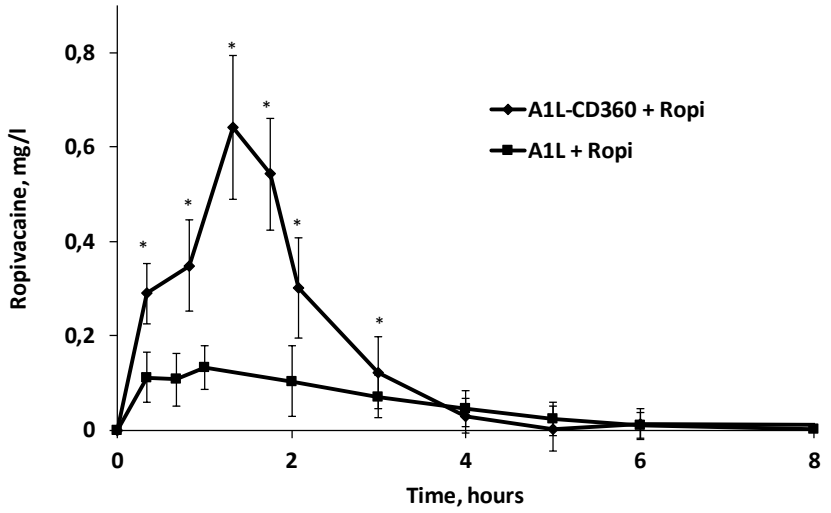

Figure 3: Mean plasma concentration time profiles of ropivacaine after implantation of drug-loaded functionalised meshes (A1L-CD360+ropi) or virgin meshes (A1L+ropi) in rats (n=10). Statistical analysis with Mann-Whitney U test (p < 0.05).*

3.2.2 Effect of *i.p.* injection of ropivacaine on pain

The analgesic effect proof of concept and the minimum effective dose by i.p. ropivacaine injection were determined on the rat CRD model. Figure 4 showed that i.p. injections of ropivacaine generate an increased pain threshold to CRD in all rats' dose-dependently. In detail, a significant pain threshold elevation of 50% (p<0.05) to CRD was found in rats by i.p. injecting 1.5mg ropivacaine (69mmHg) *vs*. control (45mmHg). Reducing the amount of ropivacaine injection to 0.5mg, an elevation of the pain tolerance (22%) was still observed (p<0.05); while under an injection dose of 0.2mg, significant analgesic effect was no longer detected. Thus, this trial study adequately validates the analgesic effect proof of concept (with a significant dose-dependency) of intraperitoneal ropivacaine delivery on visceral pain in the rat model.

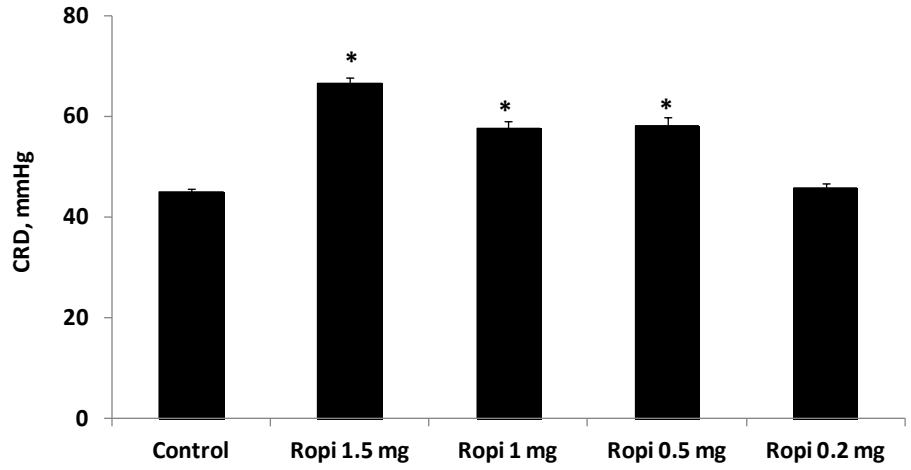

Figure 4: Dose-response relationship between visceral analgesic effect and the i.p. injection dose of ropivacaine in the rat colorectal distension model. Statistical analysis with Mann-Whitney U test (p < 0.05).*

3.2.3 Effect of ropivacaine loaded on meshes on pain

The virgin and functionalised meshes (1.5 × 1.5 cm) loaded (or not) with the same amount of ropivacaine (1mg/mesh) were implanted into 10 rats/group, which started to receive CRD manipulation 1 day after mesh implantation for evaluating the visceral pain threshold till the 8th day. No postoperative death was observed.

As shown in Figure 5, a lower pain threshold (38mmHg *vs*. the base level 46mmHg) was immediately observed on the first day after implantation of non-drug carrying virgin or functionalised meshes (p<0.05). This low pain tolerance proved the surgery-induced visceral pain and absence of an analgesic effect of both non-drug carrying meshes. It has persisted over time till D8 (42.7 $\pm$ 1.7mmHg). Thus, it is clear that the polyHPβCD coating on the meshes did not affect the pain threshold of rats.

Regarding the drug-loaded meshes, virgin meshes plus 1mg ropivacaine injection (A1L+ropi) did somewhat relieve the surgery-induced pain. The pain threshold (46mmHg), which persisted throughout the duration of the study though, was significantly higher (p<0.05) than that of non-drug carrying virgin meshes (38mmHg) on the first two days after implantation; whereas the notable and significant (p=0.015) pain relieving effect was found on the ropivacaine-loaded functionalised meshes (A1L-CD360+ropi). The pain threshold (53$\pm$1.71mmHg) was significantly elevated (p<0.05) during the first 4 days after implantation. This clearly demonstrated the contribution of the polyHPβCD to drug release behaviour. Although both drug-loading groups have the same loading amount (1mg) of ropivacaine, the drug-loaded functionalised meshes were significantly more efficient than the virgin meshes in terms of both height and duration of pain tolerance. It again indicates the special role that the polyHPβCD

plays in controlling the local drug delivery (prolonged drug release), as we found above in the pharmacokinetic study of drug plasma concentration in rat blood.

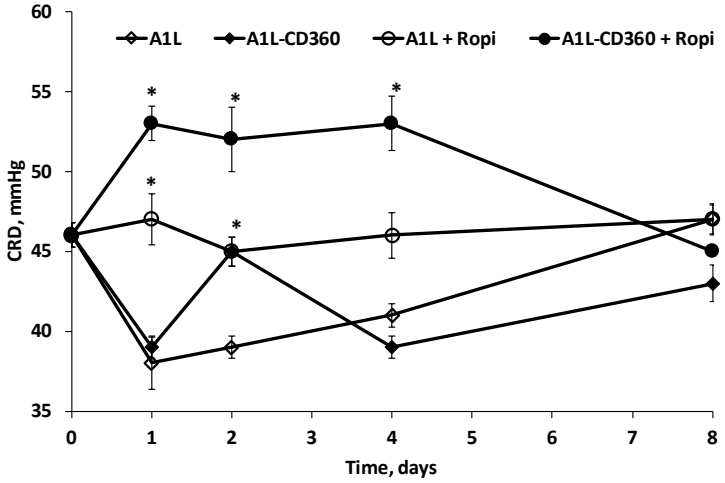

Figure 5: Study of the visceral analgesic effect of virgin and functionalised meshes (1.5×1.5 cm) loaded with (or without) the same amount of ropivacaine (1mg/mesh). Statistical analysis with Kruskal–Wallis ANOVA test ($p < 0.05$).*

3.2.4 Histopathological analysis

A detailed histological analysis of the explanted tissues from the sacrificed rats after the above CRD study was performed to examine the tissue response (particularly inflammatory reaction) to mesh implants.

The macroscopic observation (Figure 6) during the mesh explantation and the adjacent tissue sampling showed a good tissue integration of the meshes with tissue adhesion in all groups: on the surface of the mesh in contact with peritoneal surface, a variable thin connective tissue was present. No mesh infection or rejection was found in any animal.

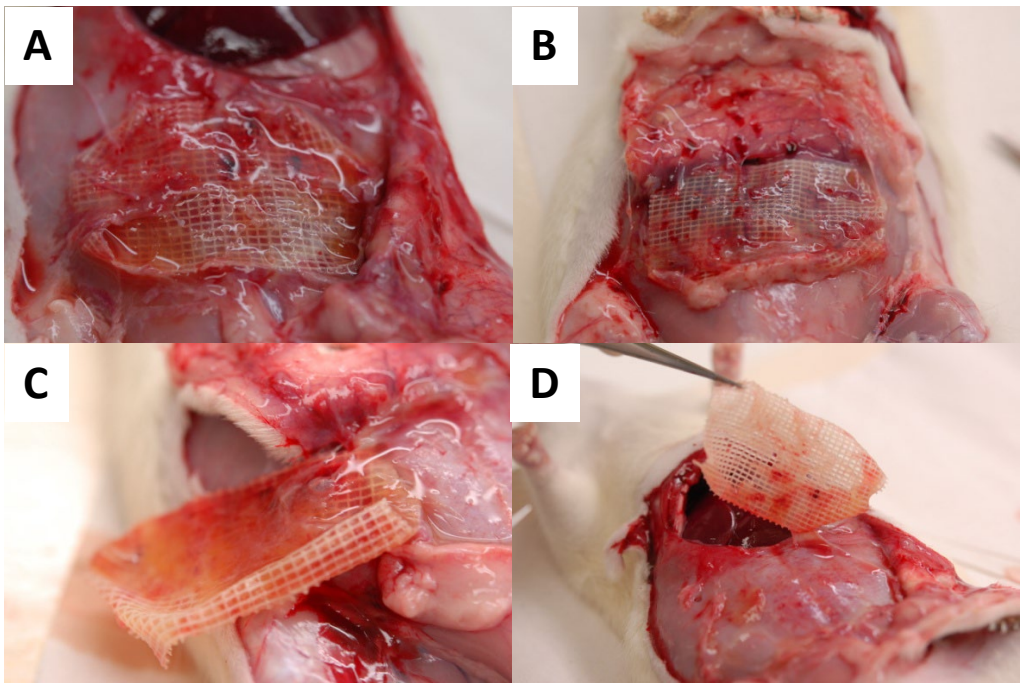

Figure 6: Macroscopic observation at 8 days of implantation site: (A: A1L; B: A1L-CD360; C: A1L + 1mg ropivacaine injection; D: A1L-CD360 meshes loaded with ropivacaine)

Representative images of H&E stained sections are shown in Figure 7, and the histological grading score of inflammation lesion were recapitulated in Figure 8. There is a moderate to marked chronic granulomatous inflammation (chronic peritonitis) found in all groups (virgin or functionalised meshes, with or without ropivacaine) without significant difference. This is a typical inflammatory foreign body reaction in the presence of the fabric material, irrelevant to the presence of polyHPβCD or ropivacaine. Secondary granulomatous inflammation reaction was noted in general in all the rats adjacent to the textile meshes by the presence of multinucleated giant cells and/or calcium deposits (basophilic deposits in extracellular matrix). We also observed on the periphery of the granulomas, a marked formation of granulation tissue from fibroplasia and new vessels (neovascularisation) and the presence of inflammatory cell (lymphocytes, plasma cells, neutrophils, macrophages) infiltration. In conclusion, the histological analysis showed no difference between the tested groups. Neither the polyHPβCD nor the loaded ropivacaine textile have shown any major impact on the inflammatory response compared to the virgin meshes.

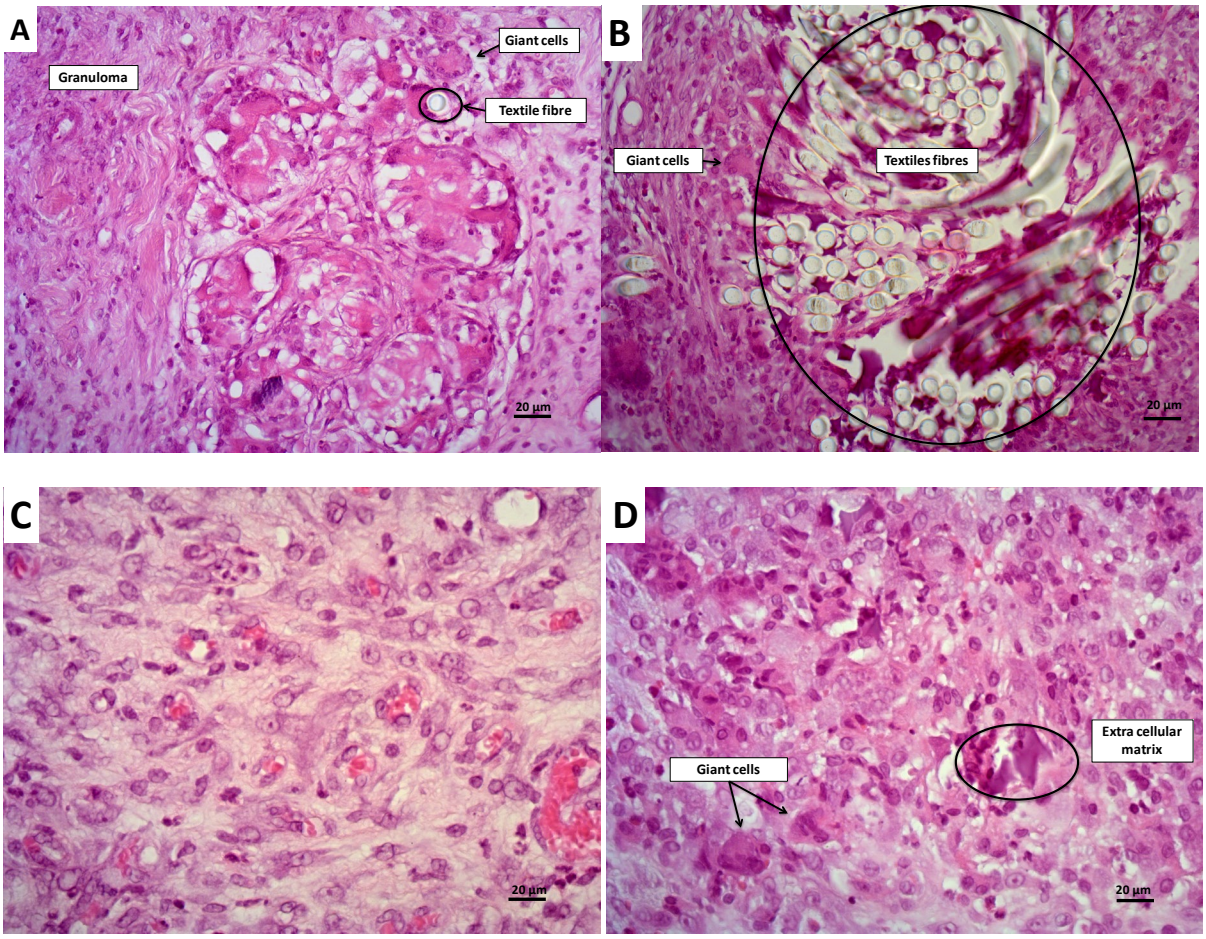

Figure 7: Representative images of H&E stained sections of explanted adjacent tissues to different visceral meshes (A: A1L; B: A1L-CD360; C: A1L plus 1mg ropivacaine injection; D: A1L-CD360 meshes loaded with ropivacaine) 8 days after implantation in rat (n=10).

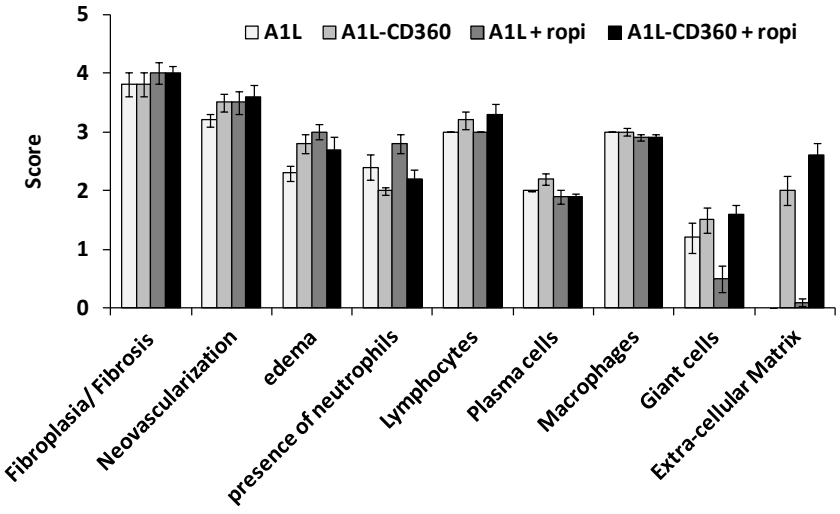

Figure 8: Histological grading score of inflammation lesion analysis of the explanted tissue surrounding different meshes 8 days after implantation in rat (n=10).

3.2.5 Degradation study of polyCD coating on meshes

A1L and A1L-CD360 mesh samples explanted from above pharmacokinetics study (1 day) and CRD study (8 days) were further analysed to evaluate the biodegradation of the polyHPβCD coating on the textile. TBO staining method was chosen due to the preferential affinity of TBO (a cationic dye) towards polyHPβCD via host-guest complexation of TBO in the cavities of cyclodextrins and via ionic interactions with the carboxylate groups of polyHPβCD [21]. No TBO adsorption onto virgin meshes A1L after 1 or 8 days after implantation. In contrast, the A1L-CD360+ropi meshes adsorbed 1.51 and 1.42mg of TBO per mesh (1.5 x 1.5 cm) after one and eight days. This result proved that the polyHPβCD coating remained stable over 8 days *in vivo*.

4 Discussion

Hernia repair (HR) is one of the most common surgeries performed worldwide. Intense pain immediately following HR is the most significant complication and can occur in 10–12% of patients. It often results in an average 2-day hospitalisation for pain management and can further develop into complicated chronic neuropathic pain. Considering how widespread, detrimental, and costly it is, prevention and treatment of pain is of utmost importance. Methods of pain control, including lidocaine or ropivacaine in patches applied to the abdomen and local anaesthetic injected during surgery to implantation sites, have been evaluated with varying success. While local anaesthetic delivered via catheter to the surgical site or local anaesthetic injected to specific sites of pain after surgery have shown some mixed results, nevertheless there remains great concern over the increased cost, plus risk of infection at the surgical site.

As most surgeons choose prosthetic material for hernia repair (open prosthetic repair or laparoscopic repair with placement of implant), we, herein, proposed an intraperitoneal delivery of a long-acting local analgesic (ropivacaine) to the operative site via a drug-eluting visceral mesh prosthesis to address the need for improved pain control after HR. Local drug delivery via hernia repair meshes have been examined previously in the context of reduction of intra-abdominal adhesion [49] or anti-infection [50]; however, its use in conjunction with an analgesic drug for pain control is new.

Cyclodextrins isolated from starch enzymatic degradation with potential use as a sustained-release drug delivery system, are ideal for the development of drug-eluting devices for pain management. As clearly evidenced by our former *in vitro* study [24], polyHPβCD coating on the PET visceral mesh has a high absorption capacity of ropivacaine and results in a sustained drug release. Because the drug loading is simply realised by soaking the mesh in drug solution in operation theatre before implantation, this strategy of surface modification of medical devices also permits greater compliance for the surgeon in choosing the specific drug adapted to the patient pathology, rather than using an imposed drug in case of a pre-impregnated

manufactured device. We demonstrated that the concentration of the ropivacaine soaking solution (2g/L and 10g/L) and the amount of polyHPβCD coating on the mesh have impacted on the drug sorption. Nevertheless, the ropivacaine loading on the functionalised mesh does not require a long soak process, i.e. 5 min soaking could be sufficient for surgeon by achieving around 90% of maximum ropivacaine adsorption. This additional time for impregnating the device in drug solution will remain acceptable in the operation theatre as this will have negligible impact on the length of time of the surgical act. However, this sorption time is very short compared to our previous study carried out on a polyHPβCD coated polylactic acid (PLLA) based visceral mesh, where 2 to 5 hours were necessary for optimally loading ciprofloxacin on the device [31]. Such features can be attributed to the different textile composition (PET versus PLLA), knitted structures and yarns compositions (i.e. diameters and number of monofilaments per yarn). Therefore, the polyHPβCD coating does not present the same distribution neither the same morphology on both types of meshes. In addition, the chemical nature of both drugs is different (ropivacaine on the one hand and ciprofloxacin on the other hand) and as a consequence adsorption of both drugs is driven i) by capillary forces, ii) by host-guest complexation in HPβCD cavities and iii) nonspecific interactions (ionic and hydrogen bonding) whose relative contributions may vary from one drug to another.

Regarding the drug desorption behaviour, we found that a large amount of ropivacaine (more than 60%) was released spontaneously in water, which seems to be coherent with above hypotheses of sorption phenomenon by capillary and nonspecific interactions. This part of the weakly bound drug on the textile support will later release quickly upon implantation resulting in a “burst effect” whereas the remaining part (40%) of ropivacaine will release slowly due to specific *host-guest* interaction. This combination of a “burst” release phase and a prolonged release phase would be more efficient to relieve the pain.

Though the *in vitro* "release profile" provided valuable insights into whether drug-carrying meshes satisfy our objective, the *in vivo* analgesic effect may not correlate with it. *In vivo* pharmacokinetic study is thus important for predicting drug efficiency and bioavailability. A precise titration of plasma drug concentration strongly depends on the choice of drug extraction techniques. The widely used protein precipitation method for processing plasma samples requires fewer samples but is unfavourable for sample testing of a low concentration. Organic solvent extraction, as reported in the literature, is more sensitive and precise for the mass analysis of biological samples. Thus, the organic solvent extraction was used as the pre-treatment method for ropivacaine plasma samples. The results of rat plasma drug titration showed that, the critical pharmacokinetic properties of drug-carrying functionalised meshes in rat blood are well associated with the treatment efficacy in CRD test. But the trials on analysing the diffusion of ropivacaine in tissue and their distribution, from 1-day sacrificed animals by

similar drug extraction of explanted implant and the surrounding tissue, detected no drug in any of the samples. This could be due to the limit of solvent extraction for animal tissue samples or sensitivity of analytical technique and is worthwhile studying further.

In order to make an objective comparison between the functionalised and raw mesh in the *in vivo* study, 1mg of ropivacaine, the same amount as that loaded on the functionalised mesh, was i.p. injected for virgin mesh upon implantation. The plasma concentration-time profiles of ropivacaine after implantation of raw meshes with drug injection showed a low-lying plain shape, and the area under the curve (AUC) represents only 25% of amount of the injected drug, while AUC of drug-loaded functionalised mesh corresponded to 90% of the loaded drug. Although the reason for this discrepancy is unclear, there may be two explanations: i) bioavailability of i.p. injected drug is much lower than polyHPβCD carried drug; ii) in our rat model, the i.p. injected drug reaches the bloodstream earlier than our first blood sampling time (10 min). The second explanation sounds more plausible to us, because, for certain very small and very water-soluble compounds, it can reach the bloodstream within 1-2 minutes in the rat [51]. If this is the case, for raw mesh group, a very high C_{max} peak appeared very rapidly (short T_{max}) after injection. Yet, it is difficult to prove it from a practical point of view as it is impossible to get blood samples any earlier than we did. In any case, the detected T_{max} for polyHPβCD coated mesh comes much later than that for raw mesh group, which is probably due to the slow dissociation of the inclusion complex formed between drug and polymerised HPβCD.

Similarly, evaluating the efficacy of analgesic devices in animals is also an important step for validating any new therapeutic strategy. The choice of the experimental model is a crucial but difficult aspect in an experiment design, particularly for assessing visceral pain. This study used a rat model of visceral pain induced by colorectal distension (CRD), which was recently validated for evaluating the efficacy of abdominal pain treatment [38,39]. Our trial study on this model, finding analgesic effect with a significant dose-dependency of intraperitoneal ropivacaine administration on visceral pain, not only supported our hypothesis on analgesic effect of local drug delivery, but also validated the translational value of this rat model. As in this model, Rousseaux *et al* has already proved an antinociceptive effect of a similar magnitude by subcutaneous administration of 1mg morphine per kg (body weight) [38]. By applying the drug-loading visceral meshes in this model, we further confirm that this pain model and its behavioural assessment could be a very useful tool for evaluating the *in vivo* efficacy of intraperitoneal devices or analgesic drugs against visceral pain. We have shown that, with the same amount of ropivacaine administration (1mg), drug-loading functionalised meshes were more efficient than virgin meshes in terms of both height and duration of pain tolerance. It is also consistent with the pharmacokinetic profile of ropivacaine found above: a 'burst' release phase followed by a sustained release. The ‘burst’ release with high-dose drug rapidly reduces

pain to an acceptable pain relief at the early stage (as with what we observed on raw mesh with i.p. drug injection); while the prolonged release drug, thanks to the slow liberation of the drug entrapped inside the cyclodextrin cavities can make pain-relief action long-lasting (8 days) and more useful in reducing inflammation.

Interestingly, Sauer et al. [52] reported recently that a randomly methylated beta cyclodextrin (RAMEB) presented intrinsic properties in the control of inflammatory pain through its capability to form inclusion complex with prostaglandin E2 (PG2), an inflammatory mediator involved in hyperalgesia. In this study, RAMEB was administered intraplantarly or intravenously to rats. Mechanical (paw pressure threshold) and thermal (paw withdrawal latency) nociceptive thresholds evidenced pain reduction associated to a decrease of PG2 content. In the present paper, our CRD animal model could not detect so clearly the same intrinsic analgesic effect of HPβCD cavities immobilised on the meshes. As a matter of fact, both curves in figure 7 displayed the same pattern, except that the pain threshold decreased after 2 days in the case of A1L-CD360 (not loaded with ropivacaine) versus 3 days for A1L control sample. So in our study, pain reduction could be associated rather to local ropivacaine release from the mesh than to the entrapment of pronociceptive agents [52]. Based on this latter study, it is possible to propose a hypothesis concerning the temporary decrease of pain threshold (expressed by the balloon pressure increase) at day 2 of the experiment on rats implanted with A1L-CD360 without ropivacaine. As a matter of fact, PG2 mediator produced by the inflammation could be captured by free HPβCD cavities present on the mesh, unlike what happened with the control sample (A1L without ropivacaine).

Safety is a primary concern when considering new excipients used in pharmaceutical formulations or new medical devices. The literature has provided many references demonstrating the use of CDs to enhance oral bioavailability of active compounds without any impairment of renal or hepatic function after implantation. Of course, it is also known that native CDs may induce shape changes and membrane invagination on human erythrocytes, and eventually induce haemolysis at higher concentrations. When the lipophilic cavity of CD is modified by certain chemical derivatisation *e.g.* HPβCD, their impact on cell membrane could be substantially modified. In our histopathological study, polyHPβCD coated mesh did not show any major impact on the inflammatory response compared to virgin meshes. This is consistent with our previous findings with implantation of the polyHPβCD coated vascular prosthesis loaded with antibiotics tested in mice [53] and dog models [22], which did not show any signs of acute or chronic, local or systemic toxicity induced by the functionalised device in the animal models from clinical data, blood-sample analysis and histological examination.

Regarding the *in vivo* degradation property of polyHPβCD coating on the mesh, we found that polyHPβCD had not degraded completely after 8 days. This is in accordance with the results

of the study in the above dog model [22], showing that HPβCD-based coating on the polyester vascular implants was still present at 1 month, and a complete biodegradation of the polyHPβCD coating occurred much later, between 3 and 6 months.

5 Conclusions

The results confirmed our hypothesis that, the polyHPβCD coated mesh presents an enhanced adsorption capacity of ropivacaine and involves a sustained drug release in rats after mesh implantation. This was further reaffirmed by an elevation of pain tolerance up to 4 days after implantation in rat CRD model compared to 1 - 2 days for raw meshes. Neither polyHPβCD nor the loaded ropivacaine have shown a major impact on the inflammatory response after 8-day implantation in rat, and polyHPβCD had not yet completely biodegraded. These findings strongly suggest that polyHPβCD functionalised visceral mesh could be a promising approach for post-operative pain control by improving the intraperitoneal drug delivery and bioavailability. Moreover, the rat CRD model could be a very useful tool for evaluating the *in vivo* efficacy of new intraperitoneal devices or drugs against visceral pain.

6 Disclosures

The authors declare no conflict of interest.

7 Acknowledgements

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