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Combination of tumor cell anti-adhesion and anti-tumor effect to prevent recurrence after cytoreductive surgery in a mice model

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Novelty and Impact

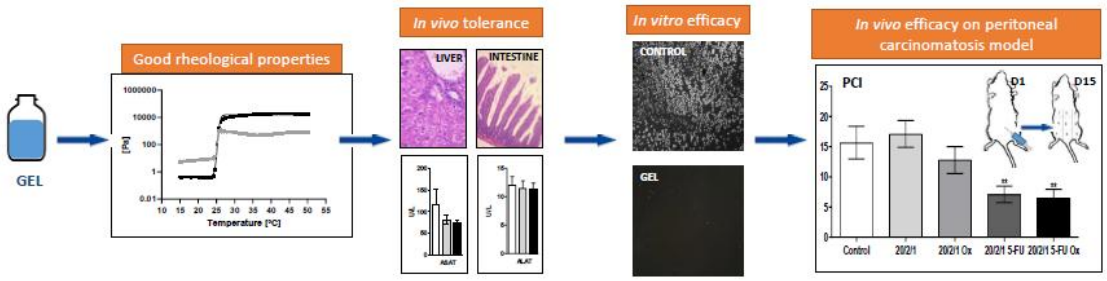
A tumor cell anti-adhesive thermogel was used as a drug delivery system to prevent tumor cells implantation in a peritoneal metastatic context. The bioavailability and the efficacy of the formulation was investigated and offered a first proof of concept in preclinical mice model.

Abstract

One of the main problems of colorectal cancer is not the treatment of the primary tumor but the metastatic stage. Means of metastatic spread is the invasion of the peritoneal cavity which leads to peritoneal metastasis (PM). PM cannot be easily cured, and the current treatments is rather heavy, combining cytoreductive surgery with intravenous and intraperitoneal chemotherapy. This therapeutic procedure is associated with significant morbidity, altered patient quality of life and poor prognosis. We postulated that development of a prophylactic treatment could be of high interest in this context. In this study, we formulated an anti-adhesive thermogel which contains chemotherapeutics to play a role of a barrier against tumor cells implantation, avoiding their adhesion and treating the remaining tumor cells with chemotherapy intraperitoneally in a mice model of PM. The bioavailability of the thermogel was tested intraperitoneally in mice. No sign of toxicity was observed in terms of change in body weight, anatomopathology and blood biomarkers. In vitro experiments proved that the thermogel induced limited adhesion of the tumor cells. Loading of oxaliplatin (Ox) and 5-Fluorouracil (5-FU) into the thermogel were able to significantly decreased peritoneal carcinomatosis index (PCI) (-58%) and ascites (-70%) in a murine model of peritoneal metastases. These pre-clinical results confirmed that smart thermogel associated with standard chemotherapy 5-FU and Ox could be a good candidate to decrease the risk of tumor cell implantation during cytoreductive surgery and prevent future metastatic process.

Keywords: Peritoneal carcinomatosis, thermogel, poloxamer, chemotherapy.

Graphical Abstract



Introduction:

Peritoneal metastasis (PM) is considered as a terminal disease for a majority of etiology. Despite improvement in the overall outcome using recent therapeutic developments and aggressive treatment strategies with cytoreductive surgery followed by hyperthermic intraperitoneal chemotherapy (HIPEC), survival remains limited for colorectal cancer PM. A pooled analysis of North central cancer treatment group Phase III trials, reported a median overall survival (OS) at 12 months shorter than for other metastatic site [1]. During the last decade many efforts have been done to understand and anticipate the risk of PM process. For primary colon cancer, risk factors have been identified as advanced tumor (T) status, perforated tumor, advanced node (N) status, ovarian tumor location and limited carcinomatosis associated resection[2–4]. For gastric cancer, risk factors are more probably related to the surgical resection and advanced tumor status [5]. Free tumor cells could adhere post- surgery to form a metastatic mass. Prevention of tumor cells adhesion post-surgery seems obvious in this context. The difficulty is that the prophylactic treatment should be easily implemented according to the clinical situation found by the surgeon.

Prevention of PM have been proposed using different strategies. For some authors, a systematic second look could be proposed and prophylactic hyperthermic intraperitoneal chemotherapy (HIPEC) delivered [6]. However, a randomized trial testing that therapeutic solution has recently failed [7]. A clinical trial is actually ongoing for gastric cancer without PM giving prophylactic HIPEC at the end of the gastric resection [8]. The major limitation of that strategy is the fact that in case of success, common cases of gastric or colonic cancer surgery will have to be performed in highly specialized teams able to perform HIPEC. Regarding the cost and the health care organization, it is not realistic for many countries. As a result, an easy-to-use preventing treatment of PM is still needed.

PM is caused for a part by free cancer cells exfoliated from tumors invading the serosal layer. If surgeons are able to detect during the surgical resection that a high risk exist, it could be possible to give at that time a preventive drug administration using a drug delivery carrier containing chemotherapy. The advantage of that strategy is to offer a cost-effective solution to all surgeons

without the need of a specific material. We, and others, have tested various chemotherapies and carriers to offer surgeons an easy to use solution to treat suspicious carcinomatosis [9]. We report here a new association of a specific gel carrier able to control tumor cell implantation, mimicking a clinical situation in an animal cancer orthotopic model.

Regarding these considerations, the pharmaceutical form was developed with a dual objective i) liquid at ambient temperature to have an easy form to handle in the surgery room ii) promote anti-adhesion of tumor cells. Smart hydrogel with a sol-gel phase transition was chosen for these properties. Thermosensitive hydrogels present numerous advantages. The sol-phase of the formulation confers a homogeneous preparation and allows easy sterilization, administration and dose adjustment. The gel-state insures *in situ* gelification and consequently local and prolonged action [10]. A review of the various delivery systems proposed in this pathology was recently submitted by our group [11]. Based on this survey and the clinical requirements, the composition of the thermogel was chosen with the following objectives i) able to gelify around 27-30°C providing both a transferrable manufacturing process and facilitating local injection of the solution in the peritoneal cavity which will turn to a gel at body temperature ii) viscoelastic properties tending towards ideally elastic (tan δ lower than 0.1) iii) prevent tumor cell adhesion iv) able to carry and release a combination of cytotoxic agents v) involving no local or systemic toxicity for safety of use.

To answer these multiple objectives, we turned to already approved pharmacopeia excipients and based our formulation on poloxamer main content to provide the thermosensitive property. Poloxamer 407 (P407) is a triblock copolymer consisting of a central hydrophobic block of polypropylene glycol of 56 repeat units flanked by two hydrophilic blocks of polyethylene glycol (PEG) constituted by 101 repeat units. It is well known for its capacity to self-assemble into micelles, than organize in a thermogel in response to increasing concentration and/or temperature[10]. Interestingly, this polymer has been proposed in the literature to reduce post-operative adhesions after infertility surgery, laminectomy or laparoscopy [12–14]. Some authors also added copolymers in the formulation to reduce post-operative pelvic adhesions. Kim et al. showed that poloxamer/alginate better prevent pelvic adhesion severity and microscopic fibrosis as compared to hyaluronic acid/cellulose [15]. More recently, Cho et al. designed P407 nanodisks charged with rapamycin or paclitaxel to prevent post-surgical adhesions and reduce tumor burden in the peritoneal cavity [16].

In this study, we proposed an original composition of poloxamers and alginate containing a combination of anticancer drugs clinically approved for colorectal cancer treatment 5-fluorouracile (5-FU) and oxaliplatin (Ox) to empeach tumor cell adhesion and dissemination during the surgery of colorectal carcinoma, and treat left inoperable metastases [17]. To answer this goal, mechanical resistance and rheofluidising properties of the thermogel were assessed. Compatibility and efficacy

of the combination of drugs embedded within the gel were determined by HPLC and in vitro. The biological compatibility and the vivo efficacy were evaluated following the development of a PM development in a model of colorectal carcinomatosis in immune competent mice.

Materials and methods

Materials

The thermogels were prepared using sterile water Versylene® purchased from Fresenius Kabi France (Sèvres, France). Kolliphor® P407 and Kolliphor® P188 of pharmaceutical use grade were a kind gift from BASF (Ludwigshafen, Germany). 5-FU (Acofarma,) and Ox (Cipla Banglore,) were of pharmaceutical grade.

Preparation of the thermogel

The necessary amounts of P407 and P188 were mixed with approximately two thirds of the targeted final solution volume of water (Millipore, 0.22 [im,Resistivity= 18.3 MH/cm] in a glass beaker. The mixture was stirred, while lying in an ice bath, during several hours with the help of a magnetic stirrer and then left overnight at 4°C. The mixture was dissolved in the upcoming day and the volume was adjusted with water to the exact desired volume (with the help of a graduated cylinder). Desired amounts of alginate (Manucol DH, FMC BioPolymer, 9005-38-3) and anticancer agents were then added to the liquid hydrogel. The gelling composition was mixed until all components were dissolved and subsequently stored at 4 °C. Gel composition will be expressed in the following experimental section as: (w/v) % P407/ (w/v) % P188/ (w/v) % alginate / (w/v) % anticancer agents.

Rheological characterization of thermogel

The rheology measurements were carried out on rheometer (Anton Paar model MCR 102, Courtaboeuf, France), using a cone-plane geometry CP50-1 (50 mm; 1 °) and an air gap of 0.10 mm. The measurements were repeated 3 times with a volume of 750 µL of thermogel. Non-destructive oscillatory measurements at 1 Hz, was used to obtain the elastic modulus (G'), the viscous modulus (G") and the phase angle (tan σ = G"/G'). The data was then processed using the associated software Anton Paar Rheo Compass 1.20.

Evaluation of 5-FU and Ox compatibility at 4°C in the thermogel

5-FU and Ox solution at the respective concentrations of 0.5 % w/v (5 mg / mL) and 0.1 % w/v (1 mg/mL) were prepared separately or in combination in water for injection (WFI). The equivalent concentration of the drugs was prepared in P407/P188/alginate 20/2/1 % thermogel. Aliquots of this

preparation (400 µL) were prepared and incubated at 4°C for the experiment. At 0, 3 and 24 hours, a sample of 20 µLwas drawn off and diluted 700 times then filtered with an Amicon 3 KDa filter (Amicon Ultra-0.5 ml, Ultracel® 3Kda). The sample was then centrifuged at 14,000 rpm for 40 min. The filtrate obtained was analyzed by HPLC to determine the concentration of each drug using a Shimadzu LC- 20AD system (Duisburg, Germany) connected to a C18 column (µBondapak C18, 10 µm, 125 A, 3,9x300 mm (Waters WAT027324). Analyses were performed at 25°C with detection at 266 nm and 250 nm (fluorescence detector, RF-10AXL, Shimadzu). Analyses were performed on 3 separate samples.

Release studies

The hydrogel (1 mL) was introduced into a 20 mL vial and placed at 5°C ± 3°C for 10 min in order to homogenize the distribution of the hydrogel at SOL state in the bottom of the vial. Afterwards, the vial was placed in an incubator set at 37°C and allowed to gelify during 30 min. Subsequently, 5 mL of pre-warmed (37°C) release medium (PBS, pH 7.3, 0.01 M), 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, 0.25, 0.5, 1, 2, 24, 48, 96, 168 and 216 hours), the release medium (200 µL) was sampled and replaced with an equal volume of pre-warmed fresh PBS. The amounts of released 5-fluorouracil and oxaliplatin were measured by HPLC, following the protocols described in the section above. All measurements were conducted in triplicate and mean values ± standard deviations were reported.

Cell line

CT26-Luc cell line, a murine colon cancer, kindly provided by Prof. Lea Eisembach Weizmann (Institute of Science Rehovot, Israel) were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10 % of fetal bovine serum (Gibco Life technologies) and 1 % of antibiotic (100 µM streptomycin, 100U/mL penicillin, Gibco Life technologies). These cell lines were cultured at 37°C in a 5 % CO_2-humidified atmosphere.

Anti-adhesive effect of the thermogel

6 well plate was coated with 300 µL of the thermogel and incubated for 30 min at 37°C. Then, 60.000 CT26-luc cells were seeded in each well. At 10, 20, 30, 45, 60, 90 & 120 minutes the cell monolayer was washed, fixed with 75 % of ethanol and finally stained with haematoxylin eosin. The adherent cells were then counted as comparison with control wells without thermogel. The evaluation of the adhesion was realized on 8 pictures and the result was expressed by mean ± SEM.

In vitro efficacy of combined Ox and 5-FU in the thermogel

Cells were seeded in Falcon™ Cell Culture Inserts (Cyclopore® Falcon® 353095) for 24h at 37°C (800 000 cells per insert in 1 mL of medium). At day 2, the apical medium was removed and 200µl of thermogel or culture medium control solution was put on the insert. At day 3, plates were incubated at 4°C for ten minutes, in order to liquefy the gel and the wells were wash two times with culture medium, to removed dead cells and thermogel residues. A solution of the 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, 298-93-1; 0.5 mg/mL) in culture medium was incubated with cells for 4 hours. The solution was removed and replaced by 100 µL of Dimethyl sulfoxide DMSO and the plate was shaken during 10 min. The absorption was subsequently measured at 562 nm (TECAN Infinite F200 Pro, Tecan Trading AG, Switzerland) in a 96-well plate (TPP Techno Plastic Products, 92024). The viability was estimated in percent of absorbance of the control wells.

In vivo tolerance of the thermogel

In vivo experiments were performed with respect to standard European-community-guideline requirements for laboratory-animal care and use. The laboratory number of national accreditation for animal experimentation is C75-10-03 (November 27-2012). The registration number of the ethics committee for the experiments is APAFIS = 3944-2015 1 22813591563 v3, the project is authorized as number 02095-03 at the date of March 25 – 2016 for a 5 years period. For the experimentation, only female mice BALB/C (Charles River, Arbresle, France) were used because of difference in tumor growth as compared to male mice. Female BALB/cJRj mice were injected with 250 µL of P407 20% thermogel (n=8) or P407/P188/alginate 20/2/1 % thermogel (n=8) in the peritoneal cavity. The weight monitoring was realized every 2-3 days. 14 days after thermogel injection, mice were anesthetized (isoflurane 2 %) and the blood was collected with cardiac puncture for the evaluation of hemotoxicity and hepatic toxicity. Then, mice were sacrificed by cervical dislocation and brain, liver, kidney, lung and intestine were removed and were fixed in phosphate buffer 4% formaldehyde for anatomopathology. The organs were included in paraffin, cut and the routine diagnostic pathology hematoxylin and eosin (HE) stain was applied onto the organ slices obtained.

Efficacy of combined Ox and 5-FU thermogel in peritoneal carcinomatosis model

A murine model of peritoneal carcinomatosis was obtained through an intraperitoneal injection (IP) of 1 x 10^4 CT26-Luc cells in order to mimic the spelling of tumor cells that can happens during a surgical procedure. Different treatments were injected 1h after injection of tumor cells, and five different groups of mice (n = 10) were studied: (i) control group: water for injection, (ii) P407/P188/alginate in 20/2/1%, (iii) P407/P188/alginate/5-FU 20/2/1/0,24% w/v (5-FU 2,4 mg /mL),

(iv) P407/P188/alginate/Ox 20/2/1/0,048 % w/v (Ox 0,48 mg /mL) (v) P407/P188/alginate/5-FU/Ox 20/2/1/0,24/0,048 % w/v. The well-being of mice was checked twice a week. At day 15, mice were killed by cervical dislocation. Then, a postmortem examination was performed through a laparotomy. Visceral and parietal peritoneum were analyzed to detect nodules and the peritoneal carcinomatosis index (PCI) was calculated according as previously described[18]Briefly, the abdomen was divided in 12 zones and a scoring was established from 0 to 3 according to the presence and the size of tumor nodules.

Statistical analysis

Analysis was performed with GraphPad Prism software. A Spearman test was applied to evaluate the correlation between shear modulus and viscosity for the 20/2/1 formulation. The 2 ways ANOVA with Bonferroni's multiple comparisons test was used for the comparison of the weight after i.p. injection of the formulation. A nonlinear fit exponential growth equation was applied on the adhesion result and Sidak's multiple comparisons statistical analysis was realized. The Cytotoxicity of the chemotherapeutic agents 5-FU and Ox and the in vivo efficacy was assessed with one way ANOVA with turkey multiple comparison. For all of the result the statistical significance was resumed as follows: Ns $P > 0.05$; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Results

Rheological characterization of the thermogel

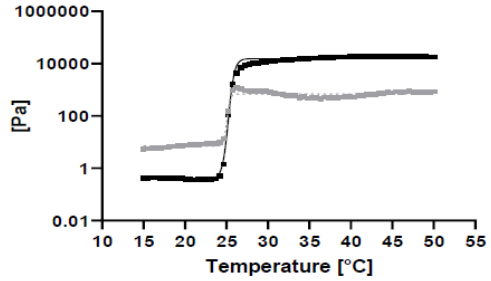
In order to determine the best formulation corresponding to the target profile, we performed rheology experiments at 37°C. The results obtained are listed in Figure 1. The measurements were carried out in a linear regime to be free from the action of the deformation and to focus only on the effect of temperature on the studied thermogel. The gelation temperature (Tgel) was determined by measuring the elastic (G') and viscous (G'') moduli of the thermogel as a function of the temperature. The crossing of the moduli indicates the sol-gel phase transition and therefore the Tgel (Figure 1A-B). The Tgel was comprised between 25.4 and 27.7°C, meaning an injectability of all formulations tested at surgery room temperature. If we consider the elasticity value, the targeted criterion was respected and was found upper than 10000 Pa. For further experiments, we chose the combination with the higher G' value (20/2 and 20/2/1). An example of the rheological curve obtained for these 2 formulations are represented in Figure 1A**Erreur ! Source du renvoi introuvable.**.

The ratio between loss and storage moduli (Tanδ) indicate the loss factor (Figure 1B). With a loss factor near 0, thermogel is assimilated to viscoelastic solid. This shows an internal mechanical

resistance towards external mechanical constraints despite the absence of gel reticulation. The loss factor obtained for this gel therefore allows to predict a good mechanical resistance at 37°C after *in situ* administration.

The inverse correlation between the viscosity and the Shear modulus indicate a pseudoplastic behavior of the formulation P407/P188/alginate 20/2/1 (Figure 1C). This property is crucial for the injectability of the thermogel, but also for a good covering of the peritoneal cavity.

A



B

P407 / P188 / Protanal %wt	Tgel (°C)	G' (Pa) 37°C	G'' (Pa) 37°C	Tanδ 37°C
20/2/0	26.0 ±0.1	18200±500	590±60	0.03±0.002
20/2/0.25	27.0 ±0.2	14500±950	620±90	0.04±0.01
20/2/0.5	26.0 ±0.1	15200±100	440±03	0.03±0.000
20/2/1	25.4 ±0.1	17400±250	500±09	0.03±0.001

C

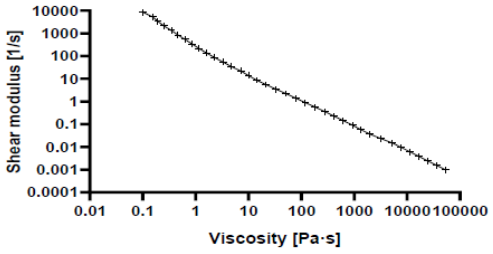
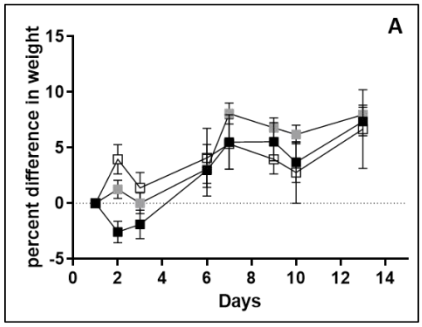


Figure 1 : P407/P188/alginate thermogel rheological results. (A) Representation of the sigmoidal fitting curve of the viscous modulus G" (grey) and the elastic modulus G' (black) for 20/2/1 thermogel. (B) Rheological parameters of the different formulations tested. n=3 mean ±SD. (C) Spearman correlation between shear modulus and viscosity for the P407/P188/alginate 20/2/1 formulation (n=6, P<0,0001****).

In vivo tolerance of the main component of the thermogel

The *in vivo* toxicity of the thermogel was first investigated. P407, main component in gel (20 % w/v), and complete gel formulation P407/P188/alginate (20/2/1) were injected in mice intraperitoneally. The weight of the mice was followed for 14 days (**Erreur ! Source du renvoi introuvable.**). Despite the slight decrease of the weight (**Erreur ! Source du renvoi introuvable.**A), observed for the complete formulation 20/2/1 48h after the injection (-2.57 %); no significant difference in weight variations was denoted between each group (**Erreur ! Source du renvoi introuvable.**B).

Fourteen days after the injection described above, several toxicities analyses were undertaken (Figure 2C-M). Regarding white blood cells, lymphocytes, monocytes, granulocytes, platelets, red blood cells and hematocrit levels, no significant difference in blood analysis could be denoted (Figure 2C-E). Similarly, no significant difference was observed in transaminases dosage between each group which is in favor of good hepatic tolerance (Figure 2F-G). Moreover, as we also expected, no toxicity was denoted in cerebellum, brain, kidney, small intestine, lung and liver anatomopatological slices (Figure 2H-M).



B

Days	Control			P407			P407/P188/Alginate 20/2/1		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	0	4	0	0	8	0	0	8
2	3,95	1,32	4	1,25	0,82	8	-2,57	0,98	8
3	1,39	1,39	4	0,00	0,94	8	-1,88	1,29	8
6	4,09	2,65	4	3,10	1,31	8	2,97	2,31	8
7	5,34	2,27	4	8,07	0,95	8	5,51	2,45	8
9	3,95	1,32	4	6,79	0,90	8	5,55	1,71	8
10	2,78	2,78	4	6,19	0,83	8	3,70	1,81	8
13	6,67	3,54	4	7,98	0,85	8	7,36	1,29	8

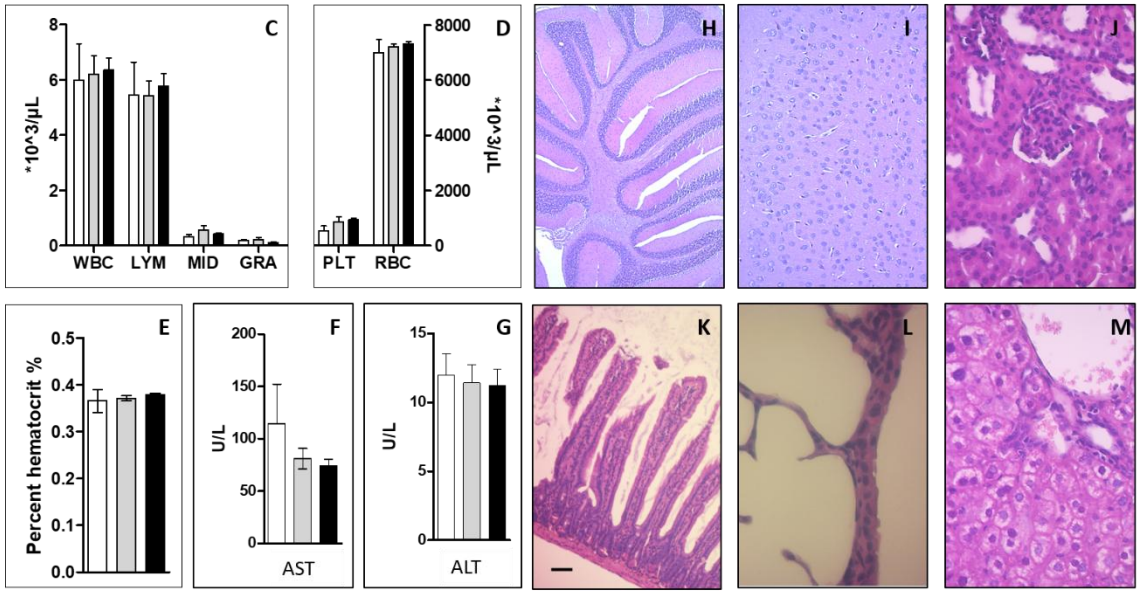


Figure 2

Figure 2: in vivo evaluation of the toxicity of the formulation in healthy mice (n=8). Longitudinal monitoring of the weight after injection of the different formulation. (A) Percentage difference in weight following 14 days of the control (white), P407 20% (grey) and P407/P188/ protanal 20/2/1 (black) groups. (B) corresponding value of the percentage difference in weight for each groups (100*value -baseline, mean,SEM and number of value). 2-way ANOVA with Bonferroni's multiple comparisons test: ns. (C, D, E) Effect of P407 20% (grey) and the thermogel 20/2/1 (black) as function of control (white) on blood component and hematocrit level where WBC: white blood cell, LYM: lymphocytes; MID: Monocytes; GRA: Granulocytes; PLT: platelet; RBC: RED blood cell. Effect of P407 (grey) and the thermogel 20/2/1 (black) as function of control (white) on hepatic enzyme AST

Aspartate Aminotransferase (F) ALT alanine transaminase (G). Effect of the key component of hydrogel: P407 on main organ, Hematoxylin eosin staining of cerebellum (H), Brain (I), kidney (J), small intestine (K), Lung (L) and Liver (M). Scale bar for images H to M corresponds to 50 µm.

In vitro anti-adhesive effect of thermogel on colon carcinoma cell line

After *in vivo* safety verification of the thermogel, its anti-adhesive effect was investigated. As shown in the pictures (Figure 3A) and in the resulting image quantification (Figure 3B), when the plate was coated with thermogel, cell adhesion was drastically reduced as compared to the control group after 90 and 120 min ($p < 0,05$ and $p < 0,001$, respectively).

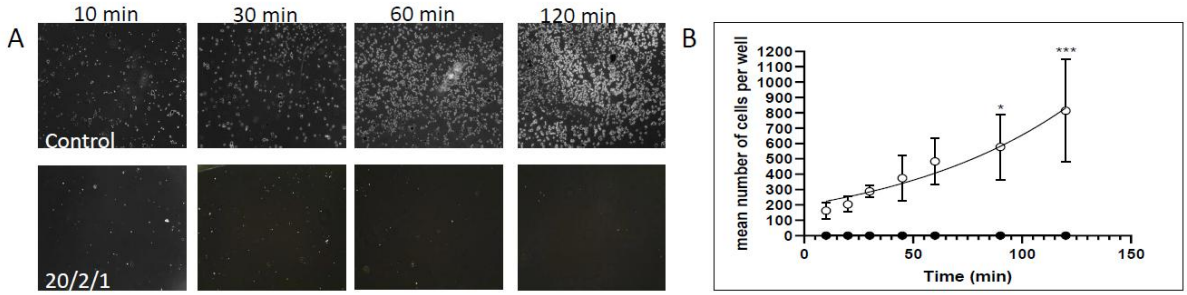


Figure 3: (A) Representative picture of CT26 cells incubated without (up) and with (down) 20/2/1 thermogel. (B) Mean cells counting in wells from 10 to 120 min after incubation with thermogel 20/2/1 (black dot) at 37°C or in control (white circle). A nonlinear fit exponential growth equation was applied on the result expressed in mean ± SEM and Sidak's multiple comparisons statistical analysis was realized $p<0.05$ *; $p<0.001$ ***.

Drug stability in thermogel

In order to combine the therapeutic outcome of our thermogel, cytotoxic agents were solubilized within the polymers network. The first step was to verify the absence of interactions between the drugs and the thermogel. By comparing retention times of drugs in the admixture 5-FU/Ox with those of 5-FU and Ox solutions (Figure 4A-B), no modification was noticed. 5-FU was eluted at 9.5 min and Ox at 20.5 min (Figure 4C).

5-FU and Ox concentrations were measured at 3 and 24 hours, in solution and thermogel. 5-FU in solution was stable over 24 hours. Ox in solution showed a steady concentration up to 3 hours post-preparation. Its concentration decreased then by 11% over 24 hours (Figure 4D). 5-FU incorporation into the thermogel had a very slight effect on its concentration. Ox concentration remained invariable up to 3 hours, though it decreased by 14% after 24 hours. The study of 5-Fluorouracil and oxaliplatin release from P407/P188/alginate/5-fluorouracil/oxaliplatin 20/2/1/0.5/0.1% w/v thermogel was conducted in vitro at 37°C. The release of the drugs from the thermogel was studied

over time in PBS (pH 7.3; 0.01 M). The data indicate a release of both Ox and 5-FU over 5h before reaching a plateau and decreasing due to degradation in particular of Ox, known to be unstable at this pH (Figure 4E).

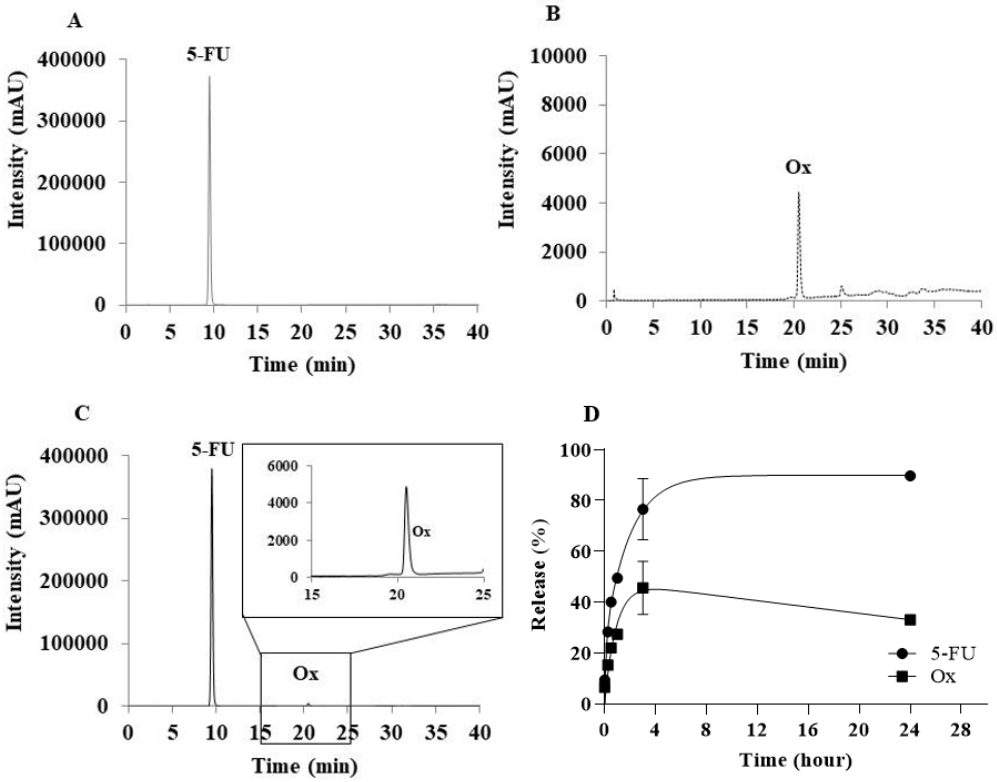


Figure 4: HPLC chromatograms of (A) 5-FU WFI, (B) Ox WFI and (C) 5-FU/Ox admixture at 266 nm (maximal wavelength of 5-FU). (D) determination of 5-FU and Ox concentrations in 5-FU/Ox admixture, prepared in WFI or thermogel, over time at 4°C (n=3).

In vitro efficacy of combined Ox and 5-FU in the thermogel

In order to validate the effect of the chemotherapeutic agent encapsulated in the 20/2/1 thermogel, the cytotoxicity of this formulation was investigated on CT26-luc cell line as compared to the chemotherapy at the same concentration in solution. The ratio was chosen based on 5-FU solubility in water at 20°C. The final drug loading content corresponded to 0.5% w/v (5 mg/mL) and 0.1% w/v (1 mg/mL) for 5-FU and Ox, respectively. As represented in Figure 5, cytotoxicity of 5-FU and Ox were maintained in thermogel and were significant as regard to the respective control (****, $p < 0.0001$). Moreover, no significant difference was observed between the solution and the thermogel with chemotherapeutic agent ($p = 0.6981$). On the other hand, thermogel without drugs had no impact on cell viability.

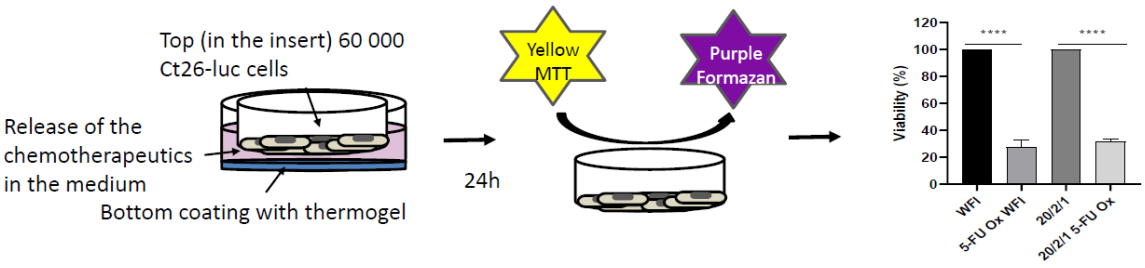


Figure 5: Cytotoxicity of the chemotherapeutic agents 5-FU and Ox embedded in the thermogel 24 h after incubation against CT26-luc colon carcinoma cell line. One way ANOVA with turkey multiple comparison **** P <0.0001.

Efficacy of combined Ox and 5-FU thermogel in peritoneal metastasis model

Based on the *in vitro* efficacy of Ox and 5-FU entrapped in thermogel, *in vivo* assay on peritoneal metastasis model was undertaken. The murine model was obtained by injection of 1 x 10^4 CT26-Luc cells in the intraperitoneal region as described [9]. The efficacy evaluation of thermogel was based on the peritoneal carcinomatosis index (PCI)[19]. Guided on the schedule shown on Figure 6A, formulations were injected 1h post cell injection to better mimic the surgical situation. As can be seen in Figure 6B, formulations were well tolerated by the mice with non-significant change in body weight until day 3 then normalizing in the following days as compared to control group. As shown in Figure 6C-D, the empty thermogel had no effect both on the PCI and the number of areas involved. Interestingly, 5-FU combined or not with Ox within the thermogel reduced significantly the PCI as compared to control group ($p < 0.005$). However, only the combination of both 5-FU and Ox within the thermogel was able to reduce the number of areas involved in peritoneal cavity ($p < 0.05$) and the number of mice with ascites (Figure 6D-E). Ascites volume was dramatically reduced using thermogel with Ox alone or combined with 5-FU (respectively $p < 0.001$ and $p < 0.005$) (Figure 6F).

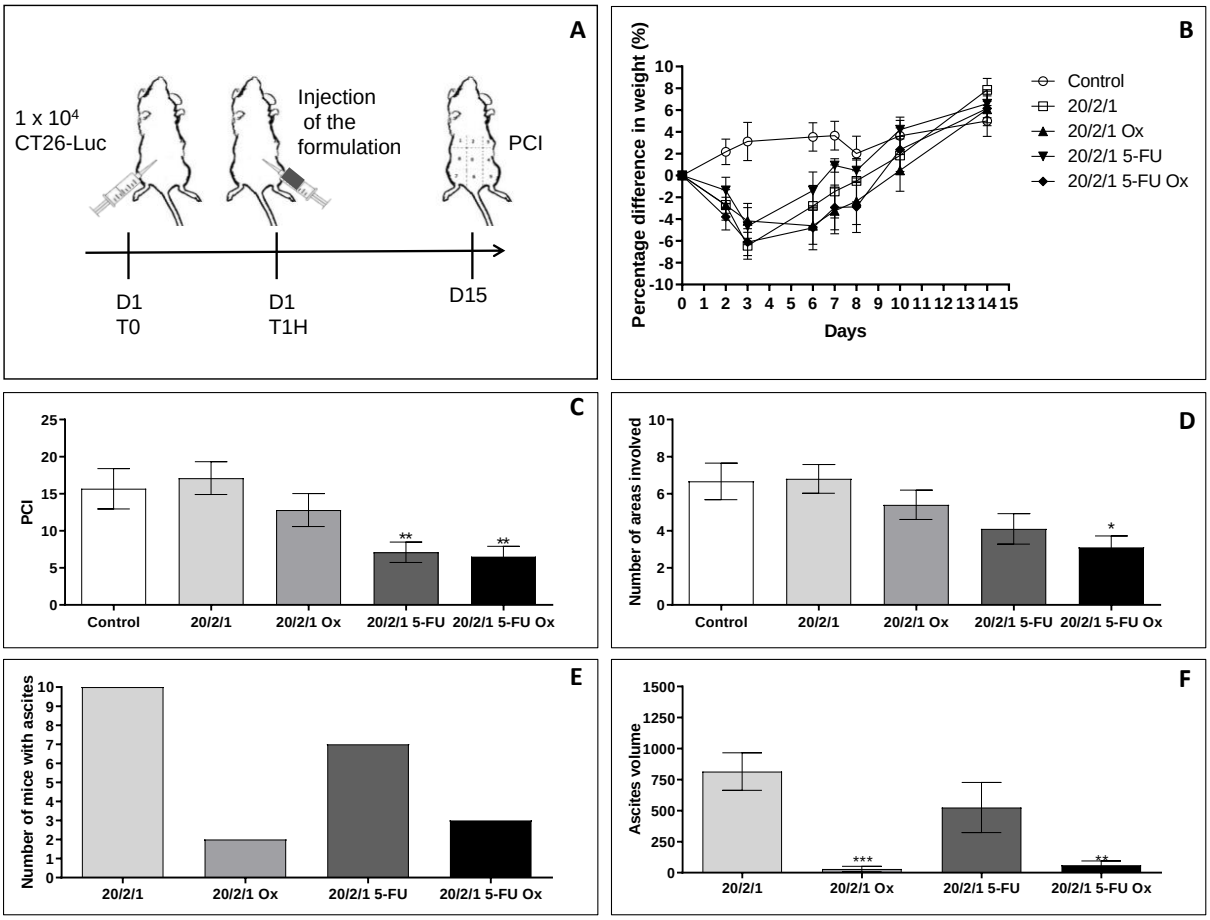


Figure 6: Evaluation of thermogel formulations efficacy on colorectal CT26-Luc peritoneal carcinomatosis model. Schedule of the experiment (A). Percentage difference in weight as function of time after formulations injection (B). PCI at day 15 after CT26-Luc cells induced carcinomatosis for the different formulations of thermogel (C). Number of areas involved at day 15 after CT26-Luc cells induced carcinomatosis for the different formulations of hydrogel (D). Number of mice with ascites (E) and mean ascites volume (F). *p < 0.05, **p < 0.005, *** p < 0.001 as compared to control group (mean ± SEM, n=9-10).

Discussion

Surgical resection of colon, ovarian or stomach cancers represents a high risk of PM or recurrence. Detachment of cancers cells during surgical procedure can lead to their implantation in peritoneal cavity where they proliferate to form new nodules scattered [5,20,21]. There is an important challenge to develop new drug delivery systems allowing sustained release associated with an anti-adhesive effect against of tumor cell implantation. Indeed, it is common when the surgeon operates that free cells are already present in the peritoneum. Moreover, when performing surgery, the surgeon also offers the proper environment for tumor implantation with the surgical scares. Fibrine is activated and fibrinogen allows tumor cell implantation during or just after surgery. These are the cells that we are targeting here using a local hydrogel just after the surgery. Actually, many surgical teams associated

after cytoreductive surgery (CRS) an intraperitoneal treatment as chemotherapy with hyperthermia (HIPEC), or postoperative intra peritoneal drug delivery using direct and repeated injections. Our objective is to offer an esay to handle drug delivery systems for all surgeons which could be used in the surgery room according to the clinical situation encountered, in particular in case of high risk of secondary peritoneal spread after surgery.

In this context, we proposed an hydrogel based on a FDA approved excipient P407. P407 has the capacity to be in solution state at low temperatures, allowing easy injectability, usability, homogeneity and sterilizability, but also turning into gel state when temperature increases. The hydrogel does not need to stay for a long time in the peritoneal cavity, neither deliver drugs for a long period, the objective is to have a sufficiently lasting gel to prevent cell adhesion which occurs within two days post-surgery,

Physico-chemical properties of poloxamers can be improved by the addition of copolymers as referred by Qiu et al.[22] which is why the composition of poloxamers was complexified in this study. Here we chose a mixture of two poloxamers P407 and P188, in a proportion leading to tunable sol-gel transition around 27-30°C [23] in combination with alginate, a gelling agent which do not cause cell adhesion [24]. Previously, it had been shown that P188 reduced the adhesion rate of erythrocytes to endothelial cells when added to a Dextran 500 kDa solution, which is a polymer known to induce cellular interaction and adhesion [25]. Steinleitner et al. also highlighted P407 ability to prevent post-surgical adhesion formation in hamster and rabbit uterine-horn injury models [12]. Those results are consistent with our *in vitro* significant findings of CT-26 cells adhesion decrease observed in presence of thermogel. Several studies also demonstrated the efficacy of alginate, in the prevention of post-surgical adhesion. Cho et al. tested various types of alginate (viscous solution, gel and film) and compared their anti-adhesion efficacy onto peritoneal surgical defect in comparison with Interceed®, a commercialized cellulose-based adhesion barrier. Alginate gel and un-crosslinked films demonstrated a higher anti-adhesion power with a similar toxicity profile as compared to control and Interceed® groups[26]. Chaturvedi et al. demonstrated also efficacy of an ultrapure alginate gel, in adhesion prevention in a rat model with caecal abrasion and peritoneal side wall excision. They did not find any difference in comparison with Seprafilm®, a sodium hyaluronate carboxymethyl cellulose based membrane which was the most effective adhesion barrier previously reported [27]. Moreover, authors proved the superiority of their ultrapure alginate gel in preventing adhesion formation on a compromised anastomotic healing rat model. These results are in favor of an antiadhesive effect of the alginate which we took advantage of to propose a mixture of poloxamer

containing alginate to further enhance the anti-adhesive effect reported for poloxamers [17]. Finally, the mucoadhesive effect of this polysaccharide [28,29] could enhance gel adhesion on peritoneal wall avoiding tissues closing as a barrier component, thus preventing post-surgery adhesion. Alginate and poloxamer also have healing properties widely described. This also attracted us to promote wounds resorption thus reducing possibility of cancer cell implantation [30,31].

Safety was expected as poloxamers are clinically used in many pharmaceutical formulations, as ophthalmic with cyclosporin eye drops Ikervis®, topical with aciclovir ointment Zovirax®, or many tablets presentations. But usually there is no medicine with poloxamer authorized for direct peritoneal use. Johnston *et al*. demonstrated a P407 toxicity dose-dependent relation after repeated IP administration in rats. No variation of hematological parameters rate was observed in rats treated IP with 10 % P 407 for 4 consecutive days compared with control group excepted a slight monocyte increase, whereas rats treated by 30 % P 407 increased almost all parameters evaluated [32]. P407 20 % ratio in our formulation appear to be a good compromise to avoid systemic toxicity after IP administration. *In vivo* tolerance of our gel was also assessed. Neither P407 20 % thermogel nor full thermogel 20/2/1 induced toxicity on blood components, transaminases or main organs such as cerebellum, brain, kidney, small intestine, lung and liver after anatomopathological evaluation. The non-significant slight change in mice body weight which had received P407/P188/alginate thermogel, also reflect the absence of systemic toxicity of our thermogel.

5-FU and Ox dosage in thermogel disclosed that drugs were stable, rejecting the assumption of a potential deleterious effect of the thermogel matrix on the drugs. Invariability of drug retention times in 5-FU/Ox admixture led us to consider that no chemical interaction occurs between both drugs considered sufficiently stable allowing the formulation and the administration of the 5-FU/Ox thermogel in mice. After verification that thermogel did not impaired 5-FU and Ox stability, *in vitro* toxicity and efficacy of 5-FU-Ox-thermogel were assessed. Obviously, longer lasting delivery systems have been developed earlier and in recent literature [33]. The evaluation of longer lasting systems could also be evaluated in future studies to see whether it would be needed in the context of tumor adhesion post-surgery, knowing that cells will not survive if they do not adhere.

Empty thermogel did not affect cell viability which is consistent with our *in vivo* toxicity results. Cytotoxicity of 5-Fu/Ox in solution group and thermogel group were similar, proving a non-inferiority of the combined drugs pharmacological effect when entrapped in the thermogel. These results are consistent with the findings of Xie et al. who developed a thermosensitive hydrogel based on polyethylene glycol and ε-caprolactone with P407 nanoparticles of honokiol. P407 showed a very low cytotoxicity on SKOV3 ovarian cells with viability upper than 80 % at 48 h as compared to untreated cells, independently of its concentration in culture medium. Nanoparticles of P407 also did not

impact honokiol cytotoxic effect considering cell proliferation curve almost equivalent with honokiol in solution after 48 h treatment[34].

Finally, *in vivo* antitumor efficacy of our 20/2/1 thermogel was evaluated on a peritoneal carcinomatosis murine model. Only the association of both Ox and 5-FU in thermogel was able to significantly decrease the PCI, the number of areas involved, the number of mice with ascites and the mean ascites volume simultaneously. Previously, Jouvin et al. developed an icodextrine based carrier solution to administrate 5-FU and Ox IP and prevent carcinomatosis apparition. In a high risk of growing peritoneal carcinomatosis of colorectal origin murin model, PCI was significantly inferior in icodextrin 5-FU/Ox group compared to 5-FU/Ox alone, icodextrine alone and control group [9]. We undertook a comparison of PCI between icodextrine + Ox treatment and thermogel 20/2/1/Ox treatment in our murine model. Results were in favor of the thermogel with a PCI 6.4 ± 1.8, the only group showing a significant difference compared to control group ($p < 0.01$) (data not shown).

Conclusion

In this work, we developed a P407/P188/alginate (20/2/1 % w/v) non-toxic and biocompatible thermogel for a safety of use. We demonstrated the capability of our thermogel of decreasing CT-26 cells adhesion *in vitro*. The addition of both Ox and 5-Fu chemotherapeutic agents in thermogel was as efficient as drugs in solution on cell viability. We therefore proved the *in vivo* antitumoral efficacy of our drug loaded thermogel on a murine model of carcinomatosis which was superior to the thermogel alone in decreasing of PCI, number areas involved, number of mice with ascites and mean ascites volume. Our poloxamer-based thermogel appears to be a promising drug delivery system to prevent post-surgical adhesion and carcinomatosis formation from primitive colorectal cancer in peritoneal cavity. Further experiments are needed to evaluate the anti-adhesion and anti-cancer efficacy of our thermogel *in vivo*.

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