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Chitosan-doxycycline hydrogel: An MMP inhibitor/sclerosing embolizing agent as a new approach to endoleak prevention and treatment after endovascular aneurysm repair

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ABSTRACT

The success of endovascular repair of abdominal aortic aneurysms remains limited due to the development of endoleaks. Sac embolization has been proposed to manage endoleaks, but current embolizing materials are associated with frequent recurrence. An injectable agent that combines vascular occlusion and sclerosing properties has demonstrated promise for the treatment of endoleaks. Moreover, the inhibition of aneurysmal wall degradation via matrix metalloproteinases (MMPs) may further prevent aneurysm progression. Thus, an embolization agent that promotes occlusion, MMP inhibition and endothelial ablation was hypothesized to provide a multi-faceted approach for endoleak treatment.

In this study, an injectable, occlusive chitosan (CH) hydrogel containing doxycycline (DOX)—a sclerosant and MMP inhibitor—was developed. Several CH-DOX hydrogel formulations were characterized for their mechanical and sclerosing properties, injectability, DOX release rate, and MMP inhibition. An optimized formulation was assessed for its short-term ability to occlude blood vessels *in vivo*.

All formulations were injectable and gelled rapidly at body temperature. Only hydrogels prepared with 0.075 M sodium bicarbonate and 0.08 M phosphate buffer as the gelling agent presented sufficient mechanical properties to immediately impede physiological flow. DOX release from this gel was in a two-stage pattern: a burst release followed by a slow continuous release. Released DOX was bioactive and able to inhibit MMP-2 activity in human glioblastoma cells. Preliminary *in vivo* testing in pig renal arteries showed immediate and delayed embolization success of 96% and 86%, respectively. Altogether, CH-DOX hydrogels appear to be promising new multifunctional embolic agents for the treatment of endoleaks.

Statement of Significance

An injectable embolizing chitosan hydrogel releasing doxycycline (DOX) was developed as the first multi-faceted approach for the occlusion of blood vessels. It combines occlusive properties with DOX sclerosing and MMP inhibition properties, respectively known to prevent recanalization process and to counteract the underlying pathophysiology of vessel wall degradation and aneurysm progression. After drug release, the biocompatible scaffold can be invaded by cells and slowly degrade.

Local DOX delivery requires lower drug amount and decreases risks of side effects compared to systemic administration.

This new gel could be used for the prevention or treatment of endoleaks after endovascular aneurysm repair, but also for the embolization of other blood vessels such as venous or vascular malformations.

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1. Introduction

Abdominal aortic aneurysms (AAAs) are abnormal dilatations of the abdominal aorta that may result in fatal rupture. They are one of the most significant causes of morbidity and mortality in the elderly [1]. Current recommendations suggest that patients with AAAs greater than ≥5.5 cm undergo treatment via open surgery or its minimally invasive alternative, endovascular aneurysm repair (EVAR). In EVAR, a stent-graft is deployed into the aorta transarterially to exclude blood flow from the aneurysmal sac [2]. While associated with an increase in initial survival [3], the long-term success of EVAR remains limited in part due to the development of endoleaks. Endoleaks are characterized by the persistent perfusion of the aneurysm sac outside of the stent-graft. They may attain pressures comparable to those found in the systemic circulation and may lead to aneurysm rupture [2].

Transarterial or translumbar needle embolization is recommended for the treatment of expanding endoleaks, especially type II endoleaks [4]. However, current embolic agents are associated with high rates of recurrence [5,6]. Recent studies have suggested that the endothelial layer plays a major role in the development and persistence of endoleaks; in animal models, aneurysms that were mechanically denuded of their endothelium showed a decrease in the persistence of endoleaks [7]. Furthermore, the underlying pathophysiology of aneurysm progression—which has been linked to persistent, localized inflammation associated with excessive activity of matrix metalloproteinases (MMPs) resulting in the progressive vessel wall destruction [8]—has yet to be addressed. Therefore, we hypothesized that an embolization agent that occludes the endoleak, inhibits MMP and promotes endothelial sclerosis would provide a multi-faceted approach for endoleak prevention and treatment.

The aim of the present study was to develop an injectable and radiopaque chitosan-based embolization agent containing doxycycline (DOX) as an adjunct to EVAR for the treatment of AAAs. DOX is one of the most investigated medications in the management of AAAs. When injected locally, DOX causes irreversible endothelial injury promoting thrombosis and occlusion [9]. It is also an MMP inhibitor that limits AAA growth by inhibiting extracellular matrix (ECM) remodelling and localized inflammation [10]. In patients with AAAs, systemic administration of DOX has been shown to decrease MMP activity at the site of the aneurysm, resulting in decreased AAA progression [11]. In a preclinical model, this effect was achieved with just local administration of DOX, which theoretically would mitigate concerns of systemic toxicity [12]. We hypothesized that the administration of DOX in an occlusive embolic matrix would maximize localized DOX exposure and could act as an adjunct to EVAR to prevent aortic expansion and to prevent or treat endoleaks.

Chitosan-based hydrogels were selected as the embolic matrix since they are desirable for their biocompatibility, biodegradability, injectability at room temperature and adhesion properties [13,14]. Also they can rapidly undergoing sol-gel transition at body temperature [15]. They have been studied extensively for intravascular embolization [16,17]. Furthermore, these gels can be prepared with commercial contrast agents to help monitor injection and migration under fluoroscopy (given that chitosan and doxycycline are not radiopaque) [18].

We hypothesized that DOX could be integrated into chitosan hydrogels while maintaining injectability and favourable mechanical properties. We expected a two-stage release of DOX from the hydrogel, including an initial burst release—to promote sclerosis and initiate MMP inhibition—and a continuous release thereafter to sustain MMP inhibition.

2. Materials and methods

2.1. Preparation of hydrogels

In situ gelling hydrogels were prepared by mixing two solutions. Shrimp shell chitosan (CH) (Marinard Biotech, MW 250 kDa, DDA 94%) was first purified as previously published [19]. Solution A was prepared by dissolving the CH powder at 3.33% (w/v) in HCl (0.1 M) solution with or without Visipaque 320® (VIS) contrast agent (GE Healthcare, Rahway, USA) at room temperature under constant magnetic stirring. It was sterilized by autoclave (AMSCO® Evolution® Steam Sterilizer, Oh, USA) at 121 °C for 20 min and stored at 4 °C until further use. Solution B contained the gelling agent with or without DOX sterilized by 0.2 μm filtration.

Several gelling agents were investigated to obtain a final gel with a rapid gelation rate and optimal mechanical properties, based on previous work [19]. Specifically, the initial G' needed to be higher than 800 Pa upon injection to resist pressurized flow up to 200 mmHg [16]. Sodium bicarbonate $NaHCO_3$ (SHC) (MP Biomedicals, Solon, OH, USA) was mixed with phosphate buffer (PB) at pH 8, which was prepared by dissolving sodium phosphate monobasic (SPM) and sodium phosphate dibasic (SPD) salts in Milli-Q water at w/w ratio of 0.06. β-Glycerophosphate disodium salt pentahydrate (BGP) (Sigma Aldrich, Oakville, ON, Canada) was chosen as a control due to its favourable gelation kinetics. BGP solutions were prepared by dissolving the salt in Milli-Q water [19]. DOX was dissolved at different concentrations in the gelling agent solutions. To increase DOX stability, sodium thiosulfate as an antioxidant (1% w/v) and creatine as a stabilizer (1% w/v) were also added [20].

To form CH-DOX and CH hydrogels, solution A and B were loaded in separate syringes joined by a Luer lock connector and then mixed just prior to their use. The abbreviations, initial and final concentration of each compound in the gels are summarized in Table 1. In the first part of the manuscript, the names of the hydrogels indicate their final composition. For example, CH/SH C0.075PB0.08/VIS50/DOX0.1 represents a hydrogel containing 2% w/v CH, SHC0.075PB0.08M as gelling agent, 50% v/v radiopaque agent (VIS) and 0.1 w/v% DOX. All hydrogels were prepared to reach a final concentration of 2% w/v CH. Starting from Section 2.3, once the final gelling agent was selected, the name of gel was simplified to indicate only DOX concentration in the gel (CH-DOX0.1 for DOX 0.1%w/v).

2.2. Characterization of hydrogels as an embolic agent

The kinetics of gelation, occlusive properties and injectability of gels were evaluated. First, gelation kinetics and occlusive properties were used to select an optimal gelling agent based on previous work [16]. Subsequently, the impact of adding VIS50% and different concentrations of DOX on these parameters was reassessed to ensure favourable mechanical properties.

2.2.1. Rheology

Rheological properties were studied using the Physica MCR 301 rheometer (Anton Paar; Saint-Laurent, QC, Canada) with co-axial cylinder geometry. Time sweep experiment was performed in the linear viscoelastic region (frequency (1 Hz), strain (1%)). The evolution of the storage (G′) and loss (G″) moduli as a function of time at body temperature (37 °C) was determined.

2.2.2. Occlusive properties

The effectiveness of the gels in occluding blood flow after different gelation times (2 and 7 min) was estimated using an *in vitro* bench test previously developed [16]. In brief, a glycerol (40%v/v)

Table 1
Initial and final concentrations (in the syringe and in the gel respectively) of each component and pH of the various hydrogels tested.

Gel name	Chitosan (%w/v)		VIS (% v/v)		DOX (% w/v)		Gelling agent Concentration (M)	Hydrogel pH
	Initial	*Final*	*Initial*	*Final*	*Initial*	*Final*	*Final*	*Final*
CH/BGP0.4	3.33	2	0	0	0	0	BGP: 0.4	7.2
CH/SHC0.075PB0.04	3.33	2	0	0	0	0	SHC:0.075 PB: 0.04	7.2
CH/SHC0.075PB0.08	3.33	2	0	0	0	0	SHC:0.075 PB: 0.08	7.2
CH/SHC0.075PB0.08/VIS50	3.33	2	83.3	50	0	0	SHC:0.075 PB: 0.08	7.2
CH/SHC0.075PB0.08/VIS50/DOX0.1	3.33	2	83.3	50	0.25	0.1	SHC:0.075 PB: 0.08	7.1
CH/SHC0.075PB0.08/VIS50/DOX0.3	3.33	2	83.3	50	0.75	0.3	SHC:0.075 PB: 0.08	7.0
CH/SHC0.075PB0.08/VIS50/DOX1	3.33	2	83.3	50	2.5	1	SHC:0.075 PB: 0.08	7.0

and water (60%v/v) solution of viscosity similar to blood (5 cps) was pushed by a syringe pump towards a tube filled with the gel with increasing pressure (up to 200 mmHg) while possible leakage was monitored. Only gels that were able to immediately resist pressured flow were assessed in subsequent experiments.

2.2.3. Gel injectability

Gel injectability by microcatheter (FasT-325, Boston Scientific; internal diameter of 0.61 mm (0.024 inch); length of 140 cm) [18] was verified by measuring the maximal force needed to eject CH gels after different gelation times (t = 0 and 5 min) at 37 °C. The force was measured using an ElectroForce® 3200 Test Instrument (TA Instrument; 225 N load cell).

2.3. DOX cytotoxic dose-response on endothelial cells

DOX cytotoxic dose-response was studied on human umbilical vascular endothelial cells (HUVECs) (Lonza, CC-2519, ON, Canada). HUVECs were cultivated in flasks coated with 1% gelatin in EGM-2 bullet kit (Lonza, CC-3156, ON, Canada) and used between passages 3 and 5. The HUVECs (9000 cells/well) were then seeded in 96-well plates and cultured until 80% confluence in culture medium containing 1% penicillin and streptomycin (Wisent, 450-204-EL, QC, Canada). The cells were then exposed to different concentrations of DOX from 0 to 0.75% (w/v) for 24 h. Fresh culture medium was used as a negative control while culture medium supplemented with 10% (v/v) dimethyl sulfoxide solution (DMSO) was used as positive control. Cell viability was evaluated via cell metabolic activity assayed using 10% AlamarBlue (Cedarlane Corp., Burlington, ON, Canada) with fluorescence measurements (Ex 560 nm/Em 590 nm).

2.4. Ex vivo de-endothelialization

The ability of prepared hydrogels containing different DOX concentrations was evaluated *ex vivo* using the aortas of dogs sacrificed in the framework of other experiments. Aortic segments were harvested, rinsed and sutured at one end and then were embolized by gel containing 0, 0.1, 0.3 or 1% w/v DOX; one aorta was kept as control. Embolized tissues and the control were incubated at 37 °C with 5% CO_2 for 3 h in RPMI 1640 (25.03 mM Hepes and 2.05 mM L-glutamine) supplemented with 20% FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptavidin, and 2.5 μg mL^{-1} gentamicin. Arteries were fixed in buffered formalin and embedded with paraffin. Histological analysis was performed to detect vessel wall damage; factor VIII staining was used to detect the presence of endothelial cells.

2.5. Indirect cytotoxicity tests

Indirect cytotoxicity tests were performed on gel extracts obtained after incubation of the gel in culture media at different timepoints (1, 2, 3 and 7 days) following ISO 10993-5:2001 standards. They aimed to confirm the desirable toxicity of extracts due to large DOX release within the first 24 h (sclerosing effect), but also the absence of gel toxicity once DOX and radiopaque agent are mostly released (next extracts). To prepare hydrogel extracts, samples (1 mL each, n = 3) were left to gel in a 12-well plate for 3 h at 37 °C in an incubator, and then 3 mL of culture medium were added on the top of each hydrogel. At days 1, 2, 3 and 6 the medium was recovered and replaced by a fresh medium. Extracts were frozen at −20°C until the assay. L929 fibroblast cells (ATCC, Manassas, VA, USA) were seeded in 96-well plates (10000 cells/well) and cultured until 90% confluence in Dulbecco's Modified Eagle's Medium (Gibco BRL, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovin serum (FBS; Medicor, Montreal, QC, Canada). The culture medium was then removed and replaced with medium containing the extract. After 24 h, the viability of the cells was estimated by measuring their metabolic activity using AlamarBlue compared to that of negative and positive controls (media without extract and media with 10% DMSO, respectively).

2.6. In vitro drug release and swelling studies

Drug release rate was studied using an USP Dissolution Apparatus I (Distek 3200). CH-DOX 0.1 gels (3 mL) were prepared (Section 2.1), allowed to gel for 5 min and then placed in the basket of the apparatus and submerged in PBS at 37 °C. DOX dissolution was monitored over 1 week with constant stirring at 30 rpm. Aliquots of sample were withdrawn at different time intervals and analyzed by spectrophotometry at 390 nm. The amount of released DOX at each time point was determined by using a calibrated curve obtained in the range of 0–50 μg/mL.

The drug release kinetics was quantified using Korsemeyer-Peppas formula:

$$M_t/M_\infty = kt^n \quad (1)$$

where $Mt/M\infty$ is the amount of drug released at t time, k is a constant including structural and geometrical characteristic of the dosage form, and n is the diffusion exponent indicative of the release mechanism [21].

Swelling behaviour of the hydrogels was also investigated, by following the weight of samples immersed in PBS at 37 °C over 7 days [21]. Samples (2 mL) were first left to gel in a cylindrical holder for 24 h. Then the slabs were placed in similar conditions as those used for drug release tests (baskets of USP apparatus I,

in PBS medium at 37 °C and 30 rpm). Samples were removed at predetermined time points, weighted after removal of the superficial liquid and reported as a function of the initial mass (1):

$$Q = (W_0 - W_t)/W_t \times 100 \quad (2)$$

where W_0 and W_t are the sample weights at time 0 and time t, respectively.

2.7. MMP inhibition

The bioactivity of DOX released from the CH-DOX hydrogels was assessed in terms of its ability to inhibit MMP-2 secreted from human U-87 glioblastoma cells (U-87MG). MMP-2 inhibition was assessed at different time points and compared to the bioactivity of DOX solutions with known concentrations. U-87MG cells (ATCC, Manassas, VA) were cultivated (2.5×10^5 cells/well) in 6-well plates in Eagle's minimum essential Medium containing 10% (v/v) calf serum (HyClone Laboratories, Logan, UT), 1 mM sodium pyruvate, 2 mM glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin. After two days incubation at 37 °C with 5% CO_2, tissue culture plate inserts (VWR®, Radnor, PA) were used to submerge the hydrogel in the media without directly contacting cells. To determine whether DOX remained bioactive for several days in the gel, these hydrogels were placed in the wells either immediately after gelation or after pre-incubation in culture media for one or three days. The cells were then incubated for 24 h in the presence of the hydrogel, before performing viability and gelatin zymography tests. In parallel, the concentration of DOX released in the media at each time point was estimated based on DOX release in PBS in similar conditions, as measured by spectrometry.

Gelatin zymography was used to assess the extent of MMP-2 gelatinolytic activity and activation status as previously described [22]. Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/mL gelatin, a substrate that is efficiently hydrolyzed by proMMP-2 and MMP-2. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H_2O. Gels were further incubated at 37 °C for 20 h in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in H_2O. Gelatinolytic activity was detected as unstained bands on a blue background.

2.8. Structural and morphological analyses

SEM and FTIR were performed on the final CH-DOX solution.

2.8.1. Scanning electron microscope (SEM)

Samples were left for 24 h of gelation at 37 °C then vacuum freeze dried. Slices of each sample were cut using a surgical blade and sputter-coated with a thin gold layer. The morphology of the samples was examined using a Hitachi 3.0 kV scanning electron microscope (S-4300SE/N) under vacuum (Hitachi High Technologies America, Pleasanton, CA, USA).

2.8.2. Fourier transform infrared spectroscopy (FTIR)

Potential interactions between CH gels and DOX were evaluated by comparing the FTIR spectra of CH, DOX and a mixture of CH and DOX (all as dry powders) with spectra obtained from lyophilized CH and CH-DOX gels (left to gel for 48 h). The FTIR spectra of all samples were recorded (32 scans at a resolution of 4 cm^{-1}) using a Thermo-Nicolet 6700 (Madison, WI, USA) FTIR spectrometer equipped with a deuterated triglycine sulfate-KBr (DTGS-KBr) detector and a diamond smart attenuated total reflection platform. Data were analyzed with Omnic software.

2.9. In vivo assays

The feasibility of using CH/SHC0.075PB0.08/VIS50/DOX0.1 gels as an embolic agent was assessed during a short-term preliminary *in vivo* study on six pigs planned for sacrifice in the framework of another experimental protocol. The animals did not undergo aneurysm surgery or EVAR. Bilateral embolization of the caudal branch of the renal artery was performed to determine whether the agent could promote an embolization of a complete arterial territory including the parent artery and its branch divisions. The renal physiology and cardiovascular anatomy of pigs are quite similar to human physiology. We chose this acute model of renal artery embolization in a cohort of pigs already enrolled for a terminal experimentation to minimize the number of animals involved and associated costs. The procedures were approved by the CHUM institutional animal care committee (CIPA).

The animals were placed under general anesthesia and intubated for the duration of the procedure. Following a femoral artery access, either the right or left renal artery was selectively catheterized with a 5 French cobra catheter (Terumo, Tolyo, Japan) and a hyperselective catheterization of the caudal segmental artery was performed with a co-axial microcatheter (FasT-325, Boston Scientific). Contrast material was injected and digital subtraction angiograms (DSAs) were taken. Subsequently, approximately 5 mL of gel was injected under fluoroscopic guidance until satisfactory occlusion up to the tip of the microcatheter. Immediately and delayed success were evaluated by DSAs taken immediately and 10–20 min post-embolization respectively, to detect possible rapid reopening of the occluded arterial territory. The procedure was repeated for the contralateral caudal renal artery.

A semi-quantitative method was employed to assess the occlusion of distal branches (modified from [23]). In this method, the downstream divisions of the caudal branch were classified from D1-D3 (See Fig. E1), where D1 referred to the immediate and largest branches of the caudal artery, following subsequent branches from this artery were labelled D2, and further branches were labelled as D3. After D3, the resolution of the arteries was too variable to be assessed systematically, thus this was the most distal division assessed. It should be noted that the diameter of the branches and their presumed location in 3-D space were also taken into consideration to determine whether a branch was D1, D2 or D3; thus, a major branch (e.g. D2) could give off multiple D3 branches before finally bifurcating into terminal D3 branches. All pre-embolization angiograms were coded for the number of divisions presents (See Fig. E1); subsequently, the immediate and delayed post-embolization angiograms were coded similarly for visible branches. Embolization success was calculated based on the following equation:

Percentage occlusion = 100% × [1 − (Branches visible post-embolization/Branches visible pre-embolization)]

Percentage occlusion was calculated for each division level as well as globally.

A few hours after *in vivo* embolization, animals were euthanized. Histological analysis of embolized kidneys was performed. Factor VIII staining was used to detect possible damage to endothelium.

2.10. Statistical analysis

Results are presented as the mean ± standard deviation (SD) except where otherwise indicated. One-way or two-way ANOVA with Tukey's multiple comparison test were performed as appropriate. Statistical significance was defined as $p < 0.05$. Statistical analysis was performed using GraphPad Prism 7.0.

3. Results

3.1. Hydrogel rheological and in vitro occlusive properties

A single gelling agent formulation was selected based on rheology and *in vitro* embolization results in order to create a hydrogel which gels rapidly and is occlusive. Fig. 1 presents the evolution of the storage modulus G' (indicative of elastic properties) with time of CH hydrogels mixed with the three different gelling agents at 37 °C. While all gels reached G' values higher than 2000 Pa after 1 h, gels with SHC0.075PB0.08M had the highest initial mechanical properties (G'_0= 2233 ± 202) at 37 °C. This was also the only formulation to be able to immediately resist flow against the mixture of glycerol and water during *in vitro* embolization tests (Fig. 2). Gels prepared with BGP0.4M and SHC0.075PB0.04M were able to withstand the pressurized flow, but only after 7 min of gelation.

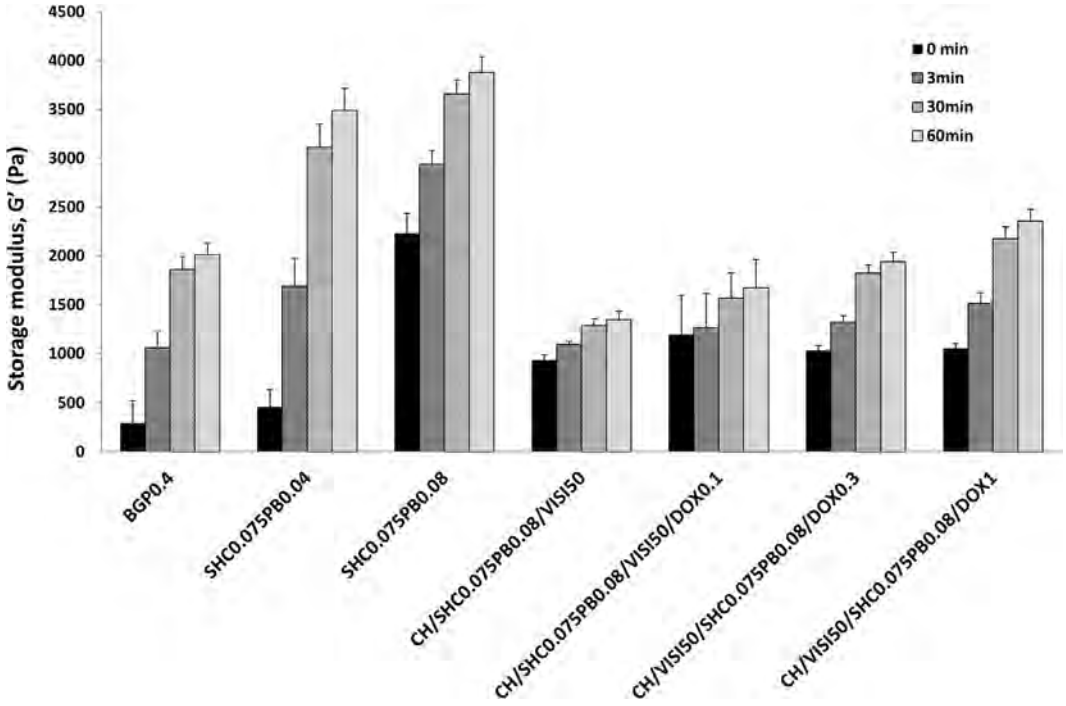

Fig. 1. Evolution of the storage modulus (G') at 37 °C as a function of time for chitosan hydrogels prepared with different gelling agents (BGP04M, SHC0.075PB0.04M and SHC0.075PB0.08M), as well as SHC0.075PB0.08M chitosan gels containing VIS (50% v/v) and DOX (0.1%, 0.3% and 1% w/v).

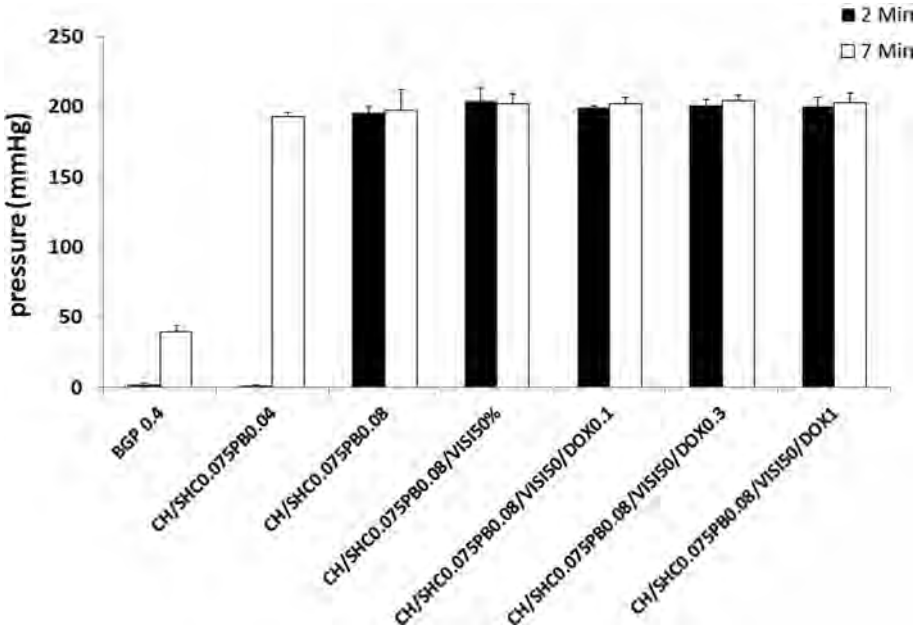

Fig. 2. Maximum liquid pressure sustained by chitosan hydrogels prepared with different gelling agents (BGP04M, SHC0.075PB0.04M and SHC0.075PB0.08 M), as well as SHC0.075PB0.08 chitosan gels with VIS (50% v/v) and DOX (0.1%, 0.3% and 1% w/v). The maximum pressure reached by the bench test was 200 mmHg.

Therefore, only gels made with SHC0.075PB0.08M as gelling agent were kept for further tests.

The addition of VIS (50% v/v) and DOX delayed the kinetics of gelation, but the initial G' values remained above the 800 Pa threshold previously identified [16] (Fig. 1). The gels were also immediately able to resist flow up to 200 mmHg in the *in vitro* embolization test (Fig. 2). While the rheological properties of the gel rapidly increased at body temperature, they remained low and stable at room temperature (22 °C) (data not shown).

The hydrogel was injectable through a 0.61 mm diameter catheter. The force required to inject the gel immediately after mixing the components (23.4 ± 0.5N) was also only slightly increased (27.1 ± 2.2N) when waiting for up to 5 min before injection. These values were still largely below the maximal force that could be applied by hand by clinicians on a 1 mL syringe, as tested by an experienced interventional radiologist.

3.2. DOX effect on endothelial cell viability

HUVECs were incubated with varied concentrations of DOX solution for 24 h. Cells showed a substantial decrease in viability when exposed to DOX concentrations above 0.5 mg/mL ($LD_{50\%}$ - ≈ 0.8 mg/mL) (Fig. 3a). Factor VIII staining on *ex vivo* embolized arteries demonstrated the removal of the endothelial layer by DOX (Fig. 3b). While injection of CH gel only slightly affected the endothelial cells layer (Fig. 3b (II)), all tested CH-DOX gels effectively removed the endothelial layer (Fig. 3b (III-V)).

3.3. Indirect cytotoxicity of CH hydrogels

The incubation of L929 cells with the hydrogel extracts showed significant cytotoxic effect of the first extract (retrieved after the first 24 h incubation) for all the hydrogels containing DOX.

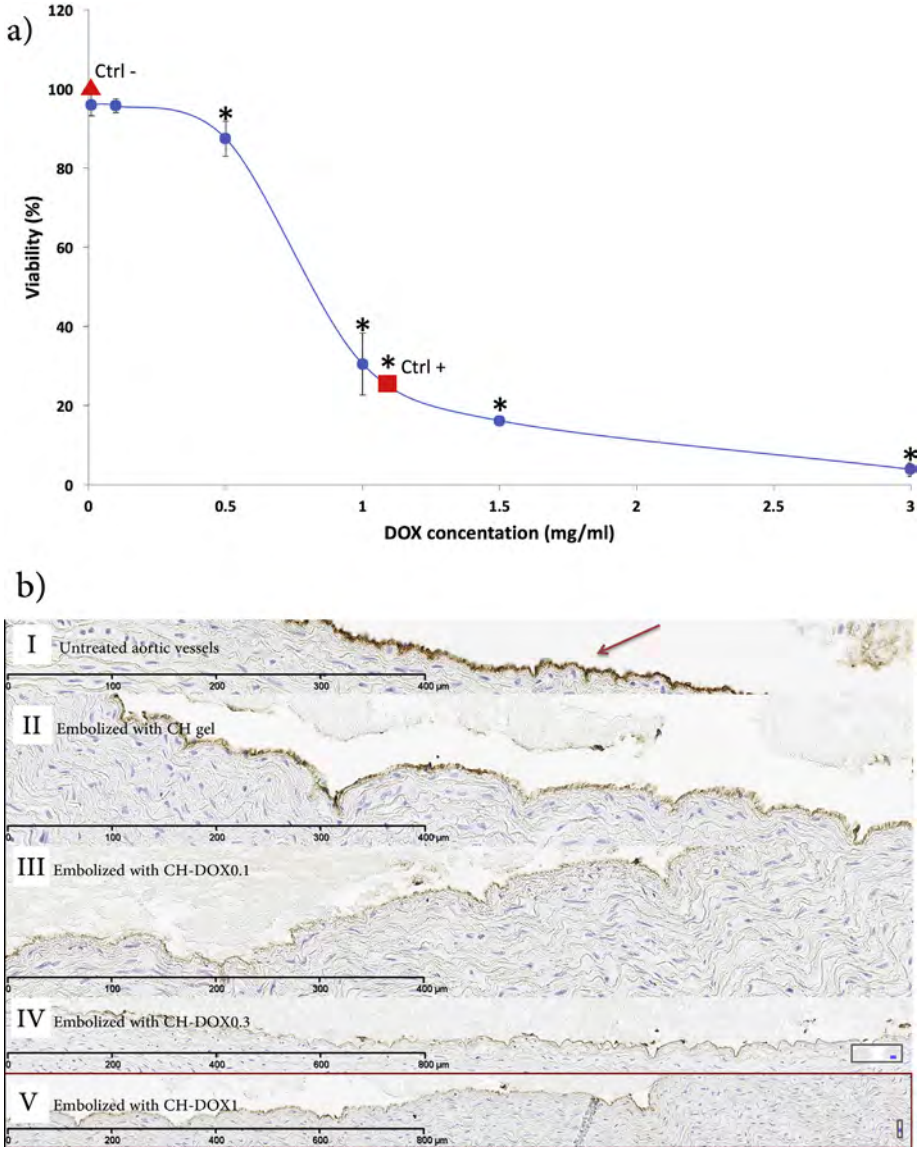

Fig. 3. a) Dose response of DOX on HUVECs showing $LD_{50\%}$ around 0.8 mg/mL in media solution. Data were normalized according to the AlamarBlue fluorescence signal of the negative control. Four samples were tested for each condition and the experiment was done in triplicate. Data are shown as mean ± SD. * $p < 0.05$ compared to negative control. b) Factor VIII immunostaining of aortic vessels: (I) untreated or embolized *ex vivo* with (II) CH gel; (III) CH-DOX0.1 gel; (IV) CH-DOX0.3 gel; (V) CH-DOX1 gel. Endothelial cells (Arrow, brown staining) are present at the lumen of untreated artery and still partially present after embolization by CH gel, but absent after embolization with DOX-containing CH gels. Embolization with each formulation was performed in triplicate.

However, extracts from the subsequent incubations (48 h, 72 h and 1 week) showed more than 85% of viability except CH-DOX0.3, which resulted in 75% of viability at 48 h (Fig. 4).

3.4. DOX in vitro release rate and hydrogels swelling

DOX release from CH-DOX0.1 and CH-DOX 1 gels exhibited a two-stage profile, with a rapid burst release observed within the first 24 h, followed by slow and continuous release for up for the next 168 h (Fig. 5a). The kinetic parameters calculated from Eq. (1) were n = 0.89 (k = 4.5) for CH-DOX 1 and n = 0.96 (k = 1.81) for CH-DOX0.1 indicating a controlled drug release close to zero-order. Swelling studies (Fig. 5b) showed that weight decrease once the gel was immersed in PBS, with the decrease being slower for CH-DOX compared to CH gel.

3.5. MMP inhibition

Fig. 6a shows the reduction of MMP-2 gelatinolytic activity of U-87 cells after 24 h of contact with DOX—either when directly added into solution (triangles, dotted line) or when released from the CH-DOX gels (squares, continuous line)—as a function of DOX concentration. In both cases, there was a concentration-dependent inhibition of MMP. The half maximal inhibitory concentrations (IC_{50}) were estimated to be 0.01 mg/mL and 0.03 mg/mL for the DOX solutions and the DOX extracts of the gels, respectively. The MMP inhibition of gels containing different concentrations of DOX—that were pre-incubated for 1 or 3 days—is displayed in Fig. 6b. Gelatin zymography images are added to the Supplementary Results section (Fig. E2). All concentrations of DOX significantly reduced MMP activity compared to control ($p < 0.05$). These results confirmed that encapsulation of DOX within a CH gel does not alter its bioactivity and that DOX remains bioactive in the gel for at least 4 days.

3.6. FTIR and SEM

Chitosan powder had a characteristic peak at 1150 cm^{-1} for C-N stretching of the amine group. DOX powder had a peak at 1661 cm^{-1} characteristic of a C=O amide and at 1604 cm^{-1} characteristic of a ketone C=O stretch. DOX also had two peaks at1574 cm^{-1} and 1556 cm^{-1} [24] characteristic of N—H bending of the amide groups; these were well distinguished in the spectrum of the powder mixture. The FTIR spectrum of the lyophilized CH-DOX gel was different than the physical mixture of their powders. The band shifted from 1661 cm^{-1} to 1691 cm^{-1} and the bands at 1556 cm^{-1} and 1574 cm^{-1} were no longer observed (Fig. E3).

SEM of freeze-dried hydrogels confirmed the presence of a homogeneous porous three-dimensional structure. As shown in Fig. 7 (a & b) the pore size was smaller for gel containing VIS and DOX compared to CH gels.

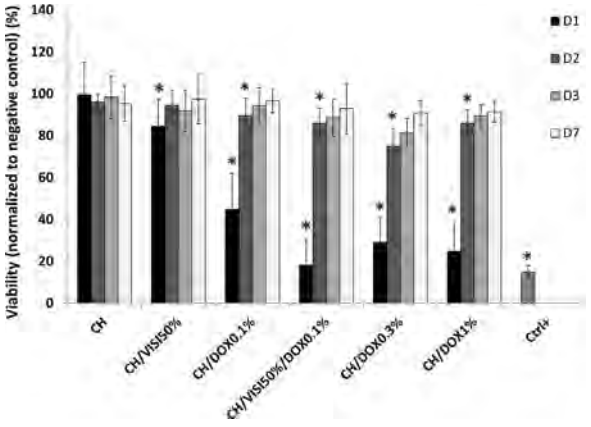

Fig. 4. Viability of L929 fibroblasts exposed to extracts recovered at days 1, 2, 3 and 7 during incubation with various CH hydrogels (n = 18, mean ± SD). (Ctrl- = cells in medium culture, Ctrl+ = cells exposed to 10% DMSO). Data are shown as mean ± SD. * $p < 0.05$ compared to control.

3.7. In vivo testing

Short term *in vivo* embolization of the renal porcine caudal polar artery with CH-DOX0.1 gels was successfully performed through the 0.61 mm internal diameter micro-catheter (Table 2). Fig. 8a and b show digital subtraction angiography (DSA) images before and after complete embolization of the artery, respectively. This gel formulation (containing 50%v/v VIS) had good visibility during injection under fluoroscopy, as confirmed by a 25-year experienced interventional radiologist (Fig. 8c). We did not observe any evidence of proximal or distal gel migration nor catheter gluing during and after embolization. The efficacy of embolization is summarized in Table 2 where the percentage of occluded vessels immediately and after a delay is indicated. Immediate success was excellent (96%), but it was reduced to 86% after 20 min. Histology of the embolized kidney confirmed that the gel was able to induce endothelial ablation (Fig. 9 b).

4. Discussion

In this study we developed an injectable, radiopaque CH hydrogel for DOX delivery. We hypothesized that the combination of the occlusive properties of CH hydrogels with the sclerosing and MMP inhibiting properties of DOX would create an embolic agent particularly well suited to manage endoleaks. To achieve this, we first optimized the mechanical properties of the CH hydrogel by mixing CH with different gelling agents [19]. We found that only gels prepared with SHC0.075PB0.08M offered sufficient initial mechanical properties suitable for embolization [16]; thus, further experimentation was performed with this gelling agent alone. Next, VIS was added to the hydrogel to confer radiopacity; the gel's mechanical properties remained adequate. Finally, DOX was added to the hydrogel to confer sclerosing and MMP-inhibiting properties.

DOX is a promising agent to prevent aneurysm progression and rupture. The pathophysiology of aneurysm progression is related to the degradation of the vascular wall by MMPs present within the inflammatory microenvironment [25]. Recent studies employing DOX have shown aneurysm suppression in experimental animal models and short term clinical trials [10,11,26,27]. The mechanism of DOX-mediated MMP inhibition is ascribed to its ability to competitively bind with zinc, which is a co-factor required for MMP function [28]. However, DOX may also prevent the progression of AAAs through other mechanisms. Indeed, AAAs could be considered an autoimmune condition given that AAA tissue shows clonal expansion of T cells [29]. It has been proposed that DOX, which can inhibit mitochondrial protein synthesis and arrest cell proliferation, also may prevent the local clonal expansion of T cells and the progression of disease [30].

While DOX therapy is promising, certain studies have concluded that oral DOX treatment is not effective for long-term or for the treatment of small AAA [31]. However, this may have been due to insufficient dosing; as high, long-term, systemic dosing may have been avoided due to the risk of negative side-effects. Local DOX delivery is an alternative to solve these issues. Local delivery was shown to provide effective short term treatment in experimental models of AAAs in rat [32], requiring lower drugs amount and theoretically avoiding dose-related side effects upon systemic

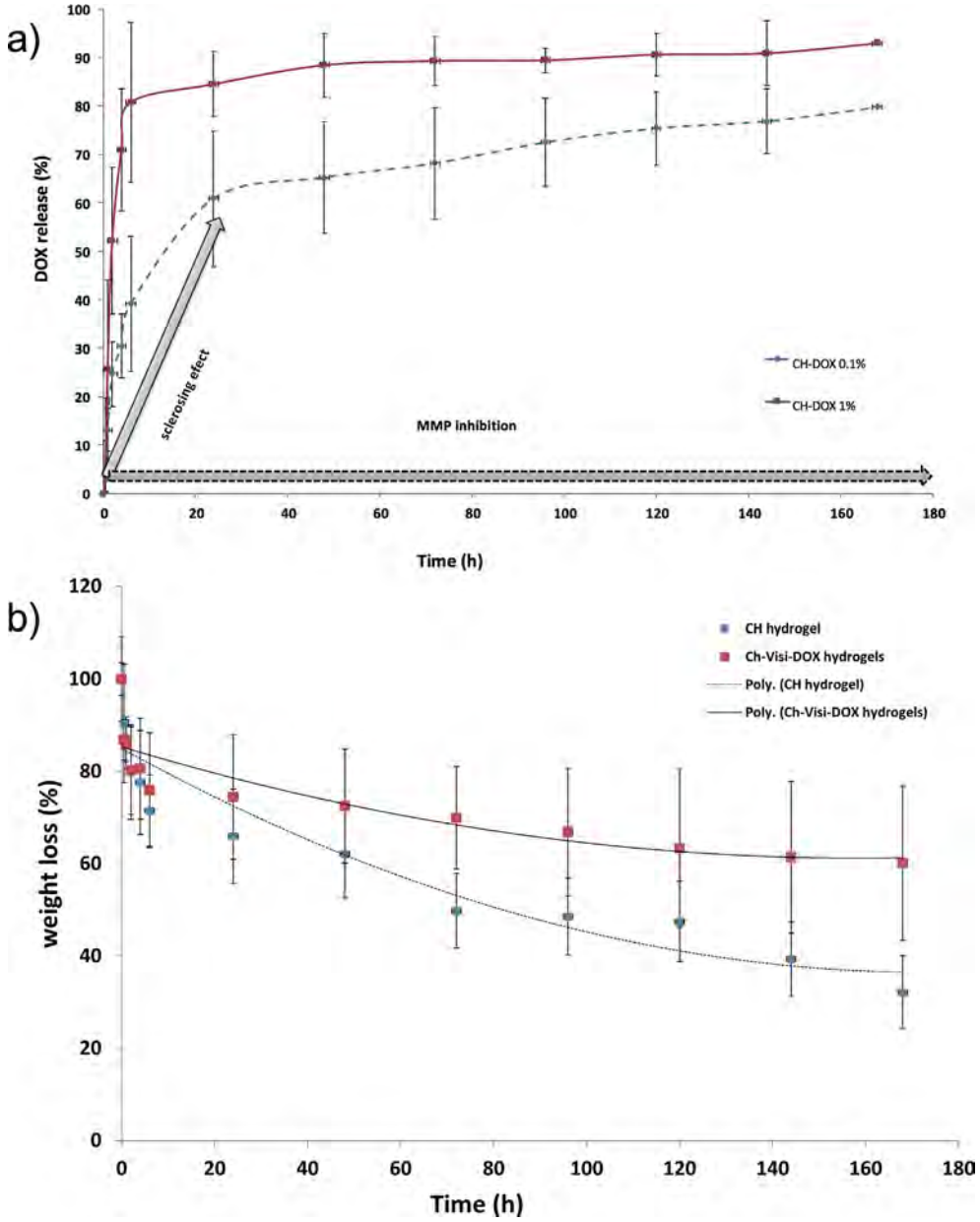

Fig. 5. a) DOX release rate from CH-DOX0.1 and CH-DOX1 gels done by release test. Aliquots of sample were withdrawn at different time intervals (0.5,1,2,4,6,24,48,72,96,120,144, 168 h). b) Evaluation of swelling behaviour of the hydrogels in PBS at 37 °C using weight loss measurements at equal time intervals to the release test. Poly (polynomial trendline) is a curved line that is used when data fluctuates.

administration [12,32]. Such side effects include pulmonary fibrosis, hair loss and pigmentation [9,33].

In addition to MMP inhibition, DOX also offers sclerosing properties. The ability of DOX (10 mg/mL in serum) to act as a sclerosing agent has been widely reported in lymphatic malformations [34,35]. The vascular endothelial layer is known to play an important role in the recanalization process and in the persistence of endoleaks [7]. Indeed, previous preclinical work has shown that an embolizing agent combining occlusive and sclerosing properties tends to reduce endoleak persistence compared to an agent with simple occlusive properties [2]. However, this agent had suboptimal mechanical properties, which were improved upon in this study with a superior gelling agent. Furthermore, neither this gel nor commercial embolic agents have sought to address the underlying pathophysiology of aneurysm progression associated with excessive activity of MMPs and resulting in progressive vessel wall destruction.

Again, the *in situ* DOX delivery within a hydrogel could help reduce the dosage of DOX required to induce endothelial sclerosis by increasing local drug retention. This is advantageous as it avoids exposure to large quantities of sclerosants.

DOX was released from the hydrogel in a two-stage pattern. There was an initial fast-burst release of DOX that followed first order kinetics within the first 24 h, followed by a slow, continuous release of DOX over the following week. We believe that immediate exposure of the vascular endothelium to high concentrations of DOX would elicit a sclerosing effect—as per the *ex vivo* studies and *in vivo* histology—and initiate MMP inhibition. The following sustained release, conversely, would sustain MMP inhibition—as per

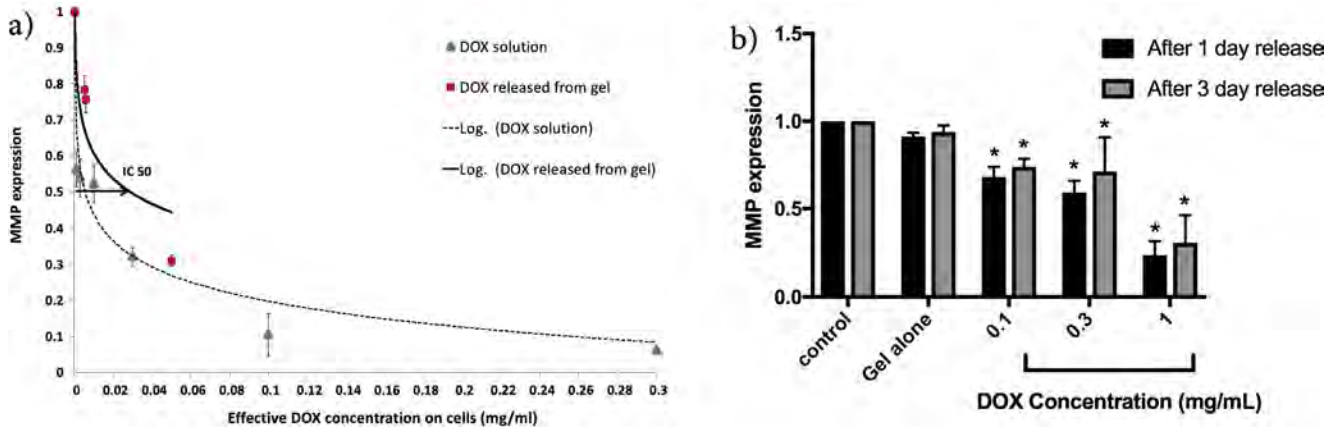

Fig. 6. a) Effect of DOX on MMP-2 gelatinolytic activity after 24 h contact with cells, when directly added in solution (blue triangles (dotted line)) or when released from the CH-DOX gels (Red squares; continuous line).The similar trend confirms that DOX remain bioactive after several days within the gel. b) MMP inhibition from gels with different concentrations of DOX that have been pre-incubated for 1 and 3 days. Data are shown as mean ± SD. * $p < 0.05$ compared to control.

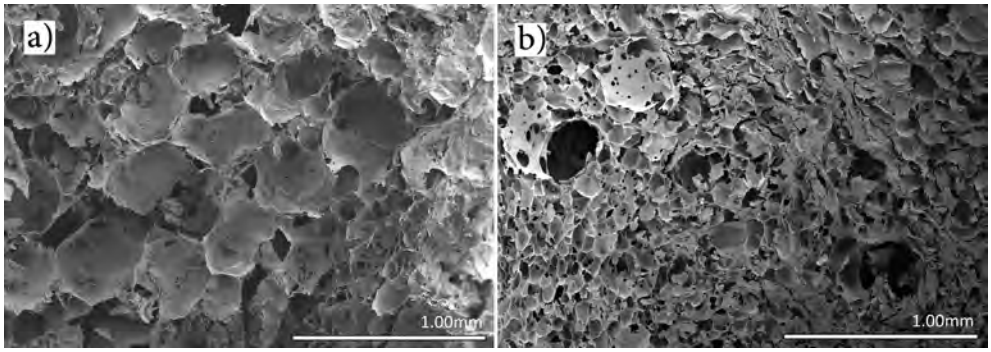

Fig. 7. SEM images of freeze dried a) CH hydrogel b) CH -DOX hydrogel.

Table 2
Percentage of immediate and delayed (after 17 min ± 5 min) successful occlusion of porcine renal caudal artery branches at various division levels and overall after embolization with CH/SHC0.075PB0.08/VIS50/DOX0.1, as measured by digital subtraction angiography.

Division	Immediate occlusion (%) Mean ± SEM (n = 11)	Delayed occlusion (%) Mean ± SEM (n = 8)
D1	87.5 ± 7.2	66.5 ± 12.6
D2	96.6 ± 2.3	83 ± 5.6
D3	97.3 ± 1.8	90.4 ± 4.1
Total:	**96.4 ± 2.4**	**85.8 ± 4.8**

the MMP inhibition zymography—while avoiding prolonged inflammatory reaction of the surrounding tissue—as evidenced by the lack of cytotoxicity after one day.

The fast burst release of DOX is likely explained by both rapid water loss and rapid diffusion of the drug from the gel surface; the continuous release can be explained by the slow diffusion of DOX from the gel core. The swelling tests showed substantial mass loss (35%) of the hydrogel during the first 24 h, which likely corresponded to both water loss and the release of VIS. The rapid release of VIS has previously been studied and occurs within hours of exposure to fluid [18]. The slow release of DOX in the second phase could be due to either its low solubility or to hydrogel-drug interactions. The similarity of the DOX release profile and swelling analysis support the role of the hydrogel in controlling the release of the agent.

Addition of DOX and VIS did not prevent gelation and led to gels with sufficient mechanical properties to impede blood circulation. Injectability of the gel through a microcatheter without risk of catheter blockage was confirmed both *in vitro* and *in vivo*. The visibility of CH-DOX on fluoroscopy was sufficient for accurate targeting and monitoring of migration. These properties demonstrated feasibility for blood vessel embolization. Furthermore, one of the appealing features of the hydrogel is that its injection does not require the use of toxic solvents, which is a requirement of some current embolic agents [36]. As previously mentioned, indirect cytotoxicity testing suggested that after the initial DOX burst release, CH-DOX 0.1 should have no toxic effect. The gel should also become a biocompatible and biodegradable scaffold that can be invaded by cells, as previously shown with a quite similar chitosan gel [37].

SEM showed no evidence of DOX crystallization on the surface of the gel, indicating the homogeneous dispersion. The smaller pore size of the CH-DOX hydrogel compared to CH alone may have been due to the presence of both VIS, which confers higher viscosity to the gel, and/or DOX, whose low solubility slows down matrix formation. These interactions may have resulted in the tighter network structure, which we suspect would be better able to control drug release. The FTIR data (i.e. the shifting of the 1661 cm^{-1} band and disappearance of the 1556 cm^{-1} and 1574 cm^{-1} bands of DOX) indicates that there are weak interactions between the amide groups of DOX and hydroxyl groups of CH, which limits vibrational motion [24]. DOX may be entrapped in the CH hydrogel network and lose molecular freedom.

The main challenge of this project was to determine the optimal DOX concentration to include in the final gel. In agreement with previous studies [28], free DOX was shown to promote a

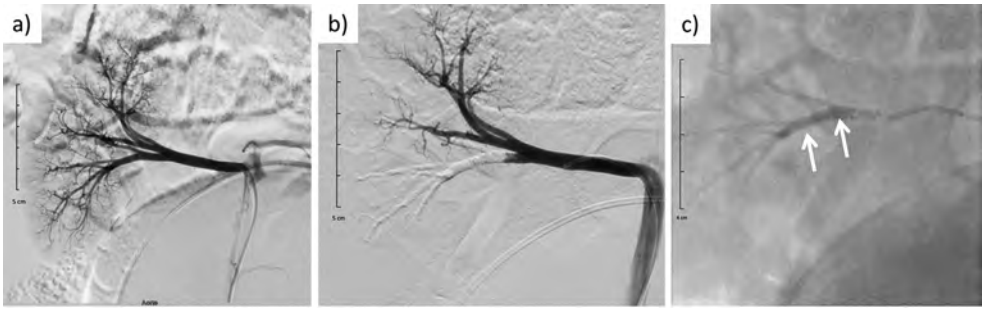

Fig. 8. DSA before a) and after b) complete embolization of the right lower polar artery of kidney by CH-DOX0.1 gels c) radiopaque embolizing gel visible without substraction (arrows).

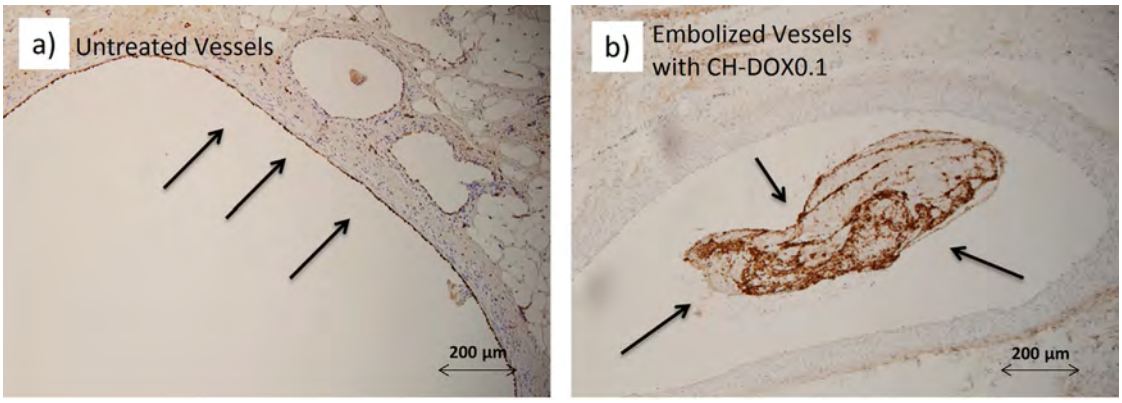

Fig. 9. Factor VIII immunostaining of *in vivo* embolized vessels: (a) untreated; (b) embolized with CH-DOX0.1. The figure shows that the endothelial lining is removed *in vivo* after embolization with CH-DOX0.1 gels, while it is clearly visible on untreated arteries (brown stain, arrows).

concentration-dependent decrease in cell viability, with about 50% survival after 24 h exposure to 0.8 mg/mL. Translation of *in vitro* data to *in vivo* situations remains difficult, however. E*x vivo* embolization was also performed and de-endothelialization was demonstrated at all DOX concentrations tested (0.1–1%w/v). MMP tests also demonstrated that 1) DOX contained in hydrogels was still bioactive after 4 days and 2) the gel containing 0.1% DOX was able to suppress MMP-2 activity by 70% after 24 h.

Based on these data, we chose to use 0.1% (w/v) DOX as the concentration tested during *in vivo* assays. This low concentration was selected to reduce risks of adverse side effect such as possible chronic inflammation (due to DOX sclerosing properties) and delayed healing [9]. Furthermore, CH-DOX with 0.1% had a lower risk of DOX oxidation which we felt would facilitate more scalable production in the future.

However, the *in vivo* data suggest that a higher DOX concentration may have been more appropriate. While CH-DOX 0.1 initially led to occlusion of 96% of target arteries, post-embolization recanalization was noted, particularly of the proximal branches (Fig. 8). This finding could be explained by inadequate thrombus formation or insufficient mechanical properties. While *ex vivo* results demonstrated that de-endothelialization occurred when the vascular lumen was exposed to DOX at 0.1% after 3 h, the *in vivo* tests showed that this concentration may not have been sufficient to promote an immediate and robust thrombotic reaction secondary to vascular sclerosis [38]. Furthermore, hydrogels containing 0.3% DOX demonstrated slightly better mechanical properties. Therefore, although this hydrogel appears promising for the treatment of aneurysms and endoleaks, further optimization of the concentration of DOX will be pursued to promote optimal de-endothelialization, leading to a faster and more robust thrombotic reaction.

Further tests will also be required to better assess the risks of off-target embolization, which is a concern for all liquid-based embolic agents. Yet, CH-DOX has several features that mitigate this risk. Its viscosity which makes it less likely to migrate in conditions of high flow and its rate of injection can be reduced to prevent distal migration, since there is no risk of catheter gluing as observed with cyanoacrylates. Embolization can also be performed through a balloon catheter to prevent not only off target embolization but reflux as well.

The present study is limited by the small number of animals and unavailability of appropriate animal models. The short-term preliminary animal study was useful as proof of concept of occlusive properties in the micro and microvasculature. However, more comprehensive animal studies in an experimental aneurysm model are needed to evaluate long term occlusion, gel degradation and severity of inflammation in the future. Such a model should mimic aneurysmal pathology (including atherosclerotic disease) in order to assess the efficacy of the product not only as an embolizing agent but also as a MMP inhibition strategy.

CH-DOX hydrogels could also potentially be used for the embolization of intracranial aneurysms and embolization of AVMs which are associated with increased MMP-2 and MM-9 expression respectively. CH-DOX could either replace or act as an adjunct with current embolic agents (especially since we believe that it can withstand systemic pressures based on our *in vitro* results and that it can reliably inhibit MMP expression) [39,40].

5. Conclusion

An injectable radiopaque chitosan-based physical hydrogel was developed as carrier system for local controlled drug (DOX) deliv-

ery and evaluated for its potential as injectable embolizing agent for blood vessel and endoleaks. The gel is injectable through a microcatheter and has sufficiently rapid gelation and mechanical properties to block tubular structures subjected to physiological pressure. The two inter-related stage DOX delivery enables the release of a large amount of drug to remove the endothelium and induce vessel thrombosis (sclerosing properties) and initiate MMPs inhibition, while the prolonged slow release stage that follows is favorable to continue MMP inhibition. This could therefore counteract ECM degradation and aneurysm progression. While preliminary short-term *in vivo* testing with DOX 0.1% concentration confirmed feasibility for blood vessel embolization and showed promising data, it also suggested that higher DOX concentration might be required to avoid recanalization of vessels after embolization. Altogether, these injectable radiopaque chitosan-based hydrogels therefore present very interesting features for blood vessel and aneurysm embolization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2017.09.021.

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