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## **Thermosensitive hydrogels for local delivery of 5-fluorouracil as neoadjuvant or adjuvant therapy in colorectal cancer**

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### **Abbreviations**

5-FU: 5-Fluorouracil; CMC: Critical micelle concentration; CMT: Critical micelle temperature; CRC: Colorectal cancer; CT26-luc: Luciferase-positive murine colon carcinoma cell line; HPLC-UV: High performance liquid chromatography with ultraviolet detection; IT: Intratumoral; P407: Poloxamer P407; P188: Poloxamer P188; PBS: Phosphate buffer saline solution; PEO: Poly(ethylene oxide); PPO: Poly(propylene oxide); SC: Subcutaneous;  $T_{sol-gel}$ : Critical SOL-GEL transition temperature.

## **Abstract**

Spurred by high risk for local tumor recurrence and non-specific toxicity of systemic chemotherapy, clinicians have recently granted a growing interest to locoregional therapeutic strategies. In this perspective, we recently developed a multipurpose thermosensitive hydrogel based on reversible thermogelling properties of poloxamers P407 and P188, a bioadhesive excipient and antineoplastic effect of 5-fluorouracil (5-FU) for the local treatment of colorectal cancer (CRC) in ectopic CT26 murine models. Antitumor efficacy was assessed in mice following intratumoral (IT) injection mimicking neoadjuvant therapy and subcutaneous (SC) application after tumor excision simulating adjuvant therapy. Rheological characterization disclosed that P407/P188/alginate 20/2/1% w/v thermosensitive hydrogel is an injectable free-flowing solution at ambient temperature that undergoes a SOL-GEL transition at  $26.0^{\circ}\text{C} \pm 0.6^{\circ}\text{C}$  and thereby forms *in situ* a non-flowing gel at physiological temperature. The generated gel presented an elastic behavior and responded according to a shear-thinning fluid upon shear rate. Although delayed by the addition of alginate 1% w/v, 5-FU is released mainly by diffusion mechanism. The local delivery of 5-FU from P407/P188/alginate/5-FU 20/2/1/0.5% w/v hydrogel in the preclinical tumor models led to a significant tumor growth delay. These results demonstrated that poloxamer-based thermosensitive hydrogels provide a simple and efficient means for local chemotherapeutics delivery.

## **Keywords**

Thermosensitive hydrogels; Poloxamers; 5-Fluorouracil; Colorectal CT26 cancer model; Local drug delivery.

## 1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer (10.2% of the total cancer cases worldwide) and the second most lethal cancer worldwide with almost 880792 deaths in 2018 according to the World Health Organization [1, 2]. Patients with locally advanced (stage II-III) rectal cancer are treated with neoadjuvant chemoradiotherapy before surgery in order to reduce tumor size and improve disease-free survival [3-5]. Moreover, according to the 1990 NIH consensus recommendations, the treatment of colon cancer patients with stage III disease (lymph node involvement) has consisted of surgery aiming to excise tumor followed by adjuvant systemic chemotherapy to eradicate micrometastases and prevent recurrence [6, 7]. Each year, approximately 230,000 patients with colon cancer are eligible for adjuvant chemotherapy [8]. The benefits of 5-fluorouracil (5-FU)-based chemotherapy in reducing the risk of relapse and prolonging survival in patients are well-established [9]. However, the administration of 5-FU by systemic route results in short plasmatic half-life (11.4 min after bolus intravenous administration) and drug clearance from plasma within 1 hour as a consequence of a very rapid metabolism by the dihydropyrimidine dehydrogenase or uracil reductase enzymes [10]. Hence, this implies low drug concentration in tumor tissue and reduced overall efficacy. In addition, severe non-specific toxicities are reported as a consequence of the high doses of chemotherapeutic drugs administered systemically.

Therefore, tumor-targeting strategy such as local delivery of chemotherapeutic drugs is thought to provide a site-specific drug delivery and a better control of the on-site drug concentration, thus improving both safety and efficacy. A study carried out by *Yi et al.* on 5-FU-loaded polyethylene glycol hydrogel showed an optimized 5-FU pharmacokinetic with an elimination half-life of 0.9 hours, 6-fold longer than the free solution of 5-FU, both administered subcutaneously. The 5-FU-loaded polyethylene glycol hydrogel group showed a

significant tumor growth inhibition in comparison to the untreated group and a significant enhanced tumor inhibition rate compared to the free solution of 5-FU group in mice bearing xenografted tumor [11].

Hydrogels are characterized by a hydrophilic polymer network capable to swell in the presence of water or physiological fluids leading to a three-dimensional structure [12, 13]. Thermosensitive hydrogels are composed of polymers responsive to a thermal stimulus. Temperature change alters the interaction between hydrophilic and hydrophobic chains in the polymer with water molecules, thus inducing a phase transition of the polymeric solution from a solution (SOL state) to a gel (GEL state). This transition occurs *in situ* and it is commonly known as SOL-GEL transition. Accordingly, *in situ*-forming thermosensitive hydrogels have attracted increasing attention as local drug delivery systems for locoregional chemotherapy [14-21]. They allow an easy administration of injectable fluid solution that forms a semi-solid gel in response to physiological temperature as soon as it is in contact with tumor tissue. Thus, they have the potential to (a) deliver chemotherapeutic drugs locally to the tumor site leading to low dose requirements, (b) provide controlled and sustained drug release within tissues, (c) ensure drug diffusion into cells, (d) reduce multiple drug administration cycles, and (e) reduce systemic toxicities due to reduced systemic drug diffusion [22, 23].

Poloxamers are tri-block copolymers of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) which exhibit excellent thermosensitive properties [23, 24]. They are non-toxic and FDA approved [25]. Below their critical micelle concentration (CMC) and critical micelle temperature (CMT), poloxamers are in the form of individual block copolymer molecules (unimers) in solution. Above their CMC and CMT, copolymers self-assemble giving rise to a process defined as “micellization”. An increasing temperature reaching their critical SOL-GEL transition temperature ( $T_{\text{sol-gel}}$ ) results in the rearrangement of micelles and gel formation [26].

Owing to its hydrophilic character, 5-FU represents an appropriate candidate to be incorporated into poloxamer hydrogel. A comparative study between 5-FU suppository and poloxamer-based *in situ*-gelling enema showed that enema provided higher rectal 5-FU concentrations for up to three hours compared to suppositories [27]. This could be mainly explained by a larger release area and a longer retention time of 5-FU in gelling enema formulation. Both suppositories and enema resulted in low 5-FU blood concentration compared to intravenous administration. In this sense, many topical and aerosolized formulations of 5-FU have been developed to treat local tumors [15, 28, 29]. Furthermore, intratumoral (IT) delivery of chemotherapeutic drugs constitutes an attractive strategy owing to its capacity to introduce the cytotoxic drugs directly into the tumor, hence limiting the systemic absorption, reducing the toxicity and increasing the overall efficacy [16, 30].

Injectable thermosensitive hydrogels provide an efficient means for IT administration. Consequently, alternative neoadjuvant and adjuvant treatments aiming to reduce tumor size prior to surgery and prevent recurrence after surgical excision of tumor, respectively, or treat small-sized tumor, thereby bypassing surgery, are highly required. Not only should said treatments increase life expectancy, but they are also expected to improve patient comfort by limiting chemotherapy toxicities. The aim of this work was to develop a multipurpose thermosensitive hydrogel capable to incorporate 5-FU for the local treatment of CRC by: 1) reducing tumor size prior to surgery, 2) preventing tumor recurrence after surgical resection in ectopic tumor models in mice. We have used Poloxamer P407 (P407) as thermosensitive gelling agent to prepare thermosensitive hydrogels. Poloxamer P188 (P188) was added to increase P407  $T_{sol-gel}$  and reduce gelling time [31]. Alginate and chitosan were included as natural, biocompatible and biodegradable polymers to impart adhesive, thickening and shear-thinning properties [32-34]. Alginate is a linear, water-soluble polysaccharide copolymer composed of longer homo-polymeric regions of mannuronate and guluronate, potentially

separated by regions of alternating structure mannuronate-gulonate. It is an anionic bioadhesive polymer which is known for its ability to create hydrogen bonds with mucin-type glycoproteins through carboxyl–hydroxyl interactions. Chitosan is a linear polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine units. As a polycationic biopolymer, chitosan is bioadhesive and readily binds to negatively charged surfaces such as mucosal membranes. Medium MW chitosan was utilized because it confers low viscosity to the hydrogel (the viscosity of a chitosan solution primarily depends proportionally on the average molecular weight of the polymer), it increases slightly the  $T_{sol-gel}$  [32] and impart a mucoadhesion with no significant difference with low and high MW chitosan [35]. Resulting formulations were carefully characterized in terms of thermogelling and rheological behaviors. 5-FU release from thermosensitive hydrogel was studied at 37°C in a flow-cell device. 5-FU release and hydrogel erosion were also tested *in vitro*. Finally, *in vivo* antitumor efficacy was assessed in two colorectal CT26 cancer model in BALB/c mice.

## 2. Materials and Methods

### 2.1. Materials

Ploxamer P407 (P407; Kolliphor<sup>®</sup> P407; PEO98–PPO67–PEO98; MW 12.6 kDa) and poloxamer P188 (P188; Kolliphor<sup>®</sup> P188; PEO80–PPO30–PEO80; MW 8.4 kDa) were purchased from BASF France. Sodium alginate (Protanal<sup>®</sup> LF 10/60 FT; M:G ratio 25:75) was a gift from FMC Biopolymer (Little Island, Ireland). Chitosan chloride (Protasan<sup>™</sup> UP CL 113; Ultrapure chitosan chloride salt; Deacetylation degree 75-90%; MW < 200 kDa) was supplied by NovaMatrix<sup>®</sup>, FMC Biopolymer (Sandvika, Norway). 5-Fluorouracil (5-FU; MW 130.08 Da) was obtained from Acofarma (Madrid, Spain). Water used here was deionized “water for injection” obtained from C.D.M. Lavoisier (Paris, France). Phosphate buffer saline solution (PBS; pH 7.3; 0.01 M) was provided by Gibco, Life technologies (Saint Aubin,

100 France). Drug and excipients were of European Pharmacopoeia grade whereas solvents were of analytical grade.

## **2.2. Thermosensitive hydrogel preparation**

### **2.2.1. P407/P188-based hydrogel preparation**

105 Thermosensitive hydrogels were prepared on a volume basis. Concentrations of all the components reported here are expressed as weight/volume percentage (% w/v). P407 and P188 were used and hydrogels are represented below as P407/P188 hydrogels. Hydrogels were prepared according to the “cold” method described by Schmolka [24]. Briefly, appropriate amounts of P407 and P188 powders were added to a volume of water. Dispersion was left under magnetic stirring overnight at  $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$  until clear solution was obtained. 110 Water was then added to adjust the final volume of the hydrogel placed in an ice bath. After homogenization, resulting hydrogels were stored at  $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$  for further studies. Three P407/P188 thermosensitive hydrogels were prepared yielding final concentrations of 17/1, 20/2 and 20/5% w/v, respectively.

### **2.2.2. Bioadhesive-contained hydrogel preparation**

115 Two bioadhesive polymers i.e. alginate and chitosan, were investigated. For that purpose, concentrated solution of alginate or chitosan was first prepared by slowly adding appropriate amount of the bioadhesive powder to vigorously stirred water in order to avoid lumping. Mixture was then stirred until complete hydration occurs and a clear viscous solution was obtained (1 hour). Afterwards, appropriate amounts of P407 and P188 powders were added 120 and subsequent steps were precisely followed as described in the section above. Obtained hydrogels contained P407/P188 at 17/1, 20/2 and 20/5% w/v, respectively, and a bioadhesive polymer at a final concentration of 0.25, 0.5 or 1% w/v.



### 2.2.3. 5-FU-loaded hydrogel preparation

In order to prepare 5-FU-loaded hydrogel, appropriate amount of 5-FU powder was weighed  
125 and added to a precise volume of P407/P188/alginate 20/2/1% w/v hydrogel. Mixture was  
protected from light and the drug was solubilized by magnetic stirring at  $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$  yielding a  
final concentration of 0.5% w/v (5 mg/mL) 5-FU. After complete solubilization, the resulting  
hydrogel was stored at  $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$  for further studies. P407/P188/alginate/5-FU 20/2/1/0.5%  
w/v hydrogel presented a pH value of 6.6 (supplementary Fig. S1).

### 130 2.3. Rheological characterization

Rheological analyses were performed using a rheometer (MCR102, Anton Paar, Germany) in  
a cone-and-plate geometry with a diameter of 50 mm (cone angle  $1^{\circ}$ ). Samples were kept at  
 $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$  until measurement. A solvent trap was used to minimize evaporation and to keep a  
solvent saturated atmosphere surrounding the sample. Measurements were performed in at  
135 least triplicate. Error bars were sometimes omitted to retain clarity.

#### 2.3.1. Oscillation mode studies

Oscillatory studies allow to determine the viscoelastic properties of hydrogels by subjecting  
the sample to a sinusoidal shear stress. The hydrogel (730  $\mu\text{L}$ ) was placed on the pre-cooled  
Peltier plate ( $15^{\circ}\text{C}$ ) immediately prior to testing and submitted to a temperature sweep in the  
140 range of  $15\text{-}45^{\circ}\text{C}$  at a heating rate of  $1^{\circ}\text{C}/\text{min}$ . A Peltier controller unit was used to control the  
sample temperature with an accuracy of  $0.1^{\circ}\text{C}$ . The test was carried out at a controlled strain  $\gamma$   
of 0.2% and constant frequency of 2 Hz. These parameters were determined prior to the test to  
define the linear viscoelastic regime where the stored elastic energy (storage or elastic  
modulus  $G'$ ) remained invariant and the sample did not undergo structural modifications  
145 (non-destructive dynamic conditions). The changes of  $G'$  modulus and the viscous dissipated  
energy (loss or viscous modulus  $G''$ ) were thus investigated. Oscillatory rheology provides the

most sensitive measurement of the main parameters  $T_{\text{sol-gel}}$ ,  $G'$  and  $G''$  moduli and the loss factor tangent  $\delta$  ( $\tan\delta = G''/G'$ ).

### 2.3.2. Rotational mode studies

150 Rotational studies allow to determine the dynamic viscosity ( $\eta$ ) and shear stress ( $\tau$ ) of hydrogels by subjecting the sample to constant or ascending shear rate. Herein, viscosity ( $\eta$ ) was measured at  $10^{-1} \text{ s}^{-1}$  shear rate at constant or gradient of temperature in the range of 15-45°C, while shear stress ( $\tau$ ) was obtained at a shear sweep between  $10^{-3}$  and  $10^4 \text{ s}^{-1}$  at 5°C and 37°C.

### 155 2.4. Hydrogel erosion

Hydrogel erosion study was performed *in vitro* at 37°C using a membrane-free release model. PBS (pH 7.3; 0.01 M) was used as release medium. Experiment met the sink conditions. In detail, under ice bath, 1 mL of the hydrogel was introduced into 20 mL transparent vials and placed at  $5^\circ\text{C} \pm 3^\circ\text{C}$  for 10 minutes in order to homogenize the distribution of the hydrogel at 160 SOL state in the bottom of the vial. Afterwards, vials were placed in an incubator set at 37°C and allowed to gel for 30 minutes. Subsequently, 5 mL of pre-warmed (37°C) release medium, were gently layered over the surface of the gel and vials were stored in an orbital shaker/incubator (Orbital Shaker, Forma Scientific, Ohio, USA) and shaken at 50 rpm at 37°C. At specific time points (0, 0.5, 1, 3, 6, 24, 48 and 96 hours), 200  $\mu\text{L}$  of the release 165 medium were sampled and replaced with an equal volume of pre-warmed (37°C) fresh release medium. The concentrations of dissolved poloxamers in samples were measured by the colorimetric method of Baleux and the times required to erode 50% (T50%) or 80% (T80%) of the hydrogels were calculated. All measurements were conducted in triplicate and mean values  $\pm$  standard deviations were reported.

170 **2.5.Hydrogel erosion and 5-FU release**

P407/P188/alginate/5-FU 20/2/1/0.5% w/v hydrogel erosion and 5-FU release from the hydrogel were conducted simultaneously *in vitro* at 37°C using a membrane-free release model. PBS (pH 7.3; 0.01 M) and water were used as release media. The experimental protocol was precisely followed as described in the section above. The concentrations of dissolved poloxamers and released 5-FU were measured by the colorimetric method of Baleux and HPLC-UV, respectively, in samples withdrawn at specific time points (0, 0.25, 0.5, 1, 3, 6, 9, 19 and 24 hours). In parallel, a solution of 5-FU (0.5% w/v) kept in the orbital shaker/incubator at 37°C was used as control to monitor the stability of 5-FU in the experiment conditions. All measurements were conducted in triplicate and mean values ± standard deviations were reported.

**2.6.Poloxamer quantification by the colorimetric method of Baleux**

To quantify dissolved poloxamers in the release medium, samples were centrifuged for 10 min at 14,000g in order to separate dissolved poloxamers from possible hydrogel fragments. After centrifugation, supernatants were collected and assayed by the colorimetric method described by Baleux [36]. Briefly, 25 µL of potassium iodide (KI<sub>3</sub>) reagent was added to 1 mL of diluted supernatant. After homogenization, optical density was quickly measured at 440 nm to avoid photobleaching phenomenon of KI<sub>3</sub> and the formed PEO-KI<sub>3</sub> complex. A solution of 1 mL of water and 25 µL of the reagent has served as a reference. Results were calculated from linear regressions of P407 and P188 generated by dissolving known amounts of these copolymers in water in the concentration ranges of 0.05-20 µg/mL (supplementary Fig. S2). The cumulative percentage of hydrogel eroded was expressed as the weight percentage of dissolved poloxamers as a function of time.

### 2.7.5-FU quantification by HPLC-UV

The amount of released 5-FU occurring simultaneously with the hydrogel erosion was measured by high performance liquid chromatography with ultraviolet detection (HPLC-UV). To this end, samples were diluted with appropriate volume of water to destroy poloxamer micelles. After 3 min of vortex, drug was separated from poloxamers and alginate through 0.5 mL Amicon Ultra centrifugal filters (cut-off 3 kDa, Millipore Corporation, Molsheim, France) by centrifugation at 14,000g for 40 min using a tabletop Eppendorf MiniSpin® plus (Hamburg, Germany). Subsequently, ultrafiltrates free of polymers were collected and 5-FU was assayed by HPLC using a LC-20AD XR HPLC system (Shimadzu, Japan) connected to a Waters  $\mu$ Bondapak® C18 column (Guyancourt, France) (reversed phase, 300 mm X 3.9 mm i.d., 10  $\mu$ m). Chromatographic conditions were the following: Isocratic elution, mobile phase of deionized water whose pH was adjusted to 4.5 with trifluoroacetic acid (0.001% v/v), flow rate 0.6 mL/min with column temperature maintained at 25°C, an injection volume of 20  $\mu$ L and an UV detection at 266 nm. Results were calculated from linear regression of 5-FU generated by dissolving known amounts of the drug in water in the concentration range of 5-50  $\mu$ g/mL. The cumulative percentage of 5-FU released over time was expressed as the weight percentage of 5-FU.

### 2.8. In vitro 5-FU release in T-cell for flow-through dissolution

This study was meant to determine *in vitro* 5-FU release behavior from poloxamer hydrogel and the consecutive effect of addition of alginate 1% w/v on drug release rate. Two hydrogels were tested: P407/P188/5-FU 20/2/0.5% w/v and P407/P188/alginate/5-FU 20/2/1/0.5% w/v. T-cell for flow-through dissolution (membrane-free model) provided by the company Sotax (Switzerland) was utilized and water was used as the release medium. Experiment met the sink conditions. In detail, 150  $\mu$ L of each hydrogel was introduced in the pit of the cell, placed in an incubator at 37°C and allowed to gel for 30 minutes. Subsequently, the cell was

reinserted in the flow-cell system where the release medium flows continuously at 37°C. At specific times (0, 1, 2, 3, 4, 5, 6, 24 and 48 hours), 500 µL of the release medium were sampled and replaced with an equal volume of pre-warmed (37°C) fresh release medium. Collected samples were analyzed as described in the previous section and the percentage of 5-FU was determined by HPLC-UV at 266 nm. All measurements were run in triplicate, data are expressed as the mean ± standard deviations.

## **2.9. *In vivo* antitumor efficacy**

*In vivo* experiments were carried out in female BALB/cJRJ mice (Janvier, St Genest de Lisle, France), aged from 6 to 7 weeks and kept under SPF (specific pathogen free) conditions for 1 week before the study with free access to standard food and water. Animal experiments were conducted in compliance with the principles of care and use of laboratory animals of the European and national guidelines and were approved by the institutional ethics committee.

### **2.9.1. Tumor models development**

The CT26.WT murine colon carcinoma cell line was purchased from American Type Culture Collection (ATCC, CRL-2638, LGC Standards, Molsheim, France). The luciferase-positive cell line (CT26-luc) was generated by transfection of CT26.WT cell line with luciferase gene as reporter. CT26-luc cells were cultured at 37°C in a 5% CO<sub>2</sub>-humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies), 1% penicillin/streptomycin and 0.08% of Geneticin (G418 sulfate, Gibco Life Technologies). CRC tumor model was developed as previously described [37]. Briefly, 10<sup>5</sup> CT26-luc cells in 100 µL of DMEM culture medium were injected subcutaneously into the right and left flanks of the mouse. Fifteen days later, the size of the subcutaneous (SC) tumor was around 1000 mm<sup>3</sup>. The tumor-bearing mouse was sacrificed, the CT26-luc tumor was resected and placed into DMEM

culture medium. Tumor was then cut into 20-30 mm<sup>3</sup> fragments and inoculated subcutaneously, through a 12 gauge trocar (38 mm), into the right and the left mouse flanks previously disinfected with alcohol. Those mice were subsequently used for the antitumor efficacy studies. In each mice, both tumors were treated identically.

### **2.9.2. IT injection of hydrogel in neoadjuvant therapy model**

On the eighth day, mice bore a SC tumor of 63.8 mm<sup>3</sup> ± 10.6 mm<sup>3</sup> mean volume. Mice were randomly separated into four groups of five mice each. Mice were anesthetized by intraperitoneal injection of 300 µL of Ketamine (80 mg/kg) and Xylazine (10 mg/kg), and the areas around their two tumors were shaved and disinfected with alcohol (Fig. 6A). During anesthesia, mice were kept at physiological temperature. Mice received a single injection of 60 µL of P407/P188/alginate/5-FU 20/2/1/0.5% w/v (corresponding to 15 mg 5-FU/kg body weight), 5-FU solution (0.5% w/v), P407/P188/alginate 20/2/1% w/v (5-FU-free hydrogel) or water (control group). Formulations were injected into the tumor via a 26Gx1/2'' needle.

### **2.9.3. SC application of hydrogel after surgical tumor excision (adjuvant therapy model)**

Fourteen days after tumor fragment implantation, mice were randomized into two groups of three mice each. Mice were anesthetized and prepared as described in the previous section. An incision of 1-2 cm was made in the proximity of the tumor and the latter was subsequently resected without leaving macroscopic residuals (Fig. 7A). In the remaining cavity, 100 µL of P407/P188/alginate/5-FU 20/2/1/0.5% w/v (corresponding to 25 mg 5-FU/kg body weight) were applied through a syringe. For the control group, 100 µL of P407/P188/alginate 20/2/1% w/v (5-FU-free hydrogel) were applied. Afterwards, the wound was closed with silk thread 5.0. 5-FU solution and water were not tested here as they cannot be retained in the tumor cavity.

#### 2.9.4. Mice follow up

The viability, status and any visible toxicity changes at the injection sites of the mice were followed throughout the experiments. The body weight of each mouse was checked every other day and recorded as a function of time. Longitudinal monitoring of the tumor growth was performed thrice weekly by optical imaging and by caliper. To this end, 20 min before imaging, mice were injected intraperitoneally with 200  $\mu$ L of 10 mg/mL luciferin solution (D-luciferin potassium salt, Interchim). Luciferin reacts with luciferase which produces a photon signal detected by the camera (Photon Imager™ Biospace Lab). Mice were thus imaged during 10 min while being under anesthesia with isoflurane. Image analysis was performed with the M3 Vision software developed by Biospace Lab. Likewise, tumor volume was measured with a caliper and the volume was estimated as follows:  $(\text{length} \times \text{width}^2)/2$ . This allowed to determine the time point for the mice killing. Mice were sacrificed as soon as their tumor volume exceeded 1000 mm<sup>3</sup>.

#### 2.10. Statistical analysis

Multiple comparison tests for different experiments were conducted. Values represent means  $\pm$  SD. ANOVA multiple comparison tests (Tukey's test and Bonferoni's test) were performed to determine significant differences. A Student's *t* test was also used to compare the viscosity of alginate-containing hydrogel *versus* the chitosan-containing hydrogel. The level of significance was taken as  $p \leq 0.05$ . Statistical analyses were performed with GraphPad Prism version 5 for Windows (GraphPad Software, La Jolla California USA).

### 3. Results and discussion

The unique property of thermosensitive hydrogels makes them efficient drug carrier for local drug delivery. Consequently, such system must meet a set of specifications compatible with the intended use. In the present context, the thermosensitive hydrogel must (a) be a fluid

290 solution (SOL) at ambient temperature, (b) be injectable with a syringe after being combined  
with 5-FU at the SOL state and this owing to its low viscosity, i.e.  $< 1$  Pa.s, which enables its  
flow and spreading over the tumor site, (c) exhibit a  $T_{\text{sol-gel}}$  around  $26^{\circ}\text{C}$  to  $28^{\circ}\text{C}$  to undergo a  
rapid SOL-GEL transition once administered in mouse, thus avoiding its dilution in  
physiological fluids [38], (d) possess a  $G'$  modulus  $> 10 \times 10^3$  Pa and a loss factor  $\tan \delta < 1.0$  at  
295  $37^{\circ}\text{C}$  to yield a strong elastic gel [39, 40], (e) provide *in situ* 5-FU release at the site of  
injection leading to a local antitumor effect.

### 3.1. Viscoelastic properties of P407/P188-based hydrogels

P407 is a water-soluble, non-ionic, thermosensitive copolymer with high CMC ( $2.8 \times 10^{-6}$  M at  
 $37^{\circ}\text{C}$ ) and  $T_{\text{sol-gel}}$  due to weak hydrophobicity of PPO block [41]. At P407 concentrations  
300 below 15% w/v, though above the CMC, SOL-GEL transition does not probably occur [27,  
42]. Increasing the concentration, the  $T_{\text{sol-gel}}$  varies inversely with the concentration with a  
gel formation arising around  $24^{\circ}\text{C}$  at 20% w/v P407 [43], thus impairing the characteristic of  
SOL state at ambient temperatures. Given that high P407 concentration is relevant to  
strengthen gel network and bioadhesion force [44], P407 is often mixed to P188 to  
305 compensate for this drawback. P188 is more hydrophilic than P407 (hydrophilic-lipophilic  
balance of 29 vs. 22 at  $22^{\circ}\text{C}$ , respectively) [41] with lower molecular weight, thereby  
increasing the  $T_{\text{sol-gel}}$  and shear-thinning behavior of P407 [27, 31]. Furthermore, it is  
demonstrated that the combination of P407 (20% w/v) and P188 (2.5 and 5% w/v) resulted in  
hydrogels presenting suitable  $T_{\text{sol-gel}}$  [27]. Therefore, based on those data, three different  
310 P407/P188 ratios were considered: 17/1, 20/2 and 20/5. Viscoelastic properties were  
determined by oscillatory rheometry and results were illustrated in Fig. 1.  $T_{\text{sol-gel}}$  was chosen  
as the temperature at which both moduli,  $G'$  and  $G''$ , were equal, reflecting similar elastic and  
viscous properties ( $G'G''$  crossover). Fig. 1A showed that, in accordance with the literature  
[27, 45], P188 tends to increase P407  $T_{\text{sol-gel}}$  yielding a value of  $26.5^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$  for the



315 P407/P188 ratio 20/2 (as regard to 24°C for 20% w/v P407), consistent with the targeted range.  $G''$  modulus values were not affected by the variation of P407/P188 ratio (Fig. 1B). Considering the storage modulus,  $G'$  was below  $10 \times 10^3$  Pa for the P407/P188 ratio 17/1, highly dispersed between  $1 \times 10^3$  Pa and  $13 \times 10^3$  Pa for the P407/P188 ratio 20/5, whilst values for the P407/P188 20/2 ratio gathered in the range  $11 \times 10^3$  to  $16 \times 10^3$  Pa (Fig. 1C). 5% w/v of  
320 P188, thus produced a decrease in  $G'$  modulus values compared to those obtained with 2% w/v. Consistently, values of the loss factor  $\tan\delta$  were lower and less dispersed for the P407/P188 20/2 ratio and more importantly below 0.1, reflecting a  $G'$  higher than  $G''$  at 37°C (Fig. 1D). The relationship between storage modulus  $G'$  and loss modulus  $G''$  highlight the change of elasticity and viscosity between a weak hydrogel ( $\tan\delta > 1.0$ ) and a strong hydrogel  
325 ( $\tan\delta < 1.0$ ). When the loss factor  $\tan\delta$  is lower than 1.0, the  $G'$  is greater than the  $G''$  and the material behaves like a strong hydrogel with elastic behavior and not like a viscous liquid. From the rheology side, a high  $G'$  is one of the inherent characteristics of solid materials [40]. Accordingly, P407/P188 20/2% w/v, made of uncrosslinked copolymers, behaves like a liquid below the  $T_{\text{sol-gel}}$ , while above this point, it turns into a gel and provides an elastic semi-solid  
330 material at 37°C.

### 3.2. Effect of bioadhesive polymers on hydrogel properties

To achieve a local drug delivery at the site of the tumor, bioadhesiveness of the hydrogel is an important point to increase its residence time on the injection site. To improve that characteristic, several methods have been exploited based mainly on covalent-cross-linking of  
335 chemical group to the poloxmer chains [46]. Bioadhesive force also increases with gel strength that depends on poloxamer concentration. Moreover, the presence of some bioadhesive polymers may also improve this property [47]. In this context, two natural bioadhesive polymers were selected, namely alginate and chitosan, and a range of concentration (i.e. 0.25, 0.5 and 1% w/v) was tested. Independently of the concentration, the

340 addition of alginate and chitosan did not modify the rheological properties of P407/P188 hydrogels (supplementary Table S1). Only a slight increase in  $T_{\text{sol-gel}}$  in line with previous works [32] was noted at low percentages. In the light of all the above, particularly the highest  $G'$  modulus and the lowest loss factor  $\tan\delta$  values obtained with P407/P188 20/2 ratio, the P407/P188 20/2% w/v hydrogel was expected to provide the strongest gel at the injection site  
345 and was thereby hereafter studied.

As alginate and chitosan exhibit viscosifying properties, their impact on hydrogel viscosity was therefore determined. Viscosity of P407/P188 20/2% w/v hydrogel was measured as a function of a temperature sweep in the range of 15-45°C at various percentages of bioadhesive, i.e. 0, 0.25, 0.5 and 1% w/v, at  $10^{-1} \text{ sec}^{-1}$  shear rate (supplementary Fig. S3).  
350 Temperature influenced viscosity with increasing heating resulting in ascending viscosity. This confirmed once again that the phase transition of poloxamer hydrogel was not altered by the addition of bioadhesives. Besides, this highlighted that below the  $T_{\text{sol-gel}}$ , hydrogel behaves like a low viscous liquid ( $\eta < 1 \text{ Pa.s}$ ), whereas above this point, it gains in viscosity converting into a viscous gel system at 37°C ( $1.4 \times 10^3 < \eta < 1.7 \times 10^3 \text{ Pa.s}$ ). Moreover,  
355 viscosity was determined at constant temperatures, i.e. 15°C and 37°C, corresponding to SOL and GEL states, respectively, and constant shear rate of  $10^{-1} \text{ sec}^{-1}$ . At the SOL state, the addition of 1% w/v of alginate (0.50 Pa.s) or chitosan (0.18 Pa.s) caused a significant viscosity-enhancing effect compared to the bioadhesive-free hydrogel (0.04 Pa.s) (Fig. 2). However, viscosity enhancement remained markedly below 1 Pa.s. No significant difference  
360 was however observed at the GEL state (37°C). It is common knowledge that bioadhesive polymers increase adhesion but decrease syringeability by raising the viscosity. Hence, the observed viscosity rise of our hydrogel at the SOL state maintained the balance between adhesiveness, syringeability and injectability. Viscosity measured at 5°C and 37°C and very low shear rate (i.e.  $10^{-3} \text{ s}^{-1}$ ) showed no significant difference between any of the tested

365 bioadhesives (supplementary Fig. S4). Finally, hydrogel erosion study was conducted on  
these hydrogels at 37°C *in vitro*. No statistical difference was obtained between bioadhesive-  
free hydrogel and alginate- or chitosan-containing hydrogel in terms of the time required to  
erode 50% (T50%) or 80% (T80%) of the hydrogels. The values for T50% and T80% were  
between 14.5 and 23.5 hours, and 23.2 and 45.9 hours, respectively (supplementary Table  
370 S2).

Given, on one hand, the comparable results between alginate and chitosan with respect to the  
resulting viscosity at 37°C and hydrogel erosion kinetics, and, on the other hand, the wide use  
of alginate in tissue engineering [48, 49], and the availability of an injectable alginate grade  
listed in the European pharmacopoeial monograph, we opted for alginate 1% w/v for the  
375 further course of the study.

### 3.3. Viscoelastic and rheological behavior of P407/P188/alginate hydrogel

P407/P188/alginate 20/2/1% w/v hydrogel was characterized by oscillatory and rotational  
rheometry.  $G'$  and  $G''$  moduli and dynamic viscosity  $\eta$  were plotted as a function of the  
temperature (Fig. 3A).  $G'$  and  $G''$  moduli were constant as long as the temperature was below  
380 a critical value where both moduli rose sharply to new steady values characterizing the GEL  
state.  $G'G''$  crossover indicated  $26.0^\circ\text{C} \pm 0.6^\circ\text{C}$  as  $T_{\text{sol-gel}}$ . Storage modulus  $G'$  was of  $15866$   
 $\text{Pa} \pm 1397 \text{ Pa}$  whilst loss factor  $\tan\delta$  was of 0.03, both at 37°C. Viscoelastic values were  
constantly assigned to a strong elastic gel at physiological temperature. In parallel, as  
temperature increased and hydrogel turned to GEL from SOL, viscosity followed a similar  
385 pattern than  $G'$  and  $G''$  moduli (Fig. 3A, right axis). Viscosity was practically constant ( $\sim 0.5$   
 $\text{Pa.s}$ ) then it increased abruptly once the temperature was higher than 25°C, reaching a new  
steady level with  $1.6 \times 10^3 \text{ Pa.s}$  at 37°C. This brutal thickening of the solution reflects the self-  
assembling of the copolymers: at low temperatures ( $< 25^\circ\text{C}$ ), interactions are mostly

intramolecular and become intermolecular at higher temperatures driving to gel formation.

390 The shear stress of P407/P188/alginate 20/2/1% w/v hydrogel (5°C and 37°C) with respect to the change in shear rate, in comparison with a Newtonian fluid, was profiled in Fig. 3B. As the shear rate was increased from  $10^{-3}$  to  $10^4$  s<sup>-1</sup>, the shear stress rose at 5°C and remained stable at 37°C around 100 Pa. A solution whose shear stress is directly proportional to the shear rate is considered as a Newtonian fluid. Therefore, the hydrogel at 5°C can be

395 assimilated to a Newtonian fluid. However, at 37°C, as expected, the hydrogel turned to a non-Newtonian fluid. Thus, the P407/P188/alginate hydrogel presented a shear-thinning flow at 37°C and this behavior confirmed the formation of gel above the  $T_{\text{sol-gel}}$ . This finding is consistent with the shear-thinning characteristics of poloxamer and alginate hydrogels due to the destruction of intermolecular bonds [31, 50]. The low viscosity and the absence of shear-

400 thinning behavior at 5°C revealed its SOL state. Likewise, this Newtonian-fluid behavior at the SOL state could be correlated to a good contact surface of the hydrogel at the injection site leading to a continual coverage and favorable bioadhesion.

### **3.4. In vitro release studies**

#### **3.4.1. Effect of hydrogel erosion on 5-FU release**

405 Prior to choosing the 5-FU concentration to be loaded in the hydrogel, two factors were taken into account: drug solubility at  $5^\circ\text{C} \pm 3^\circ\text{C}$  and drug therapeutic dose. 5-FU has a temperature-dependent solubility (11.9 mg/mL at 20°C) which is considerably lowered at low temperature resulting in its crystallization [51]. Moreover, 5-FU therapeutic dose in mice is documented to be between 5 and 85 mg/kg body weight [11, 52]. Hence, we opted for a concentration of 5

410 mg 5-FU/mL hydrogel (0.5% w/v) which provides an adequate drug dose in mice while avoiding insolubility matter at  $+5^\circ\text{C} \pm 3^\circ\text{C}$ . It is worth noting that 5-FU (0.5% w/v) addition to the P407/P188/alginate 20/2/1% w/v hydrogel allows to maintain a  $T_{\text{sol-gel}}$  in the targeted

range, as demonstrated by differential scanning calorimetry (supplementary Fig. S5). *In vitro* erosion behavior and concomitant 5-FU release from P407/P188/alginate 20/2/1% w/v hydrogel was studied at 37°C using a T-cell for flow-through dissolution. Cumulative 5-FU release and hydrogel erosion were presented on the left and right axis of the Fig. 4A, respectively. Results showed an initial fast release with 50% of 5-FU released in the first one hour and reached a total release after 9 hours. To better understand the release mechanism, the erosion of the hydrogel was also followed by measuring the poloxamer dissolved in the release medium using the Baleux dosage. The calibration curve is given in supplementary Fig. S2. Hydrogel exhibited a slow erosion with 50% of the poloxamers dissolved within 15 hours and a complete erosion measured after 24 hours. These two informations put together indicate that the major mechanism governing 5-FU release is diffusion. Similar results were obtained when water was used as release medium (data not shown). It is worth mentioning that the various mechanisms involved in drug release have been reviewed by *Siepmann and Siepmann* [53]. 5-FU release from hydrogel is thereby reported to be rapid and characterized by a burst effect. *Narasimhan and Langer* showed that burst release was controlled by the solubility of drug, the drug diffusion coefficient and the initial drug distribution within the polymeric carrier [54]. 5-FU is a small hydrophilic ( $\text{Log}P_{\text{octanol/water}} = -0.89$ ) drug molecule and the hydrogel network is a highly hydrated microscale environment. Thereby, it is expected that 5-FU diffuse freely through the extracellular water channels of the hydrogels matrix. Our result is thus in agreement with previous works on 5-FU release from poloxamer-based hydrogels where total release was achieved within few hours (90% in 1h [27]). It is known that the diffusion coefficient of a drug in the hydrogel decreases with increasing P407 content and bioadhesive material content, consistent with a consequent increase in viscosity and gel rigidity. However, more concentrated poloxamer-based hydrogel, i.e. 30% and 35% w/v P407, were reported to be very viscous and have very low syringeability, so they were not

suitable carriers for parenteral administrations [55]. Relating to what was previously mentioned, a balance between administration and handling easiness, thermogelling and drug  
440 release rate from hydrogel is sought.

Meanwhile, 5-FU stability in solution and the hydrogel was monitored over time. 5-FU solution (0.5% w/v) was stable at 37°C along the study and served as a control (Fig. 4A). When compared with 5-FU solution, the total released amount of 5-FU (97%) (Fig. 4A left  
445 axis) and the 5-FU chromatogram at 24 hours time point (Fig. 4B), showed that hydrogel composition did not compromise 5-FU stability at 37°C. Drug was noteworthy stable in both, solution and hydrogel matrices, for at least up to three days (data not shown).

### **3.4.2. Effect of alginate on 5-FU release**

Our hydrogel is meant to be administered intratumorally or to be applied subcutaneously after tumor excision. It is well known that after tissular injection, drug first diffuses from the  
450 vehicle then it is transported by convection phenomenon through the blood flow. To simulate the intended application, 5-FU release from hydrogel was carried out using a flow-cell model. Fig. 5 illustrated that in P407/P188 20/2% w/v hydrogel, the release of 50% of the loaded 5-FU occurred within 2.8 hours, whereas, in presence of 1% w/v alginate, the 50% release took place after 6.6 hours. Alginate, though devoid of viscosity-enhancing effect on the P407/P188  
455 20/2% w/v hydrogel at 37°C, was able to delay 5-FU release by two fold. Furthermore, the flow-cell model better mimics the tissular area by submitting the sample to the sole effect of the diffusion mechanism which explains the retardation of 5-FU release obtained here when compared to the Fig. 4A. To further retard release, the initial drug loading amount in hydrogel could be adjusted. In PEG-PCL-PEG hydrogel, doubling 5-FU amount resulted in delaying  
460 release from 83% to 66% in 24 hours [15], whereas in P407 (25% w/w) hydrogel, an increase in initial 5-FU concentration from 0.1 to 1.0% w/v increased the drug release rate [56]. Drug concentration increase is known to increase the diffusion coefficient of the drug and thereby

its release rate. In addition, using poloxamer hydrogels conjugated to nanoparticles encapsulating the 5-FU would be also beneficial to delay the drug release [57].

### 465 **3.5. *In vivo* antitumor efficacy of P407/P188/alginate/5-FU 20/2/1/0.5% w/v hydrogel**

Subsequent to physicochemical characterization of the hydrogel, antitumor efficacy was assessed on CT26-luc CRC model following two different treatment modalities.

#### **3.5.1. Hydrogel efficacy in the context of neoadjuvant therapy**

470 All the tested formulations were injected in the primary tumor implanted subcutaneously. Mice weight was stable following the injections (Fig. 6B), thus indicating the absence of systemic toxic effect due to the injected formulation. The drastic weight loss noted on day 18 in the control group ( $-11.6\% \pm 6.1\%$ ) and in the group which received the 5-FU-free hydrogel ( $-21.2\% \pm 2.8\%$ ) was due to the toxicity of the tumor itself which is known to be harmful  
475 towards mouse at advanced stage (mean tumor volumes  $599.9 \text{ mm}^3 \pm 50.3 \text{ mm}^3$  and  $574.5 \text{ mm}^3 \pm 62.7 \text{ mm}^3$ , respectively) (Table 1). In addition, significant tumor growth delay was seen in the group which received 5-FU hydrogel starting the day 13, i.e. five days after receiving the treatment, as compared to the control group ( $p \leq 0.05$ ) (Fig. 6C, supplementary Tables S3 and S4). The optical images of mice taken on day 15 showed a nearly disparition of  
480 the bioluminescence signal of both tumors in mice treated with 5-FU hydrogel compared to mice of the control group and mice which received the 5-FU-free hydrogel (Fig. 6D, supplementary Fig. S6). Since 5-FU-free hydrogel did not induce a reduction of the tumor size, the improved antitumor efficacy of 5-FU hydrogel was proven here to be due to the release of 5-FU. Likewise, in the 5-FU solution group, the mean tumor volume at day 18 was  
485 relatively similar to that of the group treated with 5-FU hydrogel ( $231.4 \text{ mm}^3 \pm 67.6 \text{ mm}^3$  and  $275.5 \text{ mm}^3 \pm 36.9 \text{ mm}^3$ , respectively) (Table 1). This discrepancy with some data of the literature showing a superior efficacy of the 5-FU hydrogel compared with the 5-FU solution

would arise from the difference in experimental parameters including the administered 5-FU dose, protocol and schedule of the treatment [11, 15, 58]. Furthermore, the toxicity expressed  
490 in mean body weight loss was lower in the 5-FU hydrogel group ( $-0.4\% \pm 2.9\%$ ) as compared to the 5-FU solution group ( $-3.6\% \pm 3.0\%$ ) at the end of the study (Table 1). Although not significant, this finding suggests that in the solution group, more drug would reach the systemic circulation. The IT delivery of 5-FU through hydrogel might reduce the chance of systemic absorption and overall increase its efficacy. Drug toxicity is then limited within a  
495 localized area where tumor cells lie. Neoadjuvant chemotherapy is prescribed for patients with locally advanced stage of rectal cancer [3-5]. The thermosensitive hydrogel presented in this work is low viscous liquid ( $\eta < 1 \text{ Pa}\cdot\text{s}$ ) and flowable formulation at ambient temperature, thus allowing an easy administration through syringe or fine catheter. Therefore, the clinical translation of the approach would be the IT injection of the hydrogel formulation containing  
500 the 5-FU at the rectal tumor site through an appropriate syringe-needle system [59]. If required, an endoscopic guidance could be applied in order to achieve accurate injection of the target lesion. Hence, tumors which are unresectable due to their big size, would benefit from local neoadjuvant chemotherapy by IT injection of the drug-loaded hydrogel before surgery.

### 505 **3.5.2. Hydrogel efficacy in the context of adjuvant therapy (after tumor excision)**

Herein, the SC tumor was removed and the mice was locally treated at the excision site with the hydrogel. Solutions such as 5-FU or water were not tested here as they cannot be retained in the tumor cavity, the group which received 5-FU-free hydrogel served as a control group. Mice weight, albeit decreased between day 14 and day 18, was stable afterwards showing a  
510 good tolerability for the treatment (Fig. 7B). This rapid weight loss happening on the day of treatment could be consecutive to the surgery procedure itself. Also here no systemic toxic effect was noted. The soft, moist surface, and affinity of the hydrogel with the tissues greatly



reduce the stimulation of the mice body. The notable weight loss noted on day 34 in the 5-FU-free hydrogel group (-21.8%) was due to the toxicity of the tumor itself as previously explained. Significant tumor growth delay of fifteen days was obtained in the group treated with 5-FU hydrogel compared to the control group ( $p \leq 0.01$ ) (Fig. 7C). Concordant images of one representative mouse of each group were shown in Fig. 7D. Moreover, mice survival was increased in the 5-FU hydrogel-treated group as compared to the 5-FU-free hydrogel group which exhibited a median of survival of 32 days (Fig. 7E). In sum, 5-FU hydrogel has proved to be very efficient in inhibiting CT26 CRC local recurrence when applied locally along with tumor resection surgery.

#### 4. Conclusion

We developed a P407/P188/alginate/5-FU 20/2/1/0.5% w/v thermosensitive hydrogel which possessed appropriate viscoelastic and physicochemical properties to ensure preclinical efficacy. At ambient temperature, hydrogel existed as free-flowing solution allowing injectability through a syringe and underwent a SOL-GEL transition at  $26.0^{\circ}\text{C} \pm 0.6^{\circ}\text{C}$  yielding a strong elastic gel at physiological temperature. While preserving drug stability, hydrogel achieved total 5-FU release between 24 and 48 hours *in vitro*. In two different local treatment modalities of CRC in mice, hydrogel demonstrated a significant tumor growth delay and local recurrence prophylaxis. After IT injection and post-excision application in neoadjuvant and adjuvant therapies, respectively, hydrogel was found to be efficient in suppressing tumor growth. Accordingly, stable drug release in the vicinity of tumor cells and localized cytotoxicity of 5-FU all contributed to facilitate the antitumor efficacy in mice models. Importantly, drug-free hydrogel, constituting the 5-FU vehicle, showed no toxic effect. P407/P188/alginate/5-FU 20/2/1/0.5% w/v thermosensitive hydrogel, though, represented an efficient local drug delivery system, further optimizations are foreseeable

regarding sustaining even greater the release rate, for instance, by adjusting 5-FU initial loading and strengthening the gel network.

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## 545 Declaration of Competing Interest

The authors declare no conflict of interest.

## References

- 550 [1] Cancer fact sheets-All cancers, International Agency for Reserach on Cancer WHO. <https://gco.iarc.fr/today/data/factsheets/cancers/39-All-cancers-fact-sheet.pdf>, 2020 (accessed 17 July 2020).
- [2] J. Ferlay, M. Ervik, F. Lam, M. Colombet, L. Mery, M. Piñeros, A. Znaor, I. Soerjomataram, F. Bray, Global cancer observatory: cancer today, Lyon, France: International Agency for Research on Cancer, (2018).
- 555 [3] K. Peeters, Dutch Colorectal Cancer Group. The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma, *Ann. Surg.*, 246 (2007) 693-701.
- [4] P. Georgiou, E. Tan, N. Gouvas, A. Antoniou, G. Brown, R.J. Nicholls, P. Tekkis, Extended lymphadenectomy versus conventional surgery for rectal cancer: a meta-analysis, *The lancet oncology*, 10 (2009) 1053-1062.
- 560 [5] A.B. Benson, A.P. Venook, T. Bekaii-Saab, E. Chan, Y.-J. Chen, H.S. Cooper, P.F. Engstrom, P.C. Enzinger, M.J. Fenton, C.S. Fuchs, Rectal cancer, version 2.2015, *J. Natl. Compr. Canc. Netw.*, 13 (2015) 719-728.
- [6] A. Carrato, Adjuvant treatment of colorectal cancer, *Gastrointestinal cancer research: GCR*, 2 (2008) S42.
- 565 [7] R.B. Hines, M. Bimali, A.M. Johnson, A.R. Bayakly, T.C. Collins, Prevalence and survival benefit of adjuvant chemotherapy in stage III colon cancer patients: Comparison of overall and age-stratified results by multivariable modeling and propensity score methodology in a population-based cohort, *Cancer Epidemiol.*, 44 (2016) 77-83.
- 570 [8] A.B. Benson III, D. Schrag, M.R. Somerfield, A.M. Cohen, A.T. Figueredo, P.J. Flynn, M.K. Krzyzanowska, J. Maroun, P. McAllister, E. Van Cutsem, American Society of Clinical

- Oncology recommendations on adjuvant chemotherapy for stage II colon cancer, *J. Clin. Oncol.*, 22 (2004) 3408-3419.
- 575 [9] T. André, C. Boni, M. Navarro, J. Taberero, T. Hickish, C. Topham, A. Bonetti, P. Clingan, J. Bridgewater, F. Rivera, A. de Gramont, Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial, *J. Clin. Oncol.*, 27 (2009) 3109-3116.
- [10] W.E. MacMillan, W.H. Wolberg, P.G. Welling, Pharmacokinetics of fluorouracil in humans, *Cancer Res.*, 38 (1978) 3479-3482.
- 580 [11] H. Yi, H.-J. Cho, S.-M. Cho, D.-G. Lee, A. Abd El-Aty, S.-J. Yoon, G.-W. Bae, K. Nho, B. Kim, C.-H. Lee, Pharmacokinetic properties and antitumor efficacy of the 5-fluorouracil loaded PEG-hydrogel, *BMC Cancer*, 10 (2010) 211.
- [12] N. Peppas, P. Bures, W. Leobandung, H. Ichikawa, Hydrogels in pharmaceutical formulations, *Eur. J. Pharm. Biopharm.*, 50 (2000) 27-46.
- 585 [13] L. Klouda, A. Mikos, Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery, *Eur. J. Pharm. Biopharm.*, 68 (2008) 34-45.
- [14] Q. Wang, H. Xu, X. Yang, Y. Yang, Drug release behavior from in situ gelatinized thermosensitive nanogel aqueous dispersions, *Int. J. Pharm.*, 361 (2008) 189-193.
- 590 [15] Y. Wang, C. Gong, L. Yang, Q. Wu, S. Shi, H. Shi, Z. Qian, Y. Wei, 5-FU-hydrogel inhibits colorectal peritoneal carcinomatosis and tumor growth in mice, *BMC Cancer*, 10 (2010) 402.
- [16] C. Ju, J. Sun, P. Zi, X. Jin, C. Zhang, Thermosensitive micelles–hydrogel hybrid system based on poloxamer 407 for localized delivery of paclitaxel, *J. Pharm. Sci.*, 102 (2013) 2707-2717.
- 595 [17] H. Cho, G.S. Kwon, Thermosensitive poly-(d, l-lactide-co-glycolide)-block-poly(ethylene glycol)-block-poly-(d, l-lactide-co-glycolide) hydrogels for multi-drug delivery, *J. Drug Target.*, 22 (2014) 669-677.
- [18] T. Ci, L. Chen, L. Yu, J. Ding, Tumor regression achieved by encapsulating a moderately soluble drug into a polymeric thermogel, *Sci. Rep.*, 4 (2014) 5473.
- 600 [19] C.-H. Chen, C.-Y. Kuo, S.-H. Chen, S.-H. Mao, C.-Y. Chang, K. Shalumon, J.-P. Chen, Thermosensitive injectable hydrogel for simultaneous intraperitoneal delivery of doxorubicin and prevention of peritoneal adhesion, *Int. J. Mol. Sci.*, 19 (2018) 1373.
- [20] D.-y. Fan, Z. Liu, Y. Tian, Injectable hydrogels for localized cancer therapy, *Frontiers in chemistry*, 7 (2019) 675.
- 605 [21] Y. Mao, X. Li, G. Chen, S. Wang, Thermosensitive hydrogel system with paclitaxel liposomes used in localized drug delivery system for in situ treatment of tumor: better antitumor efficacy and lower toxicity, *J. Pharm. Sci.*, 105 (2016) 194-204.

- [22] J.B. Wolinsky, Y.L. Colson, M.W. Grinstaff, Local drug delivery strategies for cancer treatment: gels, nanoparticles, polymeric films, rods, and wafers, *J. Control. Release*, 159 (2012) 14-26.
- 610 [23] A. Fakhari, J.A. Subramony, Engineered in-situ depot-forming hydrogels for intratumoral drug delivery, *J. Control. Release*, 220 (2015) 465-475.
- [24] I.R. Schmolka, Artificial skin I. Preparation and properties of pluronic F-127 gels for treatment of burns, *J. Biomed. Mater. Res.*, 6 (1972) 571-582.
- [25] R. Rowe, P. Sheskey, S. Owen, *Pharmaceutical Handbook of pharmaceutical excipients*, in, London UK and American Pharmaceutical Association, Washington, USA, 2005.
- 615 [26] R.E. Goldstein, On the theory of lower critical solution points in hydrogen-bonded mixtures, *J. Chem. Phys.*, 80 (1984) 5340-5341.
- [27] L.-L. Wang, W.-S. Zheng, S.-H. Chen, X.-Q. Fang, Development of in situ gelling and bio adhesive 5-fluorouracil enema, *PLoS One*, 8 (2013) e71037.
- 620 [28] S.-H. Paek, J.-J. Xuan, H.-G. Choi, B.C. Park, Y.-S. Lee, T.-C. Jeong, C.H. Jin, Y.-K. Oh, J.-A. Kim, Poloxamer 188 and propylene glycol-based rectal suppository enhances anticancer effect of 5-fluorouracil in mice, *Biol. Pharm. Bull.*, 29 (2006) 1060-1063.
- [29] A.P. Rokhade, N.B. Shelke, S.A. Patil, T.M. Aminabhavi, Novel hydrogel microspheres of chitosan and pluronic F-127 for controlled release of 5-fluorouracil, *J. Microencapsul.*, 24 (2007) 274-288.
- 625 [30] Z. Lin, W. Gao, H. Hu, K. Ma, B. He, W. Dai, X. Wang, J. Wang, X. Zhang, Q. Zhang, Novel thermo-sensitive hydrogel system with paclitaxel nanocrystals: High drug-loading, sustained drug release and extended local retention guaranteeing better efficacy and lower toxicity, *J. Control. Release*, 174 (2014) 161-170.
- 630 [31] J.Y. Chang, Y.-K. Oh, H.-g. Choi, Y.B. Kim, C.-K. Kim, Rheological evaluation of thermosensitive and mucoadhesive vaginal gels in physiological conditions, *Int. J. Pharm.*, 241 (2002) 155-163.
- [32] H.-J. Cho, P. Balakrishnan, E.-K. Park, K.-W. Song, S.-S. Hong, T.-Y. Jang, K.-S. Kim, S.-J. Chung, C.-K. Shim, D.-D. Kim, Poloxamer/cyclodextrin/chitosan-based thermoreversible gel for intranasal delivery of fexofenadine hydrochloride, *J. Pharm. Sci.*, 100 (2011) 681-691.
- 635 [33] S. Cafaggi, R. Leardi, B. Parodi, G. Caviglioli, E. Russo, G. Bignardi, Preparation and evaluation of a chitosan salt-poloxamer 407 based matrix for buccal drug delivery, *J. Control. Release*, 102 (2005) 159-169.
- 640 [34] M. Davidovich-Pinhas, H. Bianco-Peled, Alginate-PEGAc: A new mucoadhesive polymer, *Acta Biomater.*, 7 (2011) 625-633.
- [35] J. Hurler, N. Škalko-Basnet, Potentials of chitosan-based delivery systems in wound therapy: Bioadhesion study, *J. Funct. Biomater.*, 3 (2012) 37-48.

- 645 [36] B. Baleux, Colorimetric determination of silver of nonionic polyoxyethylene surface using iodo-iodated solution, *Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences Serie C*, 274 (1972) 1617-1620.
- [37] J. Seguin, B.-T. Doan, H. Latorre Ossa, L. Jugé, J.-L. Gennisson, M. Tanter, D. Scherman, G.G. Chabot, N. Mignet, Evaluation of nonradiative clinical imaging techniques for the longitudinal assessment of tumour growth in murine CT26 colon carcinoma, *Int. J. Mol. Imaging*, 2013 (2013).
- 650 [38] G. Dumortier, J.L. Grossiord, F. Agnely, J.C. Chaumeil, A review of poloxamer 407 pharmaceutical and pharmacological characteristics, *Pharm. Res.*, 23 (2006) 2709-2728.
- [39] K. Almdal, J. Dyre, S. Hvidt, O. Kramer, Towards a phenomenological definition of the term 'gel', *Polymer Gels and Networks*, 1 (1993) 5-17.
- 655 [40] J. Chen, R. Zhou, L. Li, B. Li, X. Zhang, J. Su, Mechanical, rheological and release behaviors of a poloxamer 407/poloxamer 188/carbopol 940 thermosensitive composite hydrogel, *Molecules*, 18 (2013) 12415-12425.
- [41] E. Batrakova, S. Lee, S. Li, A. Venne, V. Alakhov, A. Kabanov, Fundamental relationships between the composition of pluronic block copolymers and their hypersensitization effect in MDR cancer cells, *Pharm. Res.*, 16 (1999) 1373-1379.
- 660 [42] G. Dumortier, N. El Kateb, M. Sahli, S. Kedjar, A. Boulliat, J. Chaumeil, Development of a thermogelling ophthalmic formulation of cysteine, *Drug Dev. Ind. Pharm.*, 32 (2006) 63-72.
- [43] G. Bonacucina, M. Spina, M. Misici-Falzi, M. Cespi, S. Pucciarelli, M. Angeletti, G.F. Palmieri, Effect of hydroxypropyl  $\beta$ -cyclodextrin on the self-assembling and thermogelation properties of Poloxamer 407, *Eur. J. Pharm. Sci.*, 32 (2007) 115-122.
- 665 [44] J.C. Gilbert, J. Hadgraft, A. Bye, L.G. Brookes, Drug release from Pluronic F-127 gels, *Int. J. Pharm.*, 32 (1986) 223-228.
- [45] N. Zeng, G. Dumortier, M. Maury, N. Mignet, V. Boudy, Influence of additives on a thermosensitive hydrogel for buccal delivery of salbutamol: Relation between micellization, gelation, mechanic and release properties, *Int. J. Pharm.*, 467 (2014) 70-83.
- 670 [46] T.R. Hoare, D.S. Kohane, Hydrogels in drug delivery: Progress and challenges, *Polymer*, 49 (2008) 1993-2007.
- [47] E. Giuliano, D. Paolino, M. Fresta, D. Cosco, Mucosal applications of poloxamer 407-based hydrogels: An overview, *Pharmaceutics*, 10 (2018) 159.
- 675 [48] G. Klöck, A. Pfeffermann, C. Ryser, P. Gröhn, B. Kuttler, H.-J. Hahn, U. Zimmermann, Biocompatibility of mannuronic acid-rich alginates, *Biomaterials*, 18 (1997) 707-713.
- [49] K.Y. Lee, E. Alsberg, D.J. Mooney, Degradable and injectable poly (aldehyde guluronate) hydrogels for bone tissue engineering, *Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, 56 (2001) 228-233.
- 680

- [50] F. Cuomo, M. Cofelice, F. Lopez, Rheological characterization of hydrogels from alginate-based nanodispersion, *Polymers*, 11 (2019) 259.
- 685 [51] M. Barberi-Heyob, M. Watelet, J. Merlin, C. Bleger, B. Schroeder, Stability of 5-fluorouracil solutions according to different parameters, *Bull. Cancer*, 82 (1995) 1025-1031.
- [52] D. Alishekevitz, R. Bril, D. Loven, V. Miller, T. Voloshin, S. Gingis-Velistki, E. Fremder, S.J. Scherer, Y. Shaked, Differential Therapeutic Effects of Anti-VEGF-A Antibody in Different Tumor Models: Implications for Choosing Appropriate Tumor Models  
690 for Drug Testing, *Mol. Cancer Ther.*, 13 (2014) 202-213.
- [53] J. Siepmann, F. Siepmann, Mathematical modeling of drug delivery, *Int. J. Pharm.*, 364 (2008) 328-343.
- [54] B. Narasimhan, R. Langer, Zero-order release of micro-and macromolecules from polymeric devices: the role of the burst effect, *J. Control. Release*, 47 (1997) 13-20.
- 695 [55] K. Derakhshandeh, M. Fashi, S. Seifoleslami, Thermosensitive Pluronic® hydrogel: prolonged injectable formulation for drug abuse, *Drug Des. Devel. Ther.*, 4 (2010) 255.
- [56] S. Miyazaki, S. Takeuchi, C. Yokouchi, M. Takada, Pluronic F-127 gels as a vehicle for topical administration of anticancer agents, *Chem. Pharm. Bull. (Tokyo)*, 32 (1984) 4205-4208.
- 700 [57] M.S.H. Akash, K. Rehman, Recent progress in biomedical applications of Pluronic (PF127): Pharmaceutical perspectives, *J. Control. Release*, 209 (2015) 120-138.
- [58] Y.S. Jung, D.-H. Koo, J.-Y. Yang, H.-Y. Lee, J.-H. Park, J.H. Park, Peri-tumor administration of 5-fluorouracil sol-gel using a hollow microneedle for treatment of gastric cancer, *Drug deliv.*, 25 (2018) 872-879.
- 705 [59] A. Marabelle, R. Andtbacka, K. Harrington, I. Melero, R. Leidner, T. de Baere, C. Robert, P.A. Ascierto, J.-F. Baurain, M. Imperiale, Starting the fight in the tumor: expert recommendations for the development of human intratumoral immunotherapy (HIT-IT), *Ann. Oncol.*, 29 (2018) 2163-2174.

## Figure captions

**Fig. 1** Effect of P407/P188 ratio on the rheological properties of P407/P188 thermosensitive hydrogel: (A)  $T_{\text{sol-gel}}$ , (B) loss (viscous) modulus  $G''$  at 37°C, (C) storage (elastic) modulus  $G'$  at 37°C, (D) loss factor  $\tan\delta$  at 37°C. Measurements were carried out in the linear viscoelastic range.  $n=13$ . (2-column fitting)

**Fig. 2** Viscosity of the thermosensitive hydrogel P407/P188 20/2% w/v upon addition of 1% w/v alginate or 1% w/v chitosan at (A) 15°C (SOL state) and (B) 37°C (GEL state). Viscosity was measured at  $10^{-1} \text{ sec}^{-1}$  shear rate.  $n=3$  mean  $\pm$  SD. One way ANOVA with Tukey's multiple comparison test ns  $P > 0.05$ , \*  $P \leq 0.05$ , \*\*\*\*  $P \leq 0.0001$ . (2-column fitting)

**Fig. 3** Rheological characterization of P407/P188/alginate 20/2/1% w/v thermosensitive hydrogel. (A) Variation of storage (elastic) modulus  $G'$  (left axis), loss (viscous) modulus  $G''$  (left axis) and viscosity  $\eta$  (right axis) as a function of a temperature sweep at 1°C/min heat rate. Viscosity was measured at  $10^{-1} \text{ sec}^{-1}$  shear rate. (B) Rheogram (flow curve) plot showing shear stress  $\tau$  as a function of shear rate at 5°C (SOL state) and 37°C (GEL state) compared to a Newtonian fluid. (2-column fitting)

**Fig. 4** (A) *In vitro* hydrogel erosion (right axis) and simultaneous 5-FU release (left axis) kinetics from P407/P188/alginate/5-FU 20/2/1/0.5% w/v in PBS at 37°C. 5-FU solution in PBS at 37°C was used as control for drug stability.  $n=3$  mean  $\pm$  SD. (B) Chromatograms of 5-FU eluted at 9.5 min from 5-FU solution and 5-FU hydrogel samples at 24 hours time point. (2-column fitting)

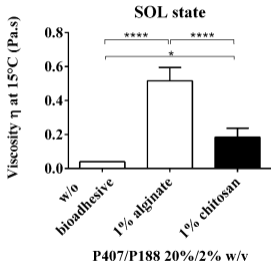
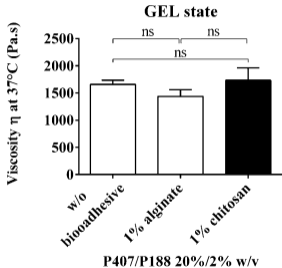
**Fig. 5** Cumulative *in vitro* release of 5-FU from P407/P188/alginate/5-FU 20/2/1/0.5% w/v hydrogel (1% alginate) compared to release from P407/P188/5-FU 20/2/0.5% w/v hydrogel (w/o alginate) in water at 37°C in T-cell for flow-through dissolution.  $n=3$  mean  $\pm$  SD. (1-column fitting)

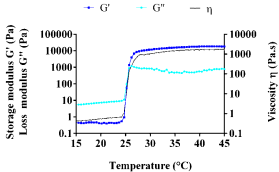
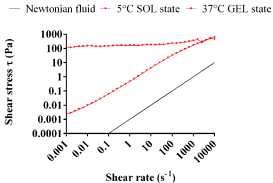


**Fig. 6** Evaluation of P407/P188/alginate/5-FU 20/2/1/0.5% w/v hydrogel antitumor efficacy after a single intratumoral injection in mice bearing subcutaneous CT26-luc tumor. (A) Schedule of the experiment. (B) Mean body weight change expressed in percentage monitored from day 4 to day 18 post-tumor implantation. (C) Longitudinal monitoring of the tumor growth over time by caliper. (D) Bioluminescence images of one representative mouse of each group on day 15 (7 days after formulation injection). Data were submitted to two way ANOVA with Tukey's multiple comparisons test, ns  $P > 0.05$ , \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$  as compared to the control group. (*2-column fitting*)

**Fig. 7** Evaluation of P407/P188/alginate/5-FU 20/2/1/0.5% w/v hydrogel antitumor efficacy after a single application after surgical excision of subcutaneous CT26-luc tumor. (A) Schedule of the experiment. (B) Mean body weight change expressed in percentage monitored from day 14 to day 34 post-tumor implantation. (C) Longitudinal monitoring of the tumor growth over time by optical imaging. (D) Bioluminescence images of one representative mouse of each group on days 11 to 29 (15 days after tumor excision and local hydrogel administration). (E) Mice survival after tumor excision and local administration of the hydrogels. Data were submitted to two way ANOVA with Tukey's multiple comparisons test, ns  $P > 0.05$ , \*\* $p \leq 0.01$  as compared to control group. (*2-column fitting*)

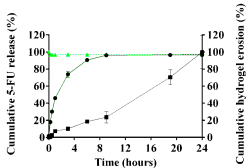
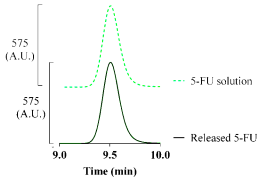


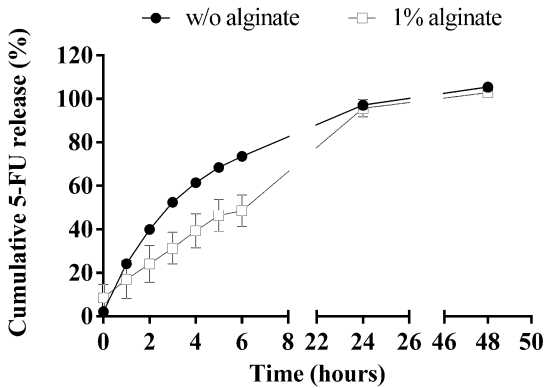
**A****B**

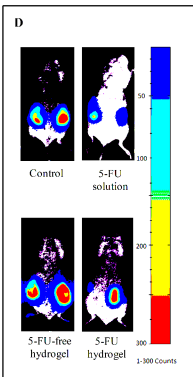
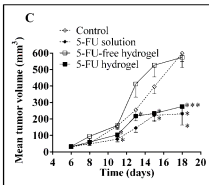
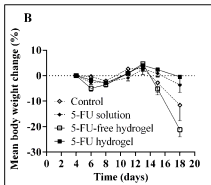
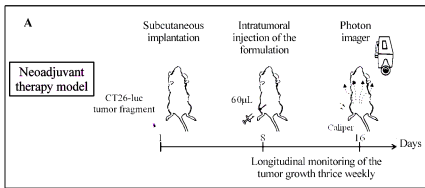
**A****B**

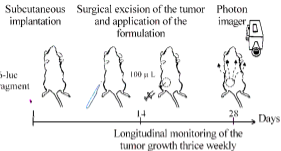
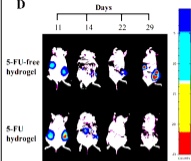
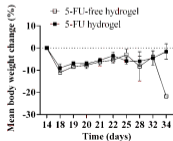
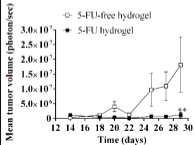
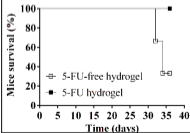
**A**

5-FU solution    Released 5-FU    Dissolved poloxamer

**B**





**A****D****B****C****E**



## Tables

**Table 1.** The change in body weight and tumor volume in mice after a single injection on the eighth day of the different formulations in neoadjuvant therapy model.

<b>Formulation</b>	<b>5-FU dosage (mg/kg)</b>	<b>Mean body weight change <math>\pm</math> SEM (%)</b>	<b>Mean tumor volume <math>\pm</math> SEM (mm<sup>3</sup>) on day 18</b>
Water (control)	0	-2.1 $\pm$ 0.6 (day 8)	599.9 $\pm$ 50.3
		-11.6 $\pm$ 6.1 (day 18)	
5-FU solution 0.5% w/v	15	-2.6 $\pm$ 1.0 (day 8)	231.4 $\pm$ 67.6
		-3.6 $\pm$ 3.0 (day 18)	
5-FU-free hydrogel	0	-3.5 $\pm$ 0.6 (day 8)	574.5 $\pm$ 62.7
		-21.2 $\pm$ 2.8 (day 18)	
5-FU hydrogel 0.5% w/v	15	-2.9 $\pm$ 1.3 (day 8)	275.5 $\pm$ 36.9
		-0.4 $\pm$ 2.9 (day 18)	

