



**HAL**  
open science

# Optimisation des propriétés théranostiques de l'émulsion de chimio-Lipiodol utilisée pour la chimio-embolisation de l'hépatocarcinome.

Frédéric Deschamps

## ► To cite this version:

Frédéric Deschamps. Optimisation des propriétés théranostiques de l'émulsion de chimio-Lipiodol utilisée pour la chimio-embolisation de l'hépatocarcinome.. Biotechnologies. Université Paris Saclay (COmUE), 2018. Français. NNT : 2018SACLS004 . tel-01977702

**HAL Id: tel-01977702**

**<https://theses.hal.science/tel-01977702>**

Submitted on 11 Jan 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Optimisation des propriétés théranostiques de  
l'émulsion de chimio-Lipiodol utilisée pour la  
chimio-embolisation de l'hépatocarcinome.**

Thèse de doctorat de l'Université Paris-Saclay  
préparée à l'Université Paris-Sud

École doctorale n°582 CBMS Cancérologie : biologie -  
médecine –santé  
Spécialité de doctorat : Sciences de la vie et de la santé

Thèse présentée et soutenue à Villejuif, le 10 janvier 2018, par

**Mr Frédéric Deschamps**

Composition du Jury :

Pr Michel DUCREUX	Oncologue, Gustave Roussy, Villejuif	Président
Pr Valérie VILGRAIN	Radiologue, Hôpital Beajon, Clichy	Rapporteur
Pr Boris GUIU	Radiologue, Hôpital St-Eloi, Montpellier	Rapporteur
Dr Laurence MOINE	Chercheur CNRS, Chatenay Malabry	Examinatrice
Pr Eric VIBERT	Chirurgien, Hôpital Paul Brousse, Villejuif	Examineur
Dr Alban DENYS	Radiologue, CHUV, Lausanne	Examineur
Pr Thierry DE BAERE	Radiologue, Gustave Roussy, Villejuif	Directeur de thèse
Pr Angelo PACI	Pharmacien, Gustave Roussy, Villejuif	Co-directeur de thèse



**Titre :** Optimisation des propriétés théranostiques de l'émulsion de chimio-Lipiodol utilisée pour la chimio-embolisation de l'hépatocarcinome.

**Mots clés :** Foie, hépatocarcinome, chimiothérapie, Lipiodol, émulsion, nanoparticules

**Résumé :** La chimio-embolisation hépatique (CHE) est recommandée pour le carcinome hépatocellulaire (CHC) non curable. L'utilisation de plateformes de release au cours des CHE permet la libération lente de la chimiothérapie dans les artères tumorales et l'exposition prolongée des cellules tumorales tout en minimisant les effets systémiques. L'émulsion lipiodolée est l'une des plateformes les plus couramment utilisées pour la CHE du CHC. Cependant, la libération de chimiothérapie se produit trop rapidement car les émulsions lipiodolées sont très instables. Tout d'abord, nous avons démontré qu'une meilleure stabilité (utilisation d'un tensioactif synthétique comme émulsifiant) améliore les propriétés thérapeutiques de l'émulsion lipiodolée dans un modèle de lapin porteur de tumeurs VX2. Une revue de la littérature indique également que l'émulsion lipiodolée doit être de type eau dans l'huile (w/o) pour une meilleure sélectivité tumorale. Ensuite, nous avons analysé les paramètres techniques qui forment des émulsions w/o avec la meilleure stabilité et sans émulsifiant supplémentaire. Nous avons constaté que l'incorporation lente de la chimiothérapie dans le Lipiodol aboutit à un type d'émulsion w/o beaucoup plus prévisible et à une plus grande stabilité.

Nous avons également constaté qu'un ratio volumique élevé Lipiodol / chimiothérapie est un paramètre important pour la stabilité de l'émulsion.

Ensuite, nous avons développé un nouveau concept d'émulsion pour CHE qui utilise des nanoparticules d'acide polylactique-co-glycolique (PLGA) (Nps) pour stabiliser le Lipiodol et la chimiothérapie (Emulsion de Pickering). L'analyse in vitro a démontré que l'émulsion de Pickering est la seule plateforme de release (versus émulsion lipiodolée ou billes chargées) qui permet une libération progressive et complète des diverses chimiothérapies testées (doxorubicine, irinotécan, oxaliplatine) mais aussi d'anticorps (anti-CTLA4), ce qui fait de l'émulsion Pickering une plateforme de release universelle, non seulement pour la CHE mais aussi pour l'immunothérapie intra-artérielle. Enfin, la CHE avec de l'oxaliplatine a été réalisée dans un modèle de lapin porteurs de tumeurs VX2 pour comparer l'émulsion de Pickering et l'émulsion lipiodolée. Grâce à son release lent, l'émulsion de Pickering a considérablement diminué l'exposition systémique, mais également augmenté de manière significative le ratio tumeur / foie de l'exposition à l'oxaliplatine.

**Title :** Optimization of the theranostic properties of the Lipiodol-emulsion used for liver trans-arterial chemo-embolization of hepatocellular carcinoma.

**Keywords :** Liver, hepatocellular carcinoma, chemotherapy, Lipiodol, emulsion, nanoparticles.

**Abstract :** Liver Trans-Arterial Chemo-Embolization (TACE) is recommended for non-resectable, intermediate Hepatocellular carcinoma (HCC). The use of drug delivery platforms during TACE allows slow release of chemotherapy into the tumors' arterial supply and prolonged exposure of the tumor cells while minimizing systemic exposures. Lipiodol-emulsion is one of the most widely used drug delivery platforms for TACE of HCC. However, the release of chemotherapy occurs very rapidly because Lipiodol-emulsions have poor stability. First, we have demonstrated that high stability (using synthetic surfactant as emulsifier) improves the therapeutic properties of Lipiodol-emulsion in a VX2-tumor rabbit model. A literature review also indicates that Lipiodol-emulsion must be water-in-oil (w/o) for better tumor' uptakes. Next, we analyzed technical parameters that formulate in vitro w/o emulsions with better stability without any additional emulsifier. We found that progressive incorporation of chemotherapy in the Lipiodol

results in much more predictable w/o type and higher stability and that higher ratio of Lipiodol/chemotherapy is also a critical parameter for stability. Then, we develop a new concept of emulsion for TACE that uses Poly(lactic-co-glycolic acid) (PLGA) nanoparticles (Nps) to stabilize Lipiodol and chemotherapy (Pickering-emulsion). In vitro analysis demonstrated that Pickering-emulsion is the only drug delivery system (versus Lipiodol-emulsion and drug eluting beads) that allows slow and complete release of various chemotherapies (doxorubicin, irinotecan, oxaliplatin) but also of antibodies (anti-CTLA4), making Pickering-emulsion an universal carrier, not only for TACE but also for trans-arterial immunotherapy. Finally, TACE with oxaliplatin were performed in a VX2-tumor rabbit model to compare in vivo Pickering-emulsion and Lipiodol-emulsion. Thanks to its slow drug release, Pickering-emulsion significantly decreased the systemic exposure but significantly increased the tumor/liver ratio oxaliplatin exposure.



# Remerciements

Je voudrais exprimer mes remerciements au Pr Valérie Vilgrain et au Pr Boris Guiu d'avoir accepté d'être mes rapporteurs de thèse.

Je souhaite exprimer ma gratitude aux membres du jury pour l'honneur qu'ils me font d'être présents lors de ma soutenance.

Je tiens à remercier mon directeur de thèse, le Pr Thierry de Baere, et mon co-directeur de thèse, le Pr Angelo Paci, pour leur soutien tout au long de ce travail.

Je remercie chaleureusement le Dr Lluís Mir pour m'avoir accueilli dans son laboratoire de Vectorologies et Thérapeutiques Anticancéreuses.

Je souhaite remercier les équipes CNRS m'ayant accueilli dans leur laboratoire pour réaliser mes différents travaux de recherche (UMR 8203 de Gustave Roussy et UMR 8612 de la Faculté de Pharmacie de Châtenay-Malabry).

A special thanks to the Interventional Radiology department at Northwestern Hospital, Chicago, IL (Pr Riad Salem, Dr Robert Lewandowski) and to the research lab at Northwestern University, Chicago, IL (Pr Andrew Larson, Kathleen Harris, Weigo Li). I really enjoyed my stay in Chicago and it was a great pleasure to work with all of you.

Un grand merci au Dr Laurence Moine et à Mr Thomas Isoardo. Travailler avec vous est un réel plaisir. J'ai hâte de continuer !

Un grand merci également à l'équipe de radiologie interventionnelle de Gustave Roussy qui m'a soutenu pendant ces années de recherche et une pensée toute particulière pour le Dr Lambros Tselikas pour son enthousiasme et son engagement dans mes travaux. « A ton tour maintenant, je suis avec toi ! ».

Je remercie Fanny, ma femme, pour son soutien quotidien, son amour et ses encouragements. Je ne l'aurai pas fait sans toi. Je remercie Sam, mon fils, et Maya, ma fille, pour leur touchante admiration. Je remercie Anny et Daniel, mes parents, qui m'ont porté jusque là et pour leur soutien rassurant et inconditionnel. C'est une grande joie de vous avoir à mes côtés.

# Table des matières

<b><u>TRANS-ARTERIAL CHEMO-EMBOLIZATION FOR HEPATOCELLULAR CARCINOMA.....</u></b>	<b>8</b>
<b>HEPATOCELLULAR CARCINOMA .....</b>	<b>8</b>
<b>TRANS-ARTERIAL CHEMO-EMBOLIZATION FOR HEPATOCELLULAR CARCINOMA .....</b>	<b>10</b>
PRINCIPLE.....	10
RESULTS.....	10
DRUG DELIVERY PLATFORMS .....	11
<b><u>OPTIMIZATION OF LIPIODOL-EMULSIONS FOR TACE .....</u></b>	<b>14</b>
<b>LITERATURE.....</b>	<b>14</b>
<b>STABILITY IS A CRUCIAL PARAMETER FOR THERANOSTIC PROPERTIES.....</b>	<b>16</b>
ABSTRACT: .....	16
INTRODUCTION: .....	18
MATERIAL AND METHODS:.....	18
RESULTS:.....	24
DISCUSSION:.....	28
LIMITATIONS:.....	30
CONCLUSION: .....	30
<b>PARAMETERS FOR STABLE WATER-IN-OIL LIPIODOL-EMULSION .....</b>	<b>31</b>
ABSTRACT: .....	31
MATERIALS AND METHODS: .....	34
RESULTS:.....	37
DISCUSSION:.....	40
LIMITATIONS:.....	42
CONCLUSIONS:.....	42
<b><u>PICKERING-EMULSIONS.....</u></b>	<b>44</b>
<b>GENERALITIES .....</b>	<b>44</b>

<b>FORMULATION OF WATER-IN-OIL PICKERING-EMULSION FOR TACE.....</b>	<b>46</b>
INTRODUCTION .....	46
MATERIALS AND METHODS.....	46
RESULTS.....	50
DISCUSSION .....	58
CONCLUSIONS .....	61
<b>PICKERING-EMULSION: IN VITRO RELEASES .....</b>	<b>62</b>
MATERIALS AND METHODS.....	62
RESULTS.....	64
DISCUSSION .....	72
LIMITATIONS.....	73
CONCLUSIONS.....	73
<b>PICKERING-EMULSION FOR LIVER TRANS-ARTERIAL CHEMO-EMBOLIZATION .....</b>	<b>74</b>
ABSTRACT: .....	74
INTRODUCTION .....	76
MATERIALS/ METHODS.....	76
RESULTS.....	80
DISCUSSION .....	84
LIMITATIONS.....	86
<b>PICKERING-EMULSION FOR LIVER TRANS-ARTERIAL IMMUNOTHERAPY. ....</b>	<b>87</b>
INTRODUCTION .....	87
MATERIALS AND METHODS: .....	89
RESULTS:.....	89
DISCUSSION:.....	91
<b><u>CONCLUSIONS:.....</u></b>	<b><u>92</u></b>
<b>PUBLICATIONS/ COMMUNICATIONS RELATED TO THIS WORK.....</b>	<b>93</b>
<b><u>BIBLIOGRAPHY .....</u></b>	<b><u>94</u></b>

## Synthèse en français

La chimio-embolisation artérielle hépatique (CHE) est le traitement de référence du carcinome hépatocellulaire inopérable. Ce traitement consiste en l'association d'une injection de chimiothérapie (en général de la doxorubicine) à une embolisation artérielle ischémique, directement dans les artères tumorales. La chimiothérapie est libérée de façon lente et prolongée dans les artères tumorales grâce à l'utilisation d'un vecteur dans lequel la chimiothérapie est préalablement « chargée ». Ce vecteur doit idéalement posséder des propriétés diagnostique (visibilité en imagerie) et thérapeutique (libération contrôlée et prolongée de la chimiothérapie). Cependant, aucun des vecteurs utilisés actuellement (Lipiodol® ou billes chargées) ne possède cette propriété théranostique (diagnostique et thérapeutique). Les billes chargées sont des microsphères à base d'alcool polyvinylique qui peuvent être chargées avec une chimiothérapie (mécanisme d'échange d'ions) et sont disponibles dans des tailles différentes allant de 70 à 700 µm. Ces billes ont déjà démontré une augmentation significative de l'exposition à la chimiothérapie tumorale et une diminution de la toxicité systémique mais elles ne contiennent pas d'agents de contraste. Ainsi, il est impossible de visualiser avec précision l'administration de la chimiothérapie pendant la procédure et de quantifier la concentration de chimiothérapie dans la tumeur après la procédure. En outre, seuls la doxorubicine et l'irinotécan peuvent être chargés dans des billes jusqu'à présent.

Le lipiodol est utilisé pour ses propriétés d'agent de contraste radio-opaque hydrophobe (huile) visible sous fluoroscopie aux rayons X et pour sa sélectivité pour les artères tumorales. La réalisation d'émulsions de chimiothérapie avec le Lipiodol permet donc d'espérer une délivrance sélective et visible de la chimiothérapie dans les tumeurs hépatiques. Cependant, les propriétés théranostiques de ces émulsions lipiodolées sont limitées par leur faible stabilité : le déphasage entre la chimiothérapie et le lipiodol se fait en quelques minutes. Très rapidement, les deux phases de l'émulsion se séparent et la

quasi-totalité de la chimiothérapie qui est soluble dans l'eau disparaît de la tumeur. En outre, comme l'émulsification est obtenue par pompage répétitif de 2 seringues (1 de chimiothérapie et 1 de lipiodol) par un robinet à trois voies, la technique n'est pas reproductible d'un opérateur à l'autre. Cette mauvaise stabilité se traduit par une concentration tissulaire tumorale aléatoire de la chimiothérapie. Bien que diverses techniques (mélangeur, ultrasons, émulsifiants) et divers types d'émulsion lipiodol (eau-dans-huile par rapport à huile-dans-eau, différents rapports de lipiodol et de chimiothérapie) ont été utilisés dans des études précliniques pour améliorer la stabilité et la reproductibilité de l'émulsion, aucune d'entre elles n'a réussi.

La stabilisation de l'émulsion de doxorubicine-lipiodol grâce à un agent tensioactif est l'objet d'un premier travail. Celui-ci a consisté en une évaluation expérimentale in vivo des propriétés théranostiques d'une émulsion eau-dans-huile (W/O) stabilisée de doxorubicine-lipiodol. Cette émulsion a été caractérisée in vitro puis une 1ère série de CHE sur lapins porteurs de tumeurs hépatiques VX2 a permis de démontrer que la sélectivité pour les artères tumorales était plus forte pour l'émulsion W/O stabilisée de doxorubicine-lipiodol que pour les émulsions préparées selon les usages cliniques habituels (W/O et O/W non stabilisées). Une 2ème phase expérimentale a comparé 3 modes d'administrations intra-artérielles hépatiques d'une même dose de doxorubicine sur lapins porteurs de tumeurs hépatiques VX2 : 1- doxorubicine seule, 2- émulsion W/O non stabilisée de doxorubicine-lipiodol et 3- émulsion W/O stabilisée de doxorubicine-lipiodol. Les analyses pharmacocinétiques ont démontré une nette diminution du pic plasmatique de doxorubicine ainsi qu'une nette augmentation de la concentration de doxorubicine intra-tumorale avec l'émulsion W/O stabilisée de doxorubicine-lipiodol par rapport aux deux autres modes d'administrations. Au total, ces 2 phases expérimentales ont permis de faire la preuve du concept qu'une stabilisation de l'émulsion W/O de doxorubicine-lipiodol permet d'en optimiser les propriétés théranostiques.

Cependant, la stabilisation d'une émulsion par un tensioactif synthétique soulève des problèmes de toxicité, incompatibles avec une utilisation médicale. La 2ème partie de ce

travail a donc été d'optimiser la technique d'emulsification sans utiliser de tensio-actifs. Ce travail a consisté à évaluer le sens des émulsions obtenues et leurs stabilités in-vitro en fonction de différents paramètres de formulations. Nous avons ainsi démontré que l'ajout progressif de la phase aqueuse (doxorubicine) dans la phase huileuse (Lipiodol) au moment du mélange permettait de favoriser très significativement le sens de l'émulsion de type W/O. Par ailleurs un fort ratio de phase huileuse et un ajout progressif de la phase aqueuse dans la phase huileuse sont deux paramètres importants pour améliorer la stabilité de l'emulsion.

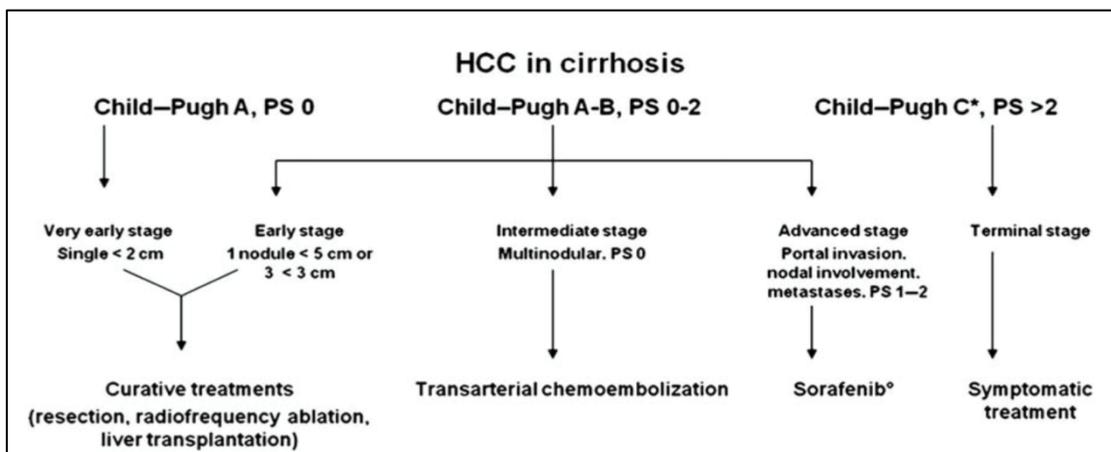
La 3eme partie de ce travail a été réalisé en collaboration avec l'Institut Galien Paris-Sud. Grâce à l'utilisation de nanoparticules de poly (acide lactique-co-glycolique) (NP de PLGA), nous sommes parvenus à résoudre le problème de l'instabilité des émulsions à base de lipiodol. Nos travaux ont démontré que nous sommes en mesure de stabiliser différents agents anticancéreux (doxorubicine/ irinotecan/ oxaliplatine/ Ipilimumab) dans des émulsions pendant plusieurs semaines et d'obtenir une libération in-vitro sur plusieurs jours de l'agent anticancéreux. Une première phase expérimentale animal a ensuite été réalisée sur modèle lapin VX2 et a démontré que l'emulsion de Pickering permettait de diminué l'exposition systémique, mais également augmenté de manière significative le ratio tumeur / foie de l'exposition lors d'une CHE à l'oxaliplatine.

# Trans-Arterial Chemo-Embolization for Hepatocellular carcinoma

## Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults and the third leading cause of cancer deaths worldwide. HCC most often develops in the background of underlying chronic liver disease, including viral hepatitis and alcoholic cirrhosis. Certain diseases, such as hemochromatosis and alpha 1-antitrypsin deficiency, markedly increase the risk of developing HCC. Metabolic syndrome and NASH (Nonalcoholic steatohepatitis) are also increasingly recognized as risk factors for HCC.

The management of HCC remains challenging due to late presentation. Most patients are ineligible for curative therapies (surgical resection, thermal-ablation or liver transplantation) due to disease burden or to severity of liver disease. Multiple classifications are available to stratify patients. The Barcelona Clinic Liver Cancer Staging (BCLC) system is the most widely used and takes into account performance status, extent of tumor involvement, and hepatic function. The BCLC system classifies HCC into five stages based on tumor burden, Child-Pugh score, and Eastern Cooperative Oncology Group (ECOG) performance status: very early and early HCC who may benefit from curative therapies (stages 0 and A), intermediate or advanced stage (stages B and C) who may benefit from palliative treatments and terminal stage (stage D).



Patients with intermediate stage disease (stage B) will be considered for trans-arterial therapies <sup>1</sup>. Compared to best supportive care, trans-arterial therapy offers a survival benefit in intermediate stage HCC and is the recommended approach for treatment <sup>1</sup>. None of the trans-arterial therapies; including bland embolization, Trans-Arterial Chemo-Embolization (TACE) or Trans-Arterial Radio-Embolization (TARE) offers a clear advantage over the other so far.

Systemic therapy is the recommended treatment for patients with advanced HCC (stage C). Cytotoxic drugs (doxorubicin or cisplatin) have demonstrated low objective response rates (<10%). This has led research away from chemotherapy to targeted molecular antagonists of pathways thought to be critical to the pathogenesis of this disease. Sorafenib is the recommended treatment, and regorafenib is an option in case of failure<sup>2</sup>. However, the modest survival benefit suggests that it is important to look for other treatment options in patients suffering from advanced HCC. In line with this, development in cancer immunotherapy with immune checkpoint blockade is a very promising approach for advanced HCC and several trials are currently on going to explore its benefit.

Median survival without therapy and expected survival improvement with specific therapies are given in the following table <sup>3</sup>.

	Natural history (median OS)	Prognosis with therapy
Very early stage (stage 0)	>36 months	Curative treatment (5-year OS= 70%–90%)
Early stage (stage A)	36 months	Curative treatemnt (5-year OS= 50%–70%)
Intermediate stage (stage B)	16 months	TACE (median OS= 20-months)
Advanced stage (stage C)	4–8 months	Sorafenib (median OS= 6–11-months)
Terminal stage D (stage D)	<4 months	

# Trans-Arterial Chemo-Embolization for Hepatocellular carcinoma

## Principle

Trans-Arterial Chemo-Embolization (TACE) was first introduced in 1977 by Dr. Yamada<sup>4</sup>, who exploited HCC preferential blood supply from the hepatic artery for the delivery of antitumor therapy. TACE is performed under image-guidance using X-ray angiography and consists in the injection of chemotherapy directly into the liver artery. An embolization is associated to prevent rapid washout of the chemotherapy and to induce ischemic necrosis. By infusing the chemotherapy directly into the tumor arterial supply, the exposure of cancer cells is increased beyond what can be safely achieved through intravenous delivery. In addition, TACE utilizes the preferential blood supply of HCC derived from the hepatic artery (~95%)<sup>5</sup>, thereby allowing local delivery of chemotherapy and embolization with minimal damage to the surrounding healthy parenchyma (primarily supplied via portal vein). The choice of chemotherapeutic agents is not standardized because no systemic chemotherapy has demonstrated a significant tumor response so far. Currently the chemotherapies injected during TACE are platinum-based agents (Cisplatin, Oxaliplatin) or anthracycline (doxorubicin, idarubicin or epirubicin).

## Results

Liver TACE is recommended with a high level of evidence in the literature for non-resectable, intermediate HCC (stage B)<sup>3</sup>. Preserved liver function and no evidence of extrahepatic metastasis nor vascular invasion are required criteria. Approximately 35%-40% of patients will achieve a 25% decrease in tumor size with response rates as high as 60%<sup>6,7</sup>. A meta-analysis of six randomized controlled trials showed that patients who underwent TACE had a 2-year improved survival rate compared to those who only had supportive therapy<sup>8</sup>. Interestingly, a meta-analysis of nine trials did not show a significant difference in survival based on chemotherapeutic agent used in TACE treatments<sup>9</sup>. Growing literature supports the efficacy of TACE for HCC downstaging and bridging to

curative treatment<sup>10,11,12</sup>. Based on response to therapy, repeat TACE treatments can be scheduled.

## Drug delivery platforms

Drug delivery platforms are currently used during TACE procedures. These act as a transient reservoir, allowing release of the chemotherapy into the tumors' supply to achieve a prolonged exposure of the tumor cells while minimizing systemic exposures. Ideally, the drug delivery platform should also act as an imaging marker in order to monitor the chemotherapy distribution during the TACE and to quantify the chemotherapy deposition within the tumor and the surrounding tissues at the end of the TACE. The term "theranostic" refers to the combination of diagnostic and therapeutic capabilities into a single agent. In clinical practice, Lipiodol and drug-eluting microspheres are the two most widely used drug delivery platforms for TACE. Both platforms have been used for the treatment of hepatocellular carcinoma<sup>3</sup> and liver metastases from neuroendocrine and less frequently for colorectal cancers.

## Lipiodol

Lipiodol is an ethyl ester of iodized fatty acids of poppy seed oil (Guerbet, France). It is an oily based radio-opaque contrast agent that also serves as a drug delivery platform because of several interesting properties. 1- When injected into the hepatic artery, Lipiodol has demonstrated a property of predominant accumulation and longer retention in HCC and in hyperarterialized liver metastases. 2- Lipiodol circulates beyond the tumor- feeding arteries into the distal portal branches through the peribiliary capillary plexus and the drainage route from the tumor<sup>13,14</sup>. 3- Lipiodol exerts a temporary embolic effect both on the hepatic artery and portal branches<sup>15</sup>. 4- Lipiodol is incorporated within the tumor cells and the endothelial cells by pinocytosis and remains in the tumor for prolonged periods<sup>16</sup>. 5- Lipiodol is highly visible under X-Ray fluoroscopy and CT-scan, making the deposit of Lipiodol an imaging biomarker for tumor response<sup>16</sup>. Lipiodol can also be used as an

imaging biomarker for survival, with studies demonstrating improved survival in patients with more complete oil retention<sup>17, 18</sup>. The loading mechanism consists in formulating a water-in-oil emulsion composed of an internal aqueous phase (containing the chemotherapy) and of an external oily phase of Lipiodol. Various chemotherapies have been emulsified in Lipiodol (doxorubicin, cisplatin, mitomycin C, streptozotocin, idarubicin,...). However, the theranostic properties of these Lipiodol-emulsions are limited in clinical practice because Lipiodol-emulsions have poor stability. Within a few minutes, the two phases of the emulsion separate, with bulk of the water-soluble chemotherapy releasing rapidly from the Lipiodol resulting in high systemic exposures to the chemotherapy, diminished deposition of the chemotherapy into the targeted tumor tissues and inadequacy between the location Lipiodol and the chemotherapy deposits<sup>19</sup>. In addition, the emulsification process is most often obtained by repetitive pumping of 2 syringes (1 of chemotherapy and 1 of Lipiodol) through a 3-ways stopcock. This technique is not reproducible from one operator to another and results in heterogeneous formulations and various pharmacological properties of Lipiodol-emulsions<sup>20</sup>.

### ***Drug-eluding beads***

To overcome the limitations of Lipiodol-emulsion, drug-eluding beads have been developed as an alternative drug delivery platform. The goal was to deliver a large amount of chemotherapy to the tumor for a prolonged period of time and to decrease the systemic exposure<sup>21,22</sup>. Drug-eluding beads are polyvinyl alcohol-based hydrogel beads. The presence of the anionic sulphonate group enables the sequestering of positively charged drugs only (Coulomb charge interactions). Thus, doxorubicin, epirubicin or irinotecan are the only chemotherapies that can be readily used in clinical practice. In addition, the ionic interaction' intensities between beads and the chemotherapy is highly variable according to the chemotherapy, resulting in variable releases: either incomplete and very slow (doxorubicin) or complete and very fast (irinotecan)<sup>23</sup>. Another disadvantage is that it is impossible to accurately monitor the deposition of the microspheres under X-ray during the TACE because

most are not radio-opaque. Recently, radio-opaque microspheres (LC Bead LUMI®, BTG) have been developed to overcome this limitation and are currently under evaluation. Finally, the beads are not only a drug delivery platform but also an embolic medical device (different sizes available, ranging from 70 up to 700  $\mu\text{m}$ ) that blocks the arterial flow. This property help to increase the tumor drug exposure but can also induce hypoxia and the associated unwanted development of new tumor blood vessels (tumor neo-angiogenesis).

Thus, it seems interesting to further improve the drug delivery platforms for TACE because neither Lipiodol-emulsion nor drug-eluting beads can be considered as ideal. For this purpose, we decided to focus this work on the ways to optimize the theranostic properties of Lipiodol-emulsion.

# Optimization of Lipiodol-emulsions for TACE

## Literature

Emulsions are defined as fine dispersions of droplets of one liquid in another immiscible liquid, which are often formed either spontaneously or due to mechanical agitation. Emulsions are made up of the continuous and dispersed phases, where the dispersed phase in the form of a droplet is typically suspended in the continuous phase. Emulsions may be classified into: i) oil-in-water (o/w) ii) water-in-oil (w/o) and iii) complex emulsions.

A review of the literature clearly indicated that best Lipiodol-emulsions for TACE should be high viscosity “water-in oil” for an optimal tumor uptake and for a better embolic effect<sup>24-27</sup>. It has been demonstrated that the ratio of tumor to non-tumorous liver uptake of Lipiodol was significantly higher with water-in-oil Lipiodol-emulsions than with oil-in-water in a VX2 model. On the other hand, it has demonstrated that water-in-oil Lipiodol-emulsion results in a superior embolic effect compared to an oil-in-water in a simulation model based on non-Newtonian fluid mechanics.

Stability of the Lipiodol-emulsion is also an important property to make Lipiodol an appropriate Theranostic drug delivery platform. Indeed, the theranostic properties of the Lipiodol-emulsion has been challenged by studies that demonstrated not correlation between the Lipiodol and the chemotherapy deposits in the tumors, suggesting that chemotherapy freely spread throughout the tumors<sup>19, 28</sup>.

To date, there is no widely accepted consensus for the formulation of Lipiodol-emulsion for TACE. Technical recommendations from experts published in 2015<sup>29</sup> suggested that the volume of chemotherapy should be lower than the volume of Lipiodol, to favor the water-in-oil emulsion type. The Lipiodol-emulsion is currently prepared by repetitive back-and-forth pumping of two syringes through a 3-ways stopcock. Vigorous mixing of the chemotherapy aqueous solution and Lipiodol via the 3-way stopcock

generates sufficient energy to decrease the size of the internal phase droplets. At least 20 pumping exchanges through the stopcock are needed to obtain an internal phase size of droplets in the range of 70–100 microns. However, the Lipiodol-emulsions formulated by the pumping technique can be very heterogeneous between operators and a recent study has reported poor stability and a limited percentage (70% for 2/1 ratio and 16% for 1/1 ratio) of water-in-oil emulsions <sup>20</sup>.

The first part of this work aims at confirming that stability is a crucial parameter for theranostic properties of Lipiodol-emulsions. The second part analyzed technical parameters that provide a reproducible and appropriate stable water-in-oil. As a result, using Pickering technology, we have developed a new concept of highly stable water-in-oil emulsions made of Lipiodol that allows a slow and controlled release of various therapeutic agents of interest in the treatment of liver tumors and especially of HCC.

## Stability is a crucial parameter for theranostic properties

### Abstract<sup>1</sup>:

#### Purpose:

To demonstrate that stability is a crucial parameter for theranostic properties of Lipiodol-emulsions during liver trans-arterial chemo-embolization.

#### Material/Methods:

We compared the theranostic properties of two emulsions made of Lipiodol and doxorubicin. Emulsion-1 reproduced one of the most commonly way of preparation (ratio of oil/water: 1/1) and emulsion-2 was designed to obtain a water-in-oil emulsion with enhanced-stability (ratio of oil/water: 3/1, plus an emulsifier). The first animal experiment compared the tumor selectivity of the 2 emulsions: 7 rabbits received left hepatic arterial infusion (HAI) of emulsion-1 and 8 received HAI of emulsion-2. 3D-CBCT acquisitions were acquired after HAI of every 0.1 mL to measure the densities' ratios between the tumors and the left liver lobes. The second animal experiment compared the plasmatic' and tumor' doxorubicin concentrations after HAI of 1.5 mg of doxorubicin administered either alone (n=3) or in emulsion-1 (n=6) or in emulsion-2 (n=6).

#### Results:

Up to 0.4 mL of emulsion infused, Emulsion-2 resulted in densities' ratios between the tumors and the left liver lobes that were significantly higher compared to emulsion-1. Plasmatic doxorubicin concentrations (at 5 minutes) were significantly lower after arterial injection of emulsion-2 (19.0µg/L) than emulsion-1 (275.3 µg/L, p<0.01) or doxorubicin

---

<sup>1</sup> Deschamps F, Farouil G, Gonzalez W, Robic C, Paci A, Mir LM, Tselikas L, de Baère T. Stabilization Improves Theranostic Properties of Lipiodol®-Based Emulsion During Liver Trans-arterial Chemo-embolization in a VX2 Rabbit Model. *CVIR* 2017 Jun;40(6):907-913.

alone (412.0  $\mu\text{g/L}$ ,  $p < 0.001$ ) and tumor' doxorubicin concentration at day-1 was significantly higher after arterial injection of emulsion-2 (20957 ng/mg) than in emulsion-1 (8093ng/g,  $p < 0.05$ ) and doxorubicin alone (2221 ng/g,  $p < 0.01$ ).

Conclusion:

Stabilization of doxorubicin in a water-in-oil Lipiodol-emulsion results in better theranostic properties.

## Introduction:

For patients with unresectable intermediate stage hepatocellular carcinoma (HCC), Trans-Arterial Chemo-Embolization (TACE) is recommended as the standard of care with a high level of evidence 1A in the literature <sup>3</sup>, either with Lipiodol or DC-Beads. Conventional TACE consists in an injection of chemotherapy mixed with Lipiodol directly into the liver arterial supply in association with embolic agents that prevent washout of the chemotherapy and induce ischemic necrosis.

The vectorization mechanism consists in formulating an emulsion of the chemotherapy (aqueous phase) and Lipiodol (oily phase). The terms “emulsion” refers to a mixture of two liquids that are normally immiscible and that will rapidly separate. This instability results in a coalescence process when droplets bump into each other and combine to form a larger droplet, so the average droplet size increases over time. Emulsion stability refers to the ability of an emulsion to resist change in its properties over time. Stabilization of the emulsion is a key to make the Lipiodol an effective theranostic carrier that will deliver the chemotherapy selectively into the feeders of the hypervascular tumors and release it slowly for a prolonged exposure and minimized systemic effects. The aim of this study is to demonstrate that stability is a crucial parameter for theranostic properties of Lipiodol-emulsions during liver trans-arterial chemo-embolization.

## Material and Methods:

### *Lipiodol-emulsions preparation*

Doxorubicin was the selected chemotherapy in this study because it is the mostly used worldwide for TACE in HCC. The Lipiodol-emulsions were prepared immediately before their hepatic intra-arterial infusion, using the three-way stopcock “back-and-forth” technique commonly used in clinical practice <sup>29</sup>.

Emulsion-1 was obtained by mixing 5 mL of Lipiodol and 50 mg of doxorubicin reconstituted in 5 mL of normal saline (ratio 1:1). No stabilization maneuver has been

performed. This emulsion was designed to reproduce the most commonly way that has been used to prepare Lipiodol-emulsions during the last twenty years <sup>30</sup>. The phase separation in the syringe was visually observed after 1h and the viscosity at 20°C was measured at 155 mPa.s.

Emulsion-2 has been designed to favor a water-in-oil emulsion with the best stability. For this purpose, we used three different interdependent parameters that have been described in the literature <sup>6,7</sup>: high Lipiodol/doxorubicin ratio, the use of an emulsifier and the use of an aqueous phase with the same density as Lipiodol for the reconstitution of doxorubicin. Thus, Emulsion-2 has been obtained by mixing 7.5 mL of Lipiodol, an emulsifier (100 mg of polyglycerol esters of polycondensed ricinoleic acids) and 50 mg of doxorubicin reconstituted in 2.5 mL of contrast medium (iobitridol, Xenetix<sup>®</sup> 250, Guerbet, Aulnay-sous-bois, France) (oil/water ratio 3:1). No phase separation is observed in the syringe within 24 hours after emulsion synthesis and the viscosity at 20°C is measured at 193 mPa.s.

### ***Study flow-chart***

We have performed two successive comparative in vivo animal experiments after injection of the 2 different emulsions. The first one was a 4D imaging of the Lipiodol uptake in tumor and the surrounding liver parenchyma and the second one was the pharmacokinetic study.

### ***Animals' experiments***

The animal experiments were performed in accordance with protocols approved by animal care and use committee from French Research Ministry under the number authorization 00441.01 for tumor uptake experiments and under the number 01267.02 for pharmacokinetic experiments. All surgeries were performed under anesthesia with a

mixture of ketamin hydrochloride, xylazin and glycopyrolate injected IM, and all efforts were made to minimize suffering.

Thirty New Zealand white rabbits (supplied by Charles River, France) weighing 3.5-4.5 kg were used for these experiments. For successful implantation of the VX2 tumor into the liver, four VX2 tumor shunks ( $1 \text{ mm}^3$ ) were thawed and first grown for three weeks on the muscles of the hind limbs of a carrier rabbit. Then, single chunks of  $1 \text{ mm}^3$  from carrier rabbit were harvested into the left lobe of liver of recipient rabbit (VX2 rabbits). The abdomen of each rabbit was shaved and prepped with providine iodine. The liver was exposed with a midline subxiphoid incision, and tumor shunks ( $1 \text{ mm}^3$ ) from the harvested minced tumors were implanted directly into the left lobe of the exposed liver of the recipient rabbits. The method allows the growth of a single, solitary, well-demarcated tumor, the liver of each recipient rabbit (VX2 model). The abdomen was closed in two layers, and proper aseptic technique was rigorously observed during each implantation. After surgery, the animals were placed in cages and monitored under the direct supervision of the technician until they recovered from anesthesia. Flunixin was administered SC for pain and distress. The tumors were allowed to grow for another  $19 \pm 1$  days.

Two successive animal experiments were performed in rabbits VX2 hepatoma models. The first experiment was a 4D imaging of the tumor Lipiodol uptake, designed to measure the tumor uptake selectivity of the two Lipiodol-emulsions. The second experiment was designed to assess the pharmacokinetic and pharmacodynamics behavior of the two types of Lipiodol-emulsions.

All the hepatic arterial infusion (HAI) procedures were performed by two experienced interventional radiologists (FD, GF) under general anesthesia in an angiography suite equipped with a Cone Beam Computed Tomography (CBCT) (GE Healthcare Discovery IGS 730, Waukesha, Wisconsin, USA). Preanesthetics were administered (buprenorphine), and anesthesia (Isoflurane 5%) was carried out as described above. First, surgical cut down was done to gain access into the common right

femoral artery, which was exposed and cannulated with a 4F vascular sheath (Cook<sup>®</sup>, Bloomington, Indiana, USA). A celiac angiogram was done to delineate the blood supply to the liver and confirm the location of the tumor. The tumor could readily be visualized as a region of hypervascular blush located on the left side of the liver. Then a 1.7F micro-catheter (Echelon<sup>®</sup>, EV3, Irvine, California, USA) was used under fluoroscopic guidance to catheterize the left branch of the hepatic artery for selective infusion of the emulsion to the tumor. After completion of the HAI, the catheter was removed, and the common femoral artery was ligated. The animals were returned to cages and all the rabbits were euthanized under deep anesthesia at day 1.

#### *4D imaging of the tumor Lipiodol uptake*

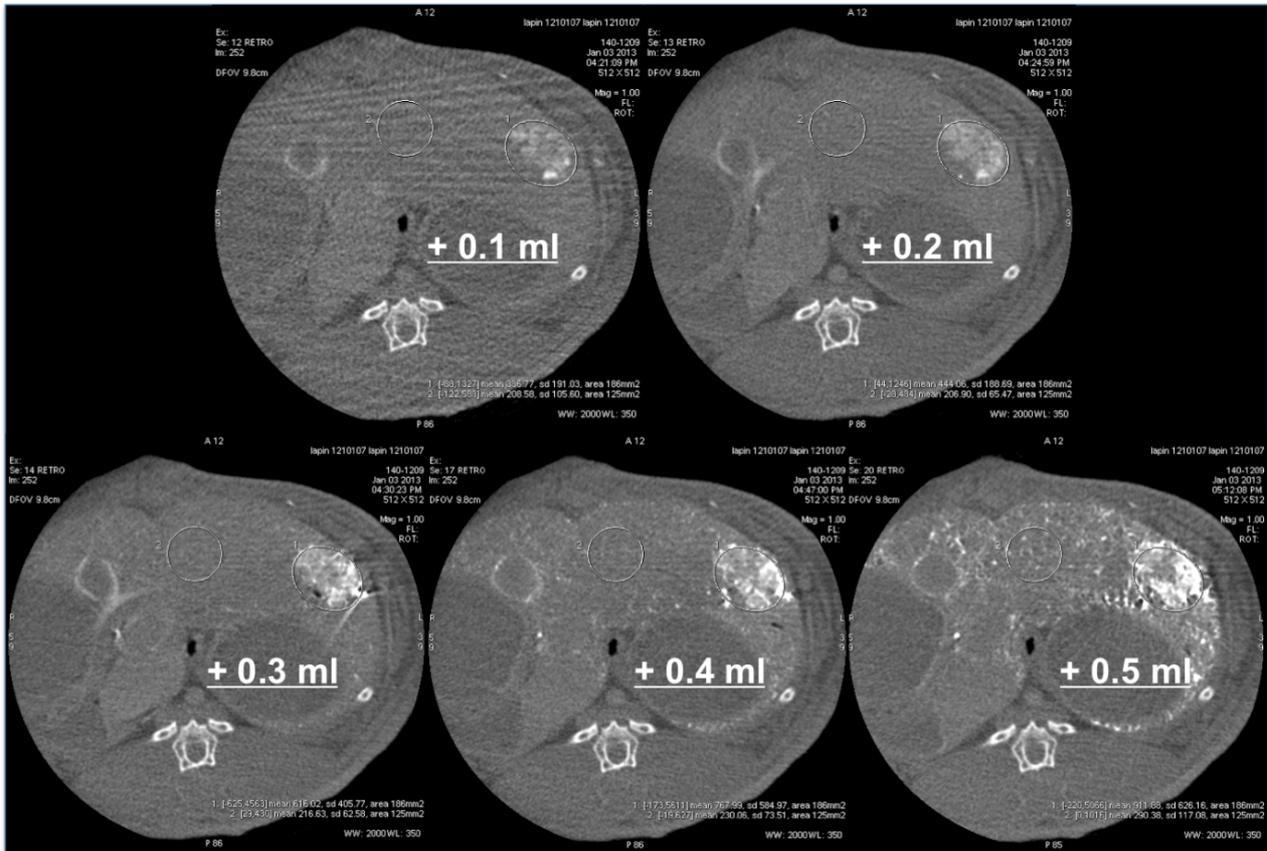
Fifteen New Zealand White rabbits, implanted with VX2 tumor in the left liver lobe, were prospectively randomized in two groups: group-1 (n=7) and -2 (n=8) received left hepatic arterial infusion (HAI) of emulsion-1 and -2 respectively.

Iterative 3D-CBCT acquisitions of the rabbit liver volume were acquired using the same table position, the same CBCT angulations and the same magnification. The very first 3D-CBCT was acquired with contrast medium injection in the left branch of the liver artery, using injector connected to the micro-catheter at a flow rate of 2 mL/min. Additional 3D-CBCTs were acquired without contrast medium injection after the HAI of every 0.1 mL of emulsion. The procedures were stopped when 0.5 mL have been injected in total. No supplementary arterial embolization was associated.

For each tumor, the 3D-CBCT images acquired during the HAI procedure were analyzed to report the following criteria:

- 1/ Volume of the tumor on the first 3D-CBCT with contrast medium injection.
- 2/ Mean densities within the tumor volume and within the left liver lobe after each incremental injection of 0.1 mL of emulsion (Figure 1). Because the ratio of Lipiodol was higher for emulsion-2, the mean density measures in group-2 were corrected

(multiplication by 2/3) to allow comparison with group-1. Ratios between the mean density within the tumor volume and the mean density within the left liver lobe were also calculated for each tumor according to the volume of emulsion injected.



*Figure 1: Successive 3D-CBCT images of Lipiodol® uptake within the tumor and the left liver lobe after each 0.1 mL of emulsion infused in the left hepatic artery branch (Measures of densities within the tumor and in the left lobe).*

- 3/ The volume of emulsion required for the visualization of Lipiodol within the portal vessels around the tumor on 3D-CBCT images (grade 1 according to Miyayama classification)<sup>31</sup> (Figure 2).



*Figure 2: Lipiodol opacification of the portal vessels around the tumor (white arrows) on 3D-CBCT images depicted after 0.4 mL of emulsion infused (same rabbit than Figure 1).*

### ***Pharmacokinetic and drug delivery properties***

Fifteen New Zealand White rabbits, implanted with VX2 tumor in the left liver lobe, received HAI of the same dose of doxorubicin (1.5 mg) in the left liver lobe. The rabbits were prospectively randomized in 3 groups: rabbits in group-A (n=6) received 0.3 mL of emulsion-1 (1.5 mg doxorubicin reconstituted in 150  $\mu$ L saline and emulsified in 150  $\mu$ L Lipiodol, oil/water ratio 1:1). Rabbits in group-B (n=6) received 0.3 mL of emulsion-2 (1.5 mg doxorubicin reconstituted in 75  $\mu$ L iobitridol at 250 mg/ml, supplemented with 3 mg of polyglycerol esters of polycondensed ricinoleic acids and emulsified in 225  $\mu$ L Lipiodol, oil/water ratio 3:1). Rabbits in group-C (n=3) received 0.3 mL of doxorubicin alone (1,5 mg doxorubicin reconstituted in 300  $\mu$ l saline without Lipiodol).

For each groups, the HAI procedures were performed in a single step using a syringe pump at a slow flow rate (0.1 mL per min) to avoid any reflux. No supplementary arterial embolization was associated.

Blood samples were collected at 5, 10, 15, 20, 30 and 45 minutes after the beginning of the HAI via a catheter inserted in an ear vein. Healthy liver and tumor were removed 24 hours after the procedures, immediately after sacrifice.

For each rabbit, doxorubicin concentrations were evaluated pathologically in the specimens of the tumor and of the liver tissues with fluorescence-HPLC method.

### ***Statistical analysis***

Results were expressed as means standard deviations for quantitative variables. Differences between groups were assessed with independent Student's t-test and Dunn's multiple comparison tests. Differences were considered statistically significant for  $p < 0.05$ .

The parameters taken into consideration and the comparison performed will be the following: VX2 tumor size, volume of emulsion for visualization of the first portal vein visualization (Miyayama grade 1), Plasmatic concentrations of doxorubicin 5 minutes after HAI and concentrations of doxorubicin within the tumor and within the left liver lobe at day-1.

### **Results:**

#### ***4D imaging of the tumor Lipiodol uptake***

All the HAI procedures were performed with success, resulting in a left lobe selective injection of 0.5 mL of emulsion per rabbit.

A total of six 3D-CBCT were performed per rabbit (one 3D-CBCT with injection of contrast medium before the injection of the emulsion and one 3D-CBCT after each subsequent injection of 0.1 mL of emulsion until 0.5 mL overall infusion).

The largest diameter of tumor was  $2.54 \pm 0.6$  cm in group-1, and  $2.57 \pm 0.7$  cm in group-2, without statistical difference between the 2 groups ( $p = 0.93$ ). The mean tumor volume was  $5.1 \pm 2.3$  and  $6.4 \pm 4.3$  cm<sup>3</sup> in group-1 and -2 respectively, without statistical difference between the 2 groups ( $p = 0.87$ ).

The mean densities within the tumor volumes and the ratio between the mean densities within the tumor volume and the left liver lobes according to the type and the volume of Lipiodol-based infused are reported in Figures 3 and 4. Briefly, the mean density within the tumor volume and the ratio between the mean densities within the tumor volume

and the left liver lobe were significantly higher in group-2 up to 0.4 mL. When more than 0.4 mL of the emulsions were infused, there was no significant difference in between the two groups.

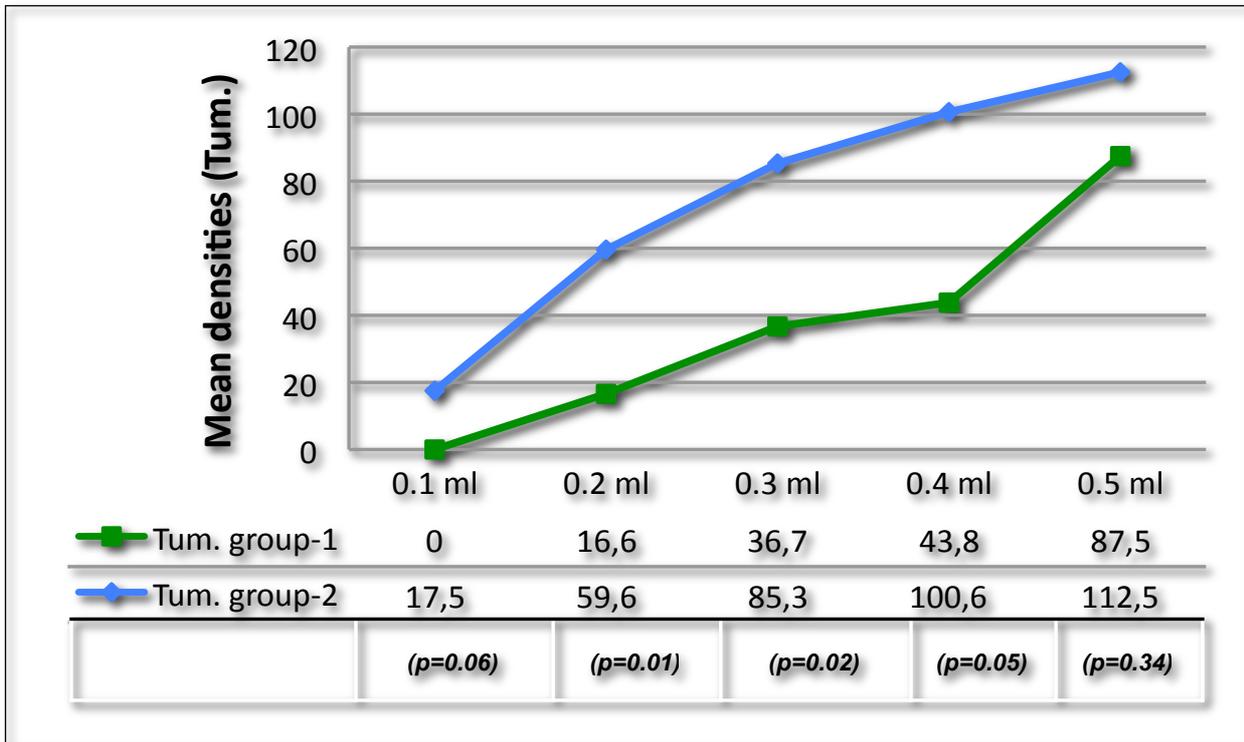


Figure 3: Mean of densities within the tumor volume according to the type and the volume of Lipiodol-emulsion infused in the left hepatic artery: Group-1 (Emulsion-1, O/W; green line), Group-2 (Emulsion-2, W/O; blue line).

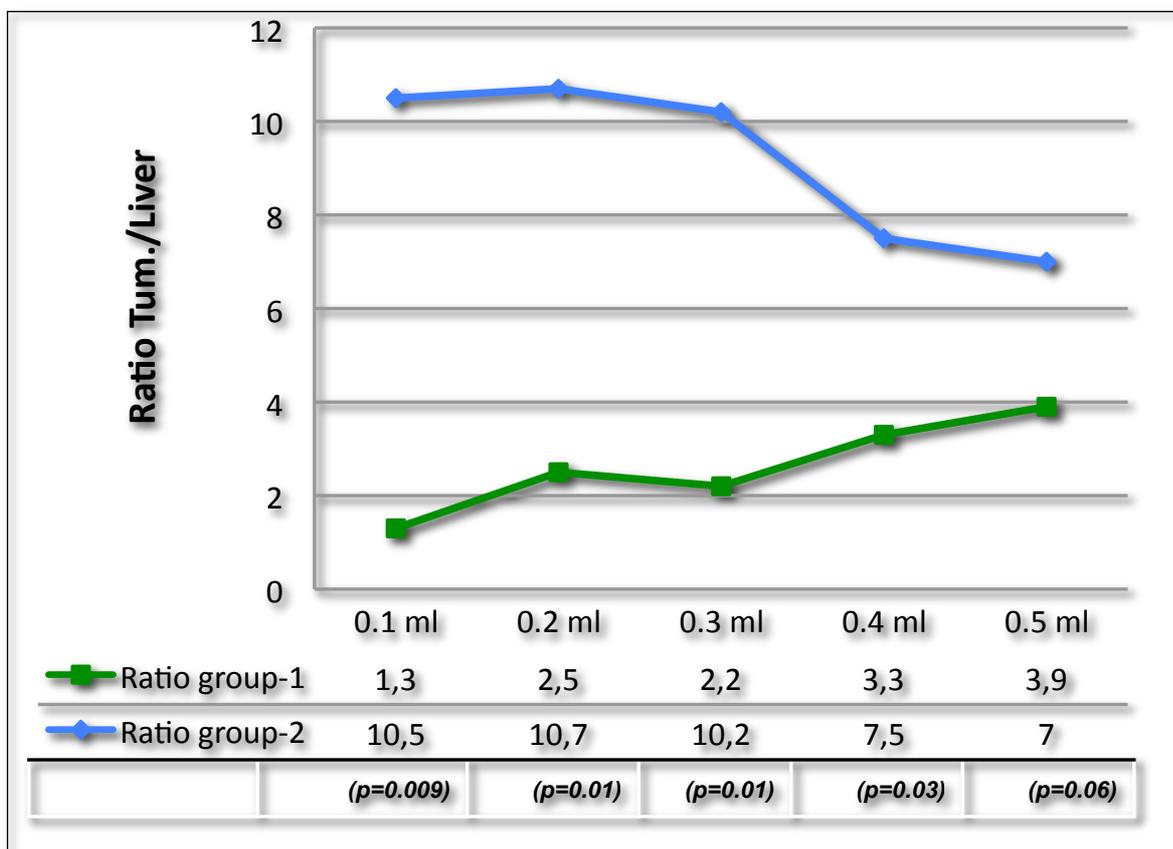


Figure 4: Ratio of densities within the tumor volume and the left liver lobe according to the type and the volume of emulsion infused in the left hepatic artery: Group-1 (Emulsion-1, O/W; green line), Group-2 (Emulsion-2, W/O; blue line).

The first portal vein visualization (Miyayama grade 1) was obtained significantly earlier in group-2 ( $0.23 \pm 0.02$  mL) than in group-1 ( $0.37 \pm 0.06$  mL,  $p=0.01$ ).

### Pharmacokinetic and drug delivery properties

All the HAI procedures were performed with success, resulting in a left lobe selective arterial injection of 1.5 mL doxorubicin per rabbit. The plasmatic doxorubicin concentrations are reported in Figure 5.

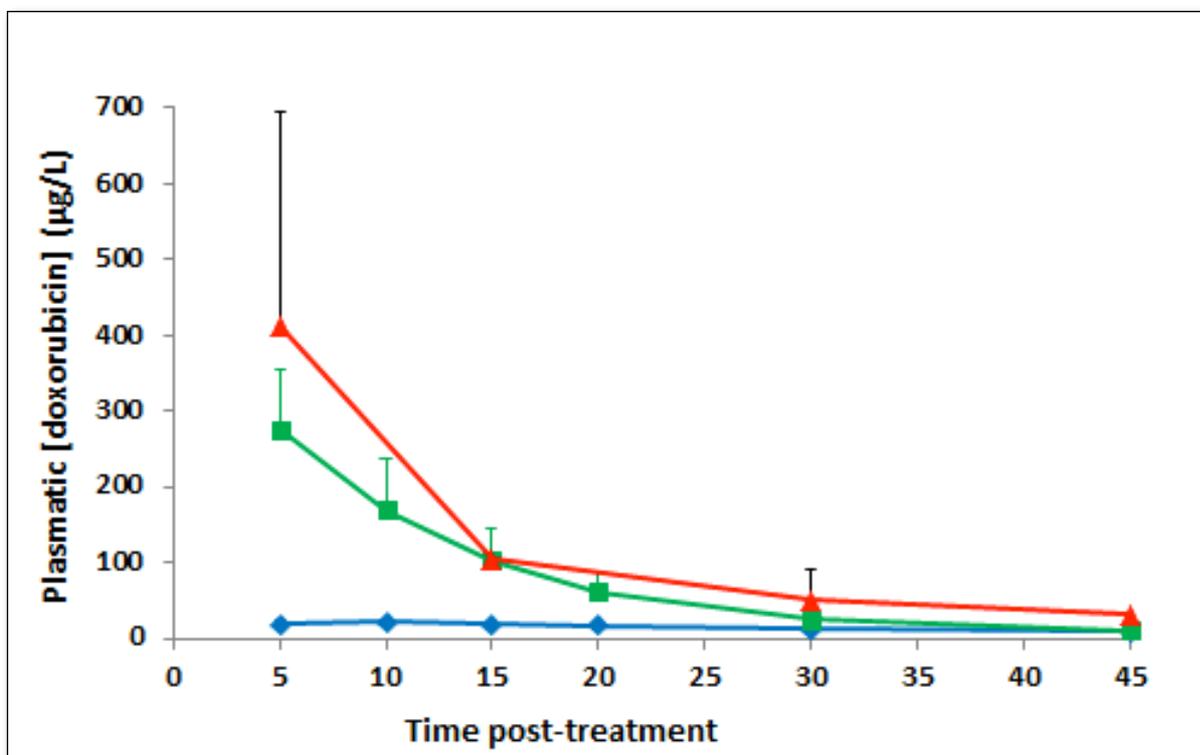


Figure 5: Plasma pharmacokinetic of doxorubicin concentration after hepatic intra-arterial infusion in the left hepatic artery: Group-A (Emulsion-1, O/W; green line), Group-B (Emulsion-2, W/O; blue line) and Group-C (HAI; red line).

Briefly, C5min was significantly lower in group-B ( $19.0 \pm 5.8 \mu\text{g/L}$ , emulsion-2) than in group-A (ne,  $p < 0.001$ ), suggesting less systemic exposure of doxorubicin using stabilized Lipiodol-emulsion.

At day-1, the concentration of doxorubicin within the tumor was significantly higher in group-B ( $20957 \pm 9022 \text{ ng/g}$  of tissue) than in group-A ( $8093 \pm 4466 \text{ ng/g}$  of tissue,  $p < 0.05$ ) and group-C ( $2221 \pm 1012 \text{ ng/g}$  of tissue,  $p < 0.01$ ) confirming a higher tumor targeting stabilized Lipiodol-emulsion correlated with very low doxorubicin release in plasma.

Doxorubicin concentrations in the left liver lobe were significantly higher in group-B ( $932 \pm 572 \text{ ng/g}$  of tissue), than in group-A ( $122 \pm 33 \text{ ng/g}$  of tissue,  $p < 0.01$ ) and group-C ( $189 \pm 62 \text{ ng/g}$  of tissue, NS).

## Discussion:

Selectivity of TACE within the tumor feeders is key to improve both; tumor response and clinical tolerance<sup>32</sup>. Selective TACE can be performed thanks to microcatheter and selective catheterism of tumor feeders. For this purpose the use of 3D-CBCT has demonstrated a real benefit in terms of detection of the tumor feeders<sup>33</sup>. In addition softwares have been developed to help the operator for depicting the tumor feeders to be treated<sup>34</sup>. However, a selective injection is not always possible either due to difficult catheterism or because of diffuse HCC tumors within the liver parenchyma. When such limitations occur, the use of a drug delivery platform able to target selectively the tumor when injected non-selectively is of utmost importance.

Lipiodol has demonstrated such targeting abilities for hypervascular liver tumors. However, Lipiodol is not an anticancer agent. Its role is to selectively deliver the chemotherapy into the tumor feeders with slow release of the chemotherapy. However, it has been proved that non-stabilized Lipiodol-emulsion results in a distribution of chemotherapy<sup>16</sup> within the liver arterial supply that is not guided by Lipiodol<sup>19</sup>. In this case, almost all of the chemotherapy disappears very rapidly in the systemic circulation, so that only Lipiodol remains in the tumor.

Thus, stabilization of the chemotherapy within the Lipiodol droplets is key for the carcinologic effect. Our results are consistent with the recent publication of Choi et al<sup>35</sup> that demonstrates that stabilized Lipiodol-emulsions provide better profiles of doxorubicin concentration within the tumor and the plasma compared to non-stabilized.

In vivo, we have demonstrated that the stabilized water-in-oil emulsion results in an increased Lipiodol tumor uptake, probably because its viscosity was higher. Indeed, the profile of Lipiodol tumor uptake is related to emulsion viscosity, with an increased tumor uptake when viscosity increases<sup>24</sup>. Since the Lipiodol tumor deposition has already been described as a good predictive factor for TACE outcome<sup>36,37,38</sup>, such stabilized emulsion could lead to better response rate in HCC patients.

Because Lipiodol is composed of iodinated ethyl esters of the fatty acids of poppy seed oil, it has been used as an imaging biomarker for therapeutic efficacy. Thanks to its iodine component, the delivery of the Lipiodol can be visualized intraprocedurally with digital subtraction angiography and quantify with computed tomography or cone-beam computed tomography. Alternatively, the fatty acids within Lipiodol should permit quantification of intrahepatic biodistribution at MRI. The accuracy of Lipiodol quantification using chemical shift–based fat-water separation techniques was recently demonstrated in a phantom model and in healthy liver tissue in rat model<sup>39</sup>. For both models, Lipiodol measurements were strongly correlated to the infused Lipiodol dose. Thanks to the stabilization, we expect that early quantification of Lipiodol deposit after TACE can be used for indirect quantification of chemotherapy exposure in liver tumors. Further studies are needed to confirm this possible correlation and its prognostic value for tumor response.

We have also demonstrated that the highly stable water-in-oil emulsion results in an earlier Lipiodol opacification of the portal vessels around the tumor. Since local recurrence has been shown to be significantly lower when portal vein opacification by Lipiodol is seen during TACE <sup>31, 36</sup>, we can hypothesize that better tumor response can be obtained using the stabilized emulsion.

In vivo, we finally demonstrated that stabilized emulsion reduces systemic doxorubicin exposure and increases early accumulation of this chemotherapy within the liver tumor. These results can be explained nonetheless by the appropriate drug delivery profile but also by the tumor selectivity of the emulsion that increases the concentration of doxorubicin in the tumor feeders and by a transient embolization that prevents the wash out of the doxorubicin after the arterial infusion. It is also important to note that the stabilized emulsion resulted in a significant increase of the doxorubicin concentration in the healthy liver around the tumor. Thus, we acknowledge that the stabilized emulsion could results in higher liver toxicity.

### **Limitations:**

This study has some limitations. The first limitation is that we didn't analyze the tumor response rate after HAI procedures. Ideally, an additional experimental study should focus on the tumor response to evaluate potential benefit of stabilized water-in-oil emulsion in term of doxorubicin concentration within the tumor. Another limitation is that we do not perform a TACE procedure but an infusion in the hepatic artery, as no complementary embolic agent has been injected. The reason for no embolization was that we wanted to avoid possible bias that can be introduced by different endpoint of an additional embolization. The last limitation is that we used an emulsifier to obtain the stabilization of emulsion-2. However the safety of this emulsifier has not been evaluated yet. Thus, its use in human patients cannot be recommended so far.

### **Conclusion:**

Stabilization of doxorubicin in a water-in-oil emulsion results in better theranostic properties: better Lipiodol tumor uptake, lower systemic exposure and higher concentration of doxorubicin within a rabbit liver tumor model.

## Parameters for stable water-in-oil Lipiodol-emulsion

### Abstract<sup>2</sup>:

#### Purpose

Direction and stability of the Lipiodol-emulsion are important properties during conventional trans-arterial chemo-embolization. Our purpose was to evaluate the influence of 3 technical parameters on these properties.

#### Material and methods

Three parameters were compared for the formulation of Lipiodol-emulsions: Lipiodol/doxorubicin volume ratio (2/1 vs. 3/1), doxorubicin concentration (10 vs. 20 mg/mL) and speed of incorporation of doxorubicin in Lipiodol (bolus vs. incremental vs. continuous). We evaluated the percentage of water-in-oil emulsion obtained using the drop-test. For water-in-oil emulsions stability was evaluated by measuring duration until complete coalescence with a static light scattering technique.

#### Results

The percentage of water-in-oil emulsions obtained was significantly higher for incremental (94%) and for continuous (100%) injections compared to bolus injection (6%) of doxorubicin. Emulsions stability was significantly longer when formulated using continuous compared to incremental injection (326 +/-309 min vs. 96 +/-101 min, p=0.018) and using 3/1 compared to 2/1 ratio of Lipiodol/doxorubicin (372 +/-276 min vs. 47 +/-43 min,when p=<0.0001).

#### Conclusion

---

<sup>2</sup> Deschamps F, Moine L, Isoardo T, Tselikas L, Paci A, Mir LM, Huang N, Fattal E, de Baère T. Parameters for Stable Water-in-Oil Lipiodol Emulsion for Liver Trans-Arterial Chemo-Embolization. *CVIR* 2017 Dec;40(12):1927-1932.

Continuous and incremental incorporations of doxorubicin in the Lipiodol result in highly predictable water-in-oil emulsion type with a significant increase of stability compared to the standard of practice (bolus injection). Higher ratio of Lipiodol/doxorubicin is also a critical parameter for stability of the emulsion.

## Introduction:

A conventional pumping method is generally used to prepare such emulsions. However, the theranostic properties of these Lipiodol-emulsions are limited by their poor stability<sup>19, 35</sup>. Within a few minutes, the two phases of the emulsion separate and almost the entire water-soluble chemotherapeutic agent disappears very rapidly from the tumor. In addition, as emulsification is obtained by repetitive pumping of 2 syringes (1 of chemotherapy and 1 of Lipiodol) through a 3-ways stopcock, the technique is not reproducible from one operator to another.

Previous researches studies have established that two properties of Lipiodol-emulsions were crucial during cTACE. The first one is the type of emulsion. Ideally, the chemotherapy should be "loaded" as droplets inside the Lipiodol, which acts as continuous phase (water-in-oil emulsion) for an optimal Lipiodol tumor uptake<sup>40, 41</sup>. Stability of the emulsion over time is the second crucial property, allowing a slow release of the chemotherapy in the feeders of the hypervascular tumors for a prolonged exposure and minimizing systemic side effects. Few options are available to formulate the right emulsion in clinical practice. The most commonly used is to increase the ratio of the Lipiodol for the formulation of the emulsion. Increasing the viscosity of the aqueous phase is another option, using contrast media instead of saline for reconstitution of the doxorubicin powder or using high concentrations of doxorubicin. The last option consists in a progressive incorporation of the chemotherapy in the Lipiodol. Indeed emulsification process is very sensitive to the time scale during which the dispersed phase is introduced into the continuous phase<sup>42</sup>.

The aim of the present study was to evaluate the influence of these 3 technical parameters on the formulation of Lipiodol-emulsion in order to fix a reliable and reproducible process and to improve the theranostic properties of the emulsions. The 3 technical parameters were: 1) ratio of Lipiodol/ doxorubicin (3/1 vs. 2/1), 2) concentration of doxorubicin (10 mg/mL vs. 20 mg/mL) and 3) speed of incorporation of doxorubicin into Lipiodol (bolus vs. incremental vs. continuous).

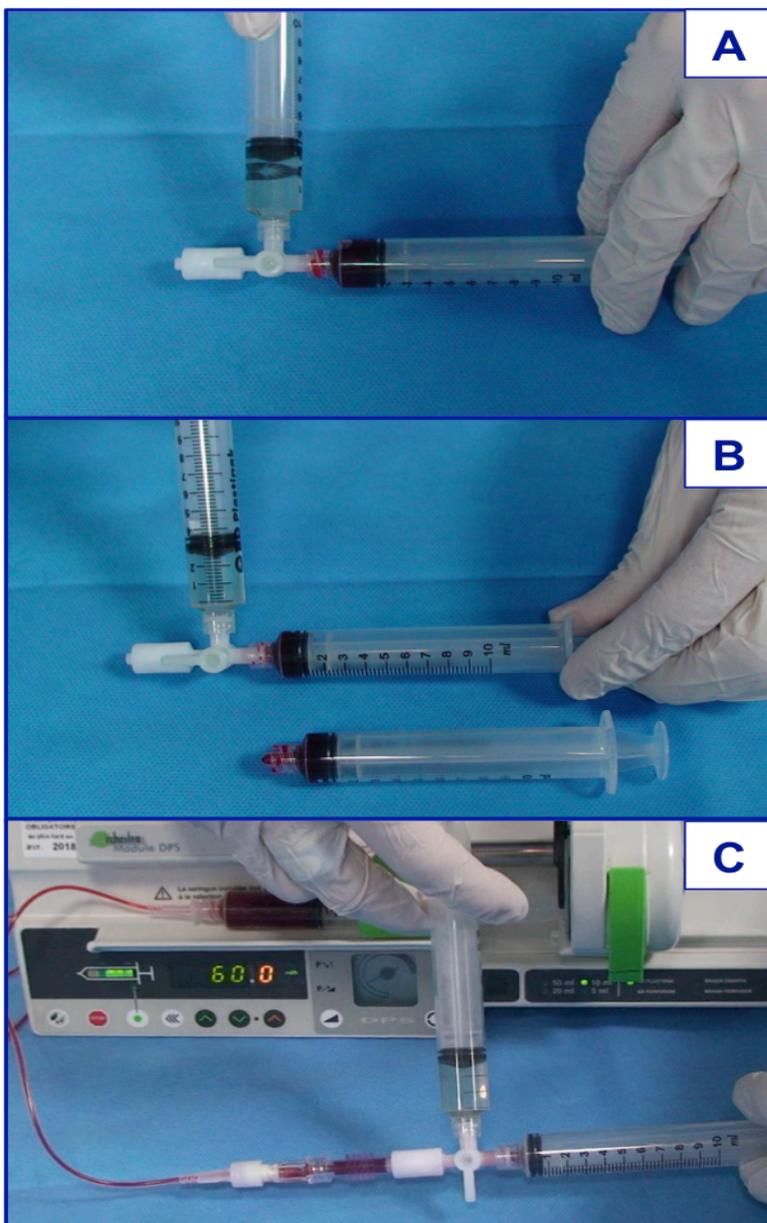
## Materials and Methods:

### *Lipiodol-emulsions formulation*

Lipiodol and hydrochloride doxorubicin reconstituted in saline (Adriblastin 50mL/25mL, Pfizer, USA) were used for the formulation of emulsions. All emulsions were formulated by repetitive pumping (70 back-and-forth) of two 10 mL syringes through a 3-ways stopcock<sup>29, 43</sup> during 70 seconds. The doxorubicin was always pushed first into the Lipiodol as recommended in guidelines<sup>29, 43</sup>.

The following 3 technical parameters were compared for the formulation of the Lipiodol-emulsions: ratio of Lipiodol/doxorubicin, doxorubicin concentration and speed of incorporation of doxorubicin in Lipiodol.

- Two volume ratios of Lipiodol/ doxorubicin were evaluated: 3/1 and 2/1. Technically, 3 mL of Lipiodol and 1 mL of doxorubicin were mixed together for ratio 3/1 and 2 mL of Lipiodol and 1 mL of doxorubicin were mixed together for ratio 2/1.
- Two concentrations were evaluated: 10 mg/mL and 20 mg/mL. For 10 mg/mL, 5 mL of saline were used for the reconstitution of one vial of doxorubicin. For 20 mg/mL, 5 mL of saline were used for the reconstitution of two vials of doxorubicin.
- Three speeds of incorporation were evaluated (Figure 6): bolus vs. incremental vs. continuous injections. Bolus injection consists in the injection of the entire volume of doxorubicin into the entire volume of Lipiodol in a “single-bolus” procedure, followed by the 70 back-and-forth during 70 seconds. The incremental injection consists in subsequent injections of aliquots of doxorubicin in the entire volume of Lipiodol. Technically, the 2 aliquots were 0.5 mL of doxorubicin, each followed by 35 back-and-forth during 35 seconds. The continuous injection consists in an incorporation of doxorubicin in the entire volume of Lipiodol using an electric syringe pump at a flow-rate of 1 mL per minute during 60 seconds. Sixty back-and-forth were performed during the incorporation of doxorubicin, followed by 10 additional back-and-forth after complete injection of doxorubicin.

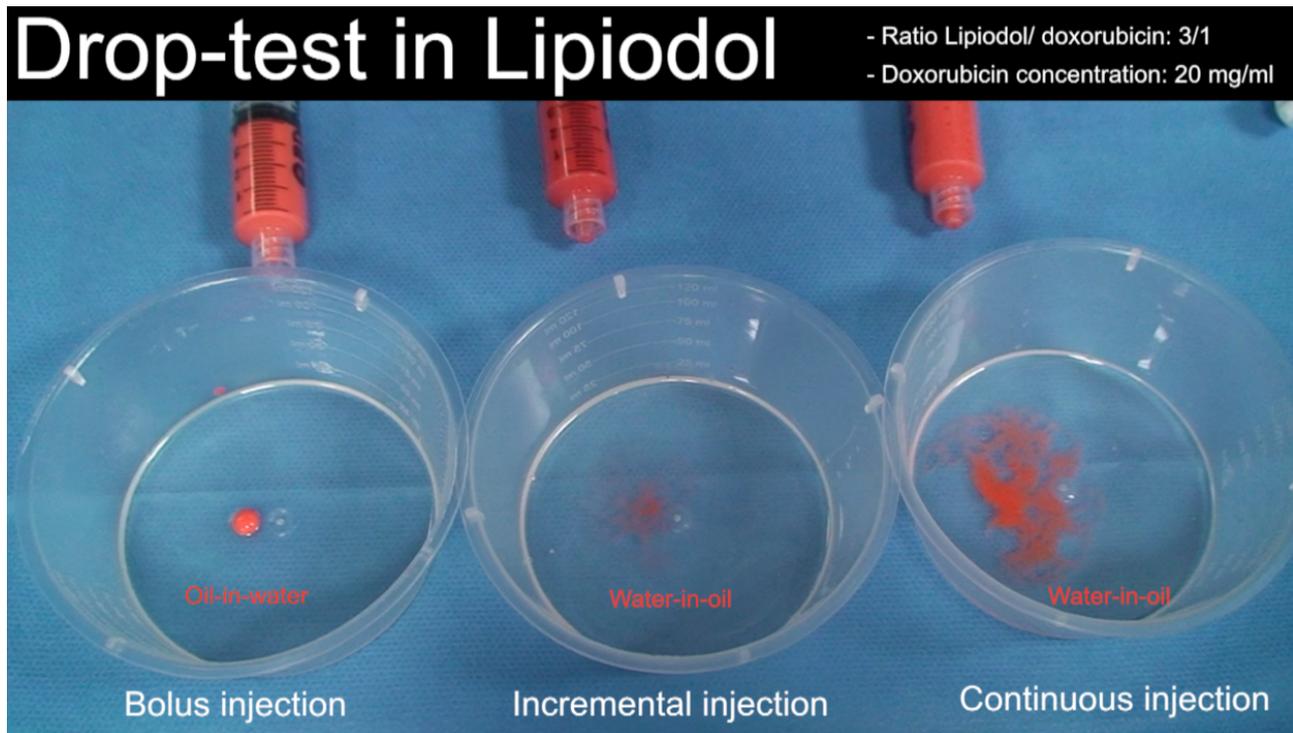


*Figure 6: Three speeds of incorporation of doxorubicin in Lipiodol (Doxorubicin concentration: 20 mg/mL, Ratio of Lipiodol/doxorubicin: 3/1). A/ Bolus injection of 1 mL of doxorubicin in 3 mL of Lipiodol followed by 70 back-and-forth through the 3-ways stopcock during 70 seconds. B/ Incremental injection of 1 mL of doxorubicin in 3 mL of Lipiodol using 2 consecutive aliquots of 0.5 mL, each followed by 35 back-and-forth through the 3-ways stopcock during 35 seconds. C/ Continuous injection of doxorubicin (1 mL during 1 minute) in 3 mL Lipiodol using an electric syringe-pump during 60 back-and-forth through the 3-ways stopcock, followed by 10 additional back-and-forth after the end of the injection.*

### ***Lipiodol-emulsions characterization***

For each emulsion formulated, the type (water-in-oil or oil-in-water) was determined by a drop-test. Observing the miscibility of an emulsion droplet dropped in a

cup containing Lipiodol revealed the continuous phase of the emulsion. The emulsion was considered as water-in-oil in case of miscibility and oil-in-water in case of immiscibility (Figure 7).



*Figure 7: Drop-test of 3 emulsions formulated with a doxorubicin concentration of 20 mg/mL and 3/1 Lipiodol/doxorubicin ratio but 3 different speeds of incorporation of doxorubicin in Lipiodol. The bolus injection (on the left) resulted in an oil-in-water emulsion (no miscibility of the droplet in the Lipiodol) whereas the incremental injection (in the middle) and the continuous injection (on the right) resulted in a water-in-oil emulsion (miscibility of the droplets in the Lipiodol).*

The durations until complete coalescence (stability) were evaluated immediately after formulation for water-in-oil emulsions only. The static light scattering technology (Turbiscan) was used for this purpose. It works on multiple light scattering in both transmission and backscattering mode, in order to analyze low and high concentration dispersions of the droplets. Transmission and backscattering signals depend on the sizes and concentrations of the droplets inside the emulsion. The Turbiscan acquires data along the sample height, thanks to a scanning reading head. Scans are repeated during sample ageing time to detect any variation of the signal due to a destabilization, such as droplets

migration and/or droplets size variation. The Turbiscan is able to measure reversible (creaming and sedimentation) and irreversible (coalescence and segregation) destabilization phenomena in the sample without any dilution and to detect these phenomena much earlier, easier and more objectively than visual analysis. The measurements were taken every minute from 5 to 10 minutes; every 5 minutes from 10 minutes to 1 hour and finally every half-hour or hour depending on the evolution of the system. This monitoring was performed until no more evolution of the intensities was noticed.

### **Statistical analysis**

Comparison between the percentage of water-in-oil emulsion and comparison between the mean stability of water-in-oil emulsions were performed using the Chi2 test and the Mann-Whitney test, respectively. Statistical significance was considered when p value was inferior to 0.05.

### **Results:**

Forty-eight emulsions of Lipiodol and doxorubicin have been formulated. Results are shown in Table 1. Thirty-two emulsions (67%) were water-in-oil emulsions and sixteen (33%) were oil-in-water emulsions. Only one emulsion (6%) formulated with the bolus injection process was water-in-oil emulsion (n=1/16), whereas the percentages of water-in-oil emulsion were 94% for incremental injection (n=15/16) and 100% for continuous injection (n=16/16).

*Table 1: Percentage of water-in-oil emulsion type and their stabilities according to the ratio of Lipiodol/doxorubicin, the concentration of doxorubicin and the speed of incorporation of doxorubicin into Lipiodol.*

		Percentage of water-in-oil emulsion	Stability of water-in-oil emulsion
Ratio of Lipiodol/doxorubicin	2/1 (n=24)	70.8% (n=17)	47 +/- 3 min  372 +/- 276 min  p<0.0001
	3/1 (n=24)	62.5% (n=15)	
Concentration of doxorubicin	10 mg/mL (n=24)	62.5% (n=15)	248 +/- 292 min
	20 mg/mL (n=24)	70.8% (n=17)	183 +/- 224 min
Speed of incorporation of doxorubicin into Lipiodol	Bolus (n=16)	6.25% (n=1)	96 +/- 101 min  326 +/- 309 min  p=0.018
	Incremental (n=16)	93.7% (n=15) p<0.0001*	
	Continuous (n=16)	100% (n=16) p<0.0001* *	

\* Incremental vs. Bolus

\*\* Continuous vs. bolus

The percentages of water-in-oil emulsion were significantly higher when the doxorubicin was incorporated incrementally (p<0.0001) or continuously (p<0.0001) compared to the bolus injection. No statistical difference was observed in the percentage of water-in-oil emulsion formulated incrementally compared to continuously (p=0.31). In addition, the percentages of water-in-oil emulsion were not statistically different when the emulsions were formulated with a 3/1 compared to a 2/1 ratio of Lipiodol/doxorubicin (71% vs. 62%, p=0.54) nor when the emulsions were formulated with a concentration of 10 mg/mL or 20 mg/mL of doxorubicin (62% vs. 71%, p=0.54).

All the water-in-oil emulsions (n=32) have been analyzed using the Turbiscan. Mean stability of water-in-oil emulsions was 215 +/- 257 min (from 5 to 1000 min). Mean stability was significantly higher when emulsions were formulated with a 3/1 compared to a 2/1 ratio of Lipiodol/doxorubicin (372 +/- 276 min vs. 47 +/- 43 min,  $p < 0.0001$ ). This higher stability was found whatever the process of formulation (incremental or continuous injection) (Figure 8). The continuous injection of doxorubicin in the Lipiodol resulted in a significant increase of the stability compared to the incremental injection (326 +/- 309 min vs. 96 +/- 101 min,  $p = 0.018$ ). This higher stability was found whatever the ratio of Lipiodol/doxorubicin used of formulation (3/1 ratio or 2/1 ratio) (Figure 8). Since only one emulsion formulated with the bolus injection process was water-in-oil emulsion, it was statistically impossible to compare the stability of this emulsion with the 2 other processes: incremental or continuous injections. The stability was not significantly different between emulsions formulated with 10 mg/mL or 20 mg/mL of doxorubicin (248 +/- 292 min vs. 183 +/- 224 min,  $p = 0.69$ ).

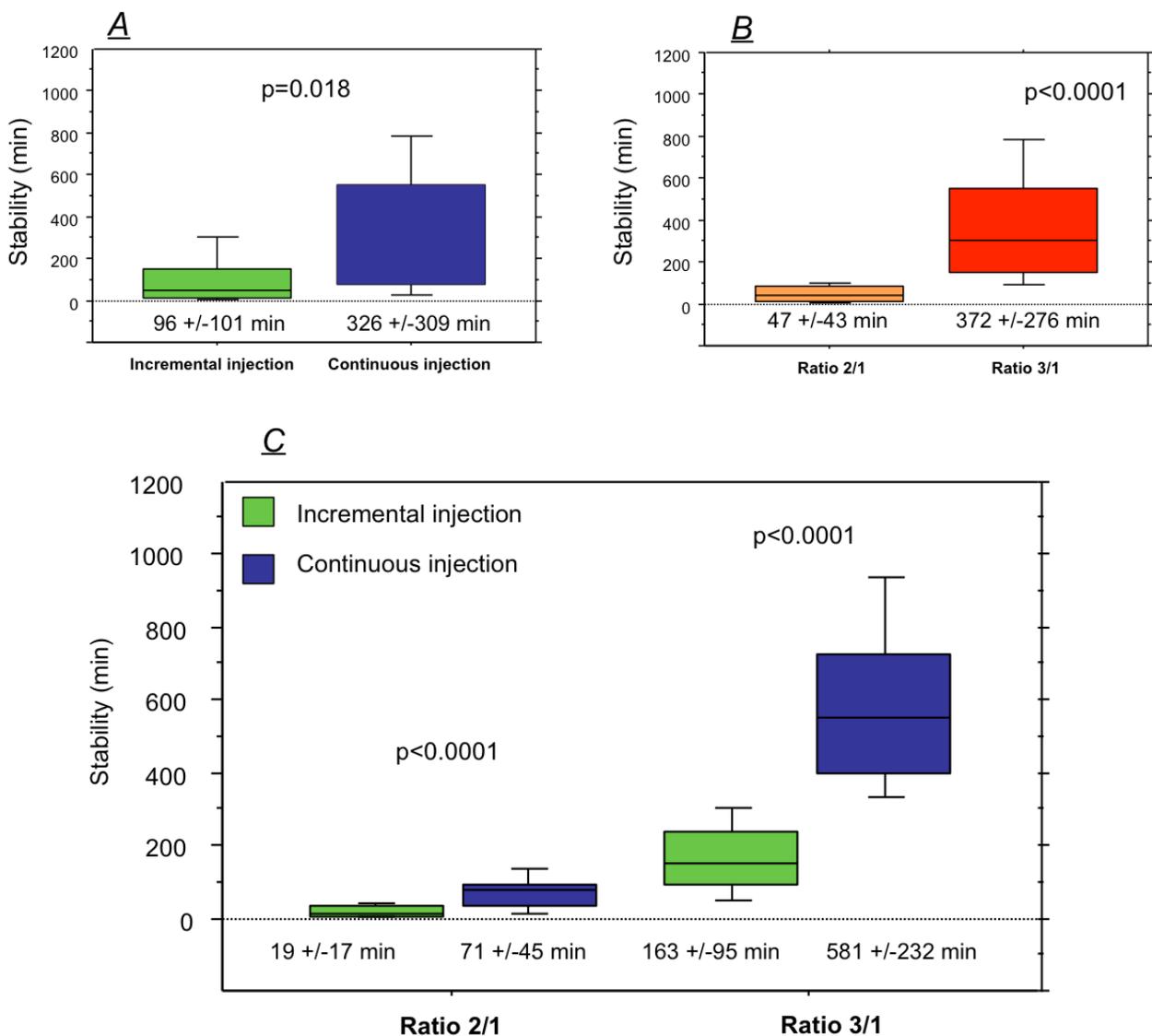


Figure 8: Comparison of duration until complete coalescences (stability). A/ Incremental injection versus continuous injection. B/ Ratio of Lipiodol/doxorubicin: 2/1 versus 3/1. C/ Incremental injection versus continuous injection for each ratio of Lipiodol/doxorubicin: 2/1 and 3/1.

### Discussion:

Technical recommendations from experts' opinion state that Lipiodol-emulsions must be stable and water-in-oil<sup>26, 41</sup>. However there are no clear data in the literature to describe the "right way" to formulate these emulsions in clinical practice, except using a higher volume ratio of Lipiodol/chemotherapy (at least 2/1) and pushing doxorubicin towards Lipiodol during the first pumping through the 3-ways stopcock. We have

investigated simple parameters that could help the interventional radiologists to make the correct emulsion in a reproducible way.

Our results confirm the technical issues that the operators face in clinical practice. We succeed in formulating water-in-oil emulsions in only two third of the cases and we obtained a huge heterogeneity between the durations until complete coalescence (from 5 to 1000 minutes). These variations represent a real concern in clinical practice because they might result in highly variable tumor responses and systemic toxicities. The knowledge of optimal parameters to formulate stable water-in-oil emulsion is crucial for improving the cTACE reproducibility and efficacy.

It is very clear from our results that progressive incorporation of doxorubicin to Lipiodol is a key parameter, and that bolus injection can hardly obtain a water-in-oil emulsion. When using progressive incorporations maneuvers (incremental and continuous injections), we succeeded in formulating water-in-oil emulsions in 97% of the cases (n=31/32), whereas bolus injections resulted in water-in-oil emulsion in only 6% (n=1/16). We found no statistical difference between the incremental and continuous injections.

As recommended by experts <sup>29</sup>, we confirm that high volume ratio of Lipiodol/chemotherapy provide longer stability. Interestingly, speed of incorporation of chemotherapy in Lipiodol is also a critical parameter for stability of the emulsion. Based on our results, it appears that the slower the doxorubicin is incorporated in the Lipiodol, the longer is the stability of the emulsion. The optimal speed of incorporation has not been investigated here and should be investigated more in details in further studies. We found that the best way for stabilizing the emulsion was the continuous injection compared to the incremental injection. For this purpose, we used an electric syringe-pump and a flow-rate of 1 mL per minute.

We believe that continuous injection of chemotherapy in Lipiodol using an electric syringe-pump provides additional great advantages. First, it could result in more reproducible formulation from different operators thanks to the constant speed of incorporation. Second, it limits the manipulations of doxorubicin contained in a syringe that

is fixed to the syringe-pump. Finally, it allows the formulation “on demand” of the desired volume of emulsion during the workflow instead of formulating the whole emulsion immediately. This seems especially interesting when several injection points are required during a cTACE, avoiding coalescence of the emulsion during the catheterization phase of the next tumor feeders.

### **Limitations:**

Our study has several limitations. The first one is that we haven't investigated all the parameters that influence both, the type of emulsion and its stability but only the 3 that we considered the most important ones. Ideally, others parameters should be analyzed: vigor, duration and number of back-and-forth through the 3-ways stopcock, diameter of 3-ways stopcock lumen, temperature of the Lipiodol, etc. In addition, we have investigated only few variations in the different parameters. We demonstrate that high ratio of Lipiodol/doxorubicin and progressive incorporation of doxorubicin in the Lipiodol are 2 critical parameters for the formulation of stable water-in-oil emulsion. However, these 2 parameters should be analyzed in more details to define the optimal ratio and the optimal speed that would formulate the appropriate emulsion in the quicker time.

We decided to not evaluate the use of contrast media for reconstitution of doxorubicin because it is “off-label”. We strongly believe that the increase of viscosity of doxorubicin provided by the contrast media is an important factor for emulsion stability. However, this practice cannot be recommended because no data support the efficacy of doxorubicin reconstituted by this way.

### **Conclusions:**

The progressive incorporation of doxorubicin in the Lipiodol results in much more predictable water-in-oil type and higher stability compared to the standard of practice

(bolus injection). We confirm that higher ratio of Lipiodol/doxorubicin is also a critical parameter for the stability of the emulsion.

# Pickering-emulsions

## Generalities

Emulsions are widely used in food, pharmaceuticals and cosmetics applications, since they allow the encapsulation of an active ingredient in the dispersed phase in order to protect it from degradation and to remain active in a sustained manner. However, emulsions are thermodynamically unstable systems requiring the use of stabilizers for their formulation and long term stability. Therefore, stable emulsions can be obtained using substances known as emulsifiers, which are able to decrease the interfacial tension between the phases, and are usually included in emulsion formulations. An emulsifier is described as chemical species with amphiphilic properties that adsorbs strongly at the oil/water interface. The adsorption of the emulsifiers at this interface leads to the formation of a protective film, which resists droplet coalescence and phase separation, thus aiding emulsion stabilization. Conventional emulsifiers include low molar mass synthetic surfactants such as sodium dodecyl sulfate or surface-active polymers. Until now, synthetic surfactants have been used for this purpose but raise directly or indirectly to toxicity issues for biomedical applications.

New stabilization approaches have been developed such as the use of biopolymers or solid particles. Very stable interfaces are obtained with such emulsifiers, while allowing to reduce, or even to avoid, the use of synthetic surfactants. An emulsion stabilized by solid particle is called a Pickering-emulsion<sup>44,45</sup>. Such emulsions display a very good stability (sometimes up to several years) thanks to their high resistance to coalescence. Indeed, the particles used are wetted by both liquids allowing them to anchor at the interface and to form a barrier against coalescence. Many types of particles (mineral or organic) can be used to prepare Pickering emulsions but most of these emulsions are not biocompatible and/or not biodegradable, due to the particles or to the oil used, which is

a problem for cosmetic and pharmaceutical applications. The main objective of this study was to formulate fully biodegradable and biocompatible Pickering-emulsions for TACE.

# Formulation of water-in-oil Pickering-emulsion for TACE

## Introduction

Pickering-emulsions retain the basic properties of classical emulsions stabilized by surfactants, so that a classical emulsion can be substituted by a Pickering-emulsion in most applications. Their “surfactant free” character makes them very attractive especially for biomedical applications<sup>46</sup>.

We therefore considered that Pickering approach may result in improvement of Lipiodol-emulsion stability. Recently, Whitby et al have reported the use of biodegradable particles as stabilizers rather than usual organic or inorganic particles such as silica, calcium carbonate, polystyrene<sup>47</sup>. These biodegradable particle made of poly(lactic-co-glycolic acid) (PLGA) offer the advantage of being biocompatible and rapidly eliminated from the body preventing potential immatory reaction by their accumulation<sup>48 49 50</sup>.

We report here the feasibility to formulate Pickering-emulsion for TACE with biodegradable nanoparticles (Nps). The impact of Nps concentration and the water-in-oil (W/O) ratio was studied by following the stability and the droplets size of the emulsion. The potential of this new system to load and release doxorubicin without affecting the global stability were then evaluated in vitro.

## Materials and methods

### Materials

Poly(lactide-co-glycolide) (PLGA, 75:25) was provided by Evonik (Germany). Poly(vinyl alcohol) (PVA, molecular weight 30,000–70,000 g/mol, 87–90% hydrolyzed), and D-trehalose (from *saccharomyces cerevisiae*, ≥ 99%) were obtained from Sigma Aldrich (France). Iron oxide nanoparticles suspension ( $\text{Fe}_3\text{O}_4$  with oleic acid coating, ~5 nm, 25 mg/mL in chloroform) was provided by Ocean NanoTech (USA). Acetone was purchased from Carlo Erba (Milan, Italy). Distilled water was obtained by purification, using a water system ultrapure MilliQ Direct Type 1 (Millipore, resistivity of 18.2 M $\Omega$ .cm, France).

Lipiodol® was purchased from Guerbet (France). Finally, doxorubicin hydrochloride (Adriblastin, 50 mg, lyophilized form) was provided by Pfizer (USA).

### ***Nanoparticles formulation***

PLGA-PVA Nps were prepared using a nanoprecipitation technique. One hundred mg of PLGA were dissolved in 10 mL of acetone. The PLGA solution was slowly added through a syringe pump into a 30 mL solution of PVA at 0.5% w/w. This mixture was stirred for 10 hours at room temperature. Then, two successive ultracentrifugations were performed to eliminate free PVA, the pellets were collected and suspended into a solution of trehalose at 5% w/w. Finally, the Nps suspension was freeze-dried and stored at -20°C.

### ***Nanoparticles characterization***

#### **Size distribution and Zeta potential**

The size distribution and zeta potential of Nps were measured with a Zetasizer (Nano ZS 90, Malvern Instruments, France, 633 nm He-Ne laser, Smoluchowski equation) operating at 25 °C with a 173° scattering angle. The Nps suspension was diluted in MilliQ water for size distribution measurements and in a 1 mM NaCl aqueous solution for zeta potential measurements. Three measurements were performed for each Nps formulation.

#### **Transmission Electron Microscopy**

Transmission Electron Microscopy (TEM) performed at I2BC (Gif-sur-Yvette, France) using a JEOL JEM-1400 operating at 80 kV. 5 µL of purified suspensions of Nps (0.5 mg/mL) were deposited for 1 min on glow-discharged copper grids covered with formvar-carbon film. Samples were then stained using 2% phosphotungstic acid (pH = 7) for 30 s. The excess solution was blotted off using a filter paper. Images were acquired using an Orius camera (Gatan Inc, USA).

## Atomic Force Microscopy (AFM)

Atomic Force Microscopy (AFM) images were collected in air at room temperature (22°C) using a commercial Multimode 8 equipped with a NanoScope V controller. The topographical imaging was carried out in Peak Force Tapping mode with a MPP-21100-10 cantilever. The nominal spring constant was 2.8 N/m and the tip radius was about 8 nm (Bruker). The scan rate was adjusted in the range of 1Hz over a selected area in dimension of 1.0  $\mu\text{m}$  x 1.0  $\mu\text{m}$ , 2.0  $\mu\text{m}$  x 2.0  $\mu\text{m}$  and 5.0  $\mu\text{m}$  x 5.0  $\mu\text{m}$ . The samples (10  $\mu\text{L}$  NP PLGA-PVA sample at 25 mg/mL) were deposited on a magnetic disk directly mounted on the top of the AFM scanner and imaged.

## Emulsion formulation

For the entire study, the aqueous phase used was a suspension of PLGA-NPs in saline and the oily phase was Lipiodol. For all formulations, the Lipiodol volume was fixed at 3 mL.

Four different Nps concentrations were tested: 5, 10, 15 and 25 mg/mL at a 3/1 water in oil (W/O) ratio (v/v). At a fixed Nps concentration of 15 mg/mL, Four different W/O ratios (v/v): 1/3 ; 1/2 ; 2/3 and 1/1 were evaluated (Table 2).

*Table 2: different Nps concentrations and different ratio (W/O) tested*

Ratio (W/O)		1/3				1/2	2/3	1/1
Volume (mL)	<i>Oil</i>	3				3	3	3
	<i>Water</i>	1				1,5	2	3
Nps Concentration (mg/mL)		5	10	15	25	15		

Emulsions were obtained by mixing through two luer-lock syringes coupled to a three-way stopcock. During emulsification, the water phase was slowly added at a flow

rate of 1 mL/min with a syringe pump. Sixty round trips per minute were done to achieve emulsification.

For doxorubicin-loaded emulsions, only the 1/3 W/O ratio at 20 mg/mL doxorubicin and 15 mg/mL Nps concentrations was tested. Although, a doxorubicin dissolution step was first required in the Nps suspension, they were obtained as the same way as other emulsions.

## *Emulsion characterization*

### *Stability*

The emulsion stability was evaluated over 24 hours at 25°C by using a Turbiscan Classic MA 2000 apparatus (Formulation, Toulouse, France). The apparatus uses a near-IR light, scans the emulsion from bottom to top and a photocell calculates the intensity of the light transmitted and scattered. The freshly produced emulsion was placed properly into a tube to avoid any perturbation of the system, The tube was not removed or even touched from the apparatus until the end of the measurement. The measures were taken every 10 minutes, the first hour then every hour until 24h.

### *Drop size analysis*

The drop size measurement was performed with a particle counter, shape and size analysis apparatus (Flowcell FC200S, Occhio, Belgium). Thanks to image analysis, the Flowcell apparatus measures the size and the morphology of droplets. The emulsion was first diluted 20 times in oil. Then 0.5 mL of the diluted emulsion was passed through a spacer of 400 µm to get a size range between 5 and 400 µm. Each sample was measured 5 times and at different days (D0, D7, D35).

### *Microscopy*

All emulsions were observed with an optical microscope (Olympus B201, Japan) at 10x optical zoom. The images were taken 1 hour, 7 days and 35 days after the emulsification.

Confocal microscopy was performed using a confocal scanning laser microscope (inverted Leica TCS SP8 – gated STED, Germany) equipped with a WLL laser (488 and 563 nm excitation wavelengths) using a HC PL APO CS2 63x/1.40 oil immersion objective lens. To prevent the deformation of emulsion droplets, the sample was placed in a curved glass slip with a cover glass slide on the top. The Nps were labelled by introduction of iron oxide nanoparticles during their preparation. Red fluorescence was observed with a 600-710 nm filter under a 563 nm laser illumination. A hybrid detector under a gated mode (0.3-6.5 ns) was used in order to avoid reflection. The green and the red fluorescence emissions were collected under a sequential mode. The pinhole was set at 1.0 Airy unit.

### ***Doxorubicin release***

0.8 mL of a freshly prepared doxorubicin loaded emulsion (equivalent to an amount of 4 mg of doxorubicin) was placed into GeBAflex dialyse tube with pores of 14 kDa (GeBa, Israel). The tubes were immersed into 40 mL of 25 mM TRIS solution containing 150 mM of NaCl (pH =7.3). Then, the whole release system was placed in an orbital shaker at 37°C for the entire duration of the experiment. Before each aliquot withdrawing, the tubes were vortexed and the medium was entirely collected and replace by fresh TRIS solution. For measurements, 100 µL of the release medium was collected and doxorubicin concentration was determined by absorption spectrometry at a wavelength of 490 nm with an Elisa microwell plate reader (LT-5000 MS, LabTech, UK).

## **Results**

### ***Nanoparticles preparation and characterization***

PLGA-Nps were formulated by a simple versatile nanoprecipitation process. AFM showed relatively homogeneous particle sizes with spherical and smooth surfaces (Figure 9). The mean hydrodynamic particles diameter was found around 150 nm with a narrow

polydispersity ( $< 0.1$ ). A slightly negative zeta potential ( $-5$  mV) was measured which is coherent for this kind of Nps covered by PVA<sup>50</sup>. For ease of handling, the Nps were freeze-dried in presence of trehalose as a cryoprotectant to facilitate their storage and then their resuspension in water phase for emulsion preparation.

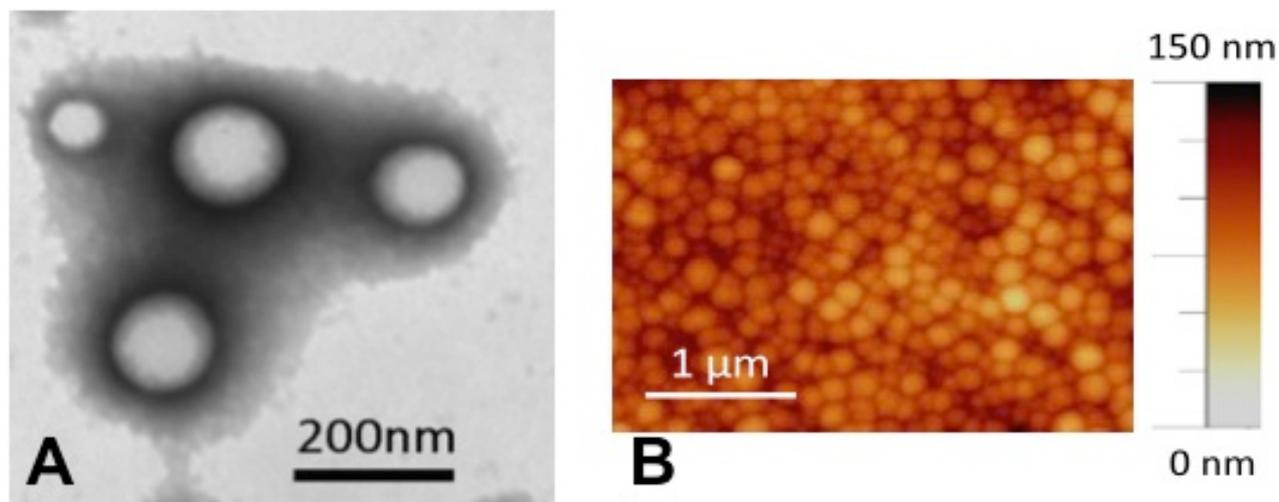


Figure 9: A/ TEM image with negative staining and B/ Topographic AFM Image ( $5.0 \mu\text{m} \times 5.0 \mu\text{m}$ ) of dried PLGA-Nps.

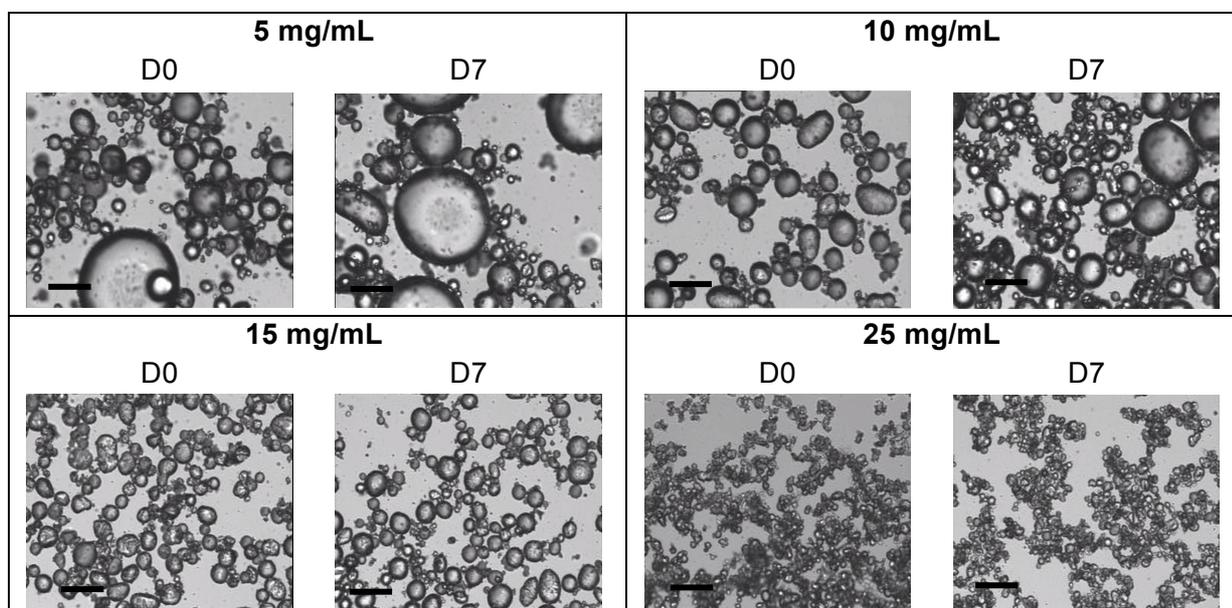
### *Emulsion formulation*

Classically, Pickering-emulsion can be prepared in a similar manner to that of classical surfactant-based emulsions. So, to respect the clinical procedure used to prepare conventional Lipiodol-emulsion, all Pickering-emulsions were formulated by repetitive pumping of two 10 mL syringes through a 3-ways stopcock<sup>20</sup>. To investigate the influence of the W/O ratio and the Nps concentration, the aqueous-phase was simply composed of saline on which the Nps were dispersed.

### *Influence of Nps concentration*

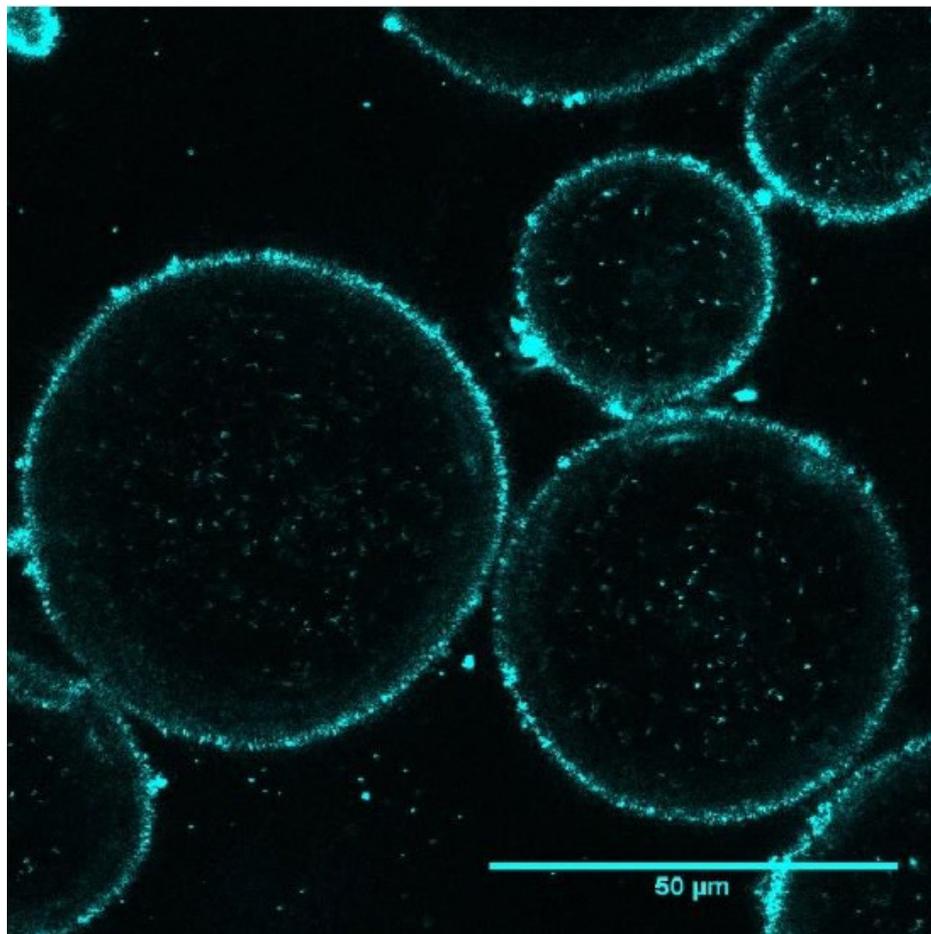
The importance of solid particles amount during formulation of Pickering-emulsions was highlighted in previous reports<sup>46 47</sup>. In particular, reducing the amount of particles in the formulation led to the formation of very large droplets since only a small interfacial area

can be stabilized. The influence of Nps concentration on the Lipiodol-emulsion formulation was therefore evaluated through the stability and droplets size of the resulting emulsions. Four different concentrations were tested: 5, 10, 15 and 25 mg/mL. The right amount of Nps were resuspended in saline before emulsification at a W/O ratio of 1/3. All the emulsions were successfully prepared and they were all water-in-oil emulsions. Microscopic aspects of the different emulsions are shown Figure 10. In all cases, aqueous droplets are relatively well dispersed in the continuous lipiodol phase. A darker interface between the aqueous core of the droplets and lipiodol could be distinguished. One can also observe that the droplets size is strongly influenced by the Nps concentration used for each formulation.



*Figure 10: Optical microscopy of the W/O lipiodol Pickering-emulsions with different Nps concentrations at D0 and D7 (scale bar = 100 μm).*

To characterize the darker interface, confocal microscopy was performed using iron oxide containing PLGA-Nps for their visualization (Figure 11). The same global aspect was found compared to optical microscopy but the surrounded of the droplets were highlighted by the presence of the Nps adsorbed at the interface.



*Figure 11: Confocal microscopy of the W/O Pickering-emulsions at 15 mg/mL of PLGA-Nps with a W/O ratio of 1/3 (scale bar = 50  $\mu\text{m}$ ).*

Immediately after formulation, evolution of the emulsion stability was followed by static light scattering (Turbiscan®) during 24h (curves are displayed in supporting information). As shown Table 3, whatever the Nps concentration, a creaming phase was rapidly observed after formulation. The duration of the creaming phase decreased with the Nps concentration while the relative height of the creaming phase increased. For the 10, 15 and 25 mg/mL formulation, phase separation was not observed during the time of analysis. Concerning the 5 mg/mL emulsion, phase separation started immediately after emulsification and stabilized after 2 h at a relative height of  $3 \pm 0.3\%$  compared to the total height of the solution.

*Table 3: Emulsion stability of the W/O Pickering-emulsions with different Nps*

	5 mg/mL	10 mg/mL	15 mg/mL	25 mg/mL
Creaming phase duration (h)	2 ± 0.2	4.5 ± 0.5	5.5 ± 0.5	9 ± 0.1
Relative height of creaming phase (%)	51.6 ± 1.9	38.2 ± 1.4	26.6 ± 1.1	9.9 ± 0.8
Image at 24h				

Determination of the average droplets size after emulsification shows a decrease with the augmentation of the Nps concentration from 60 to 20 µm (Figure 12). Seven days after the emulsification, the droplet sizes remain stable for the 10, 15 and 20 mg/mL (stat) while the size of the 5 mg/mL emulsion significantly rises from 60 to 90 µm. At 35 days, no significant change was observed compared to the 7 days measurements.

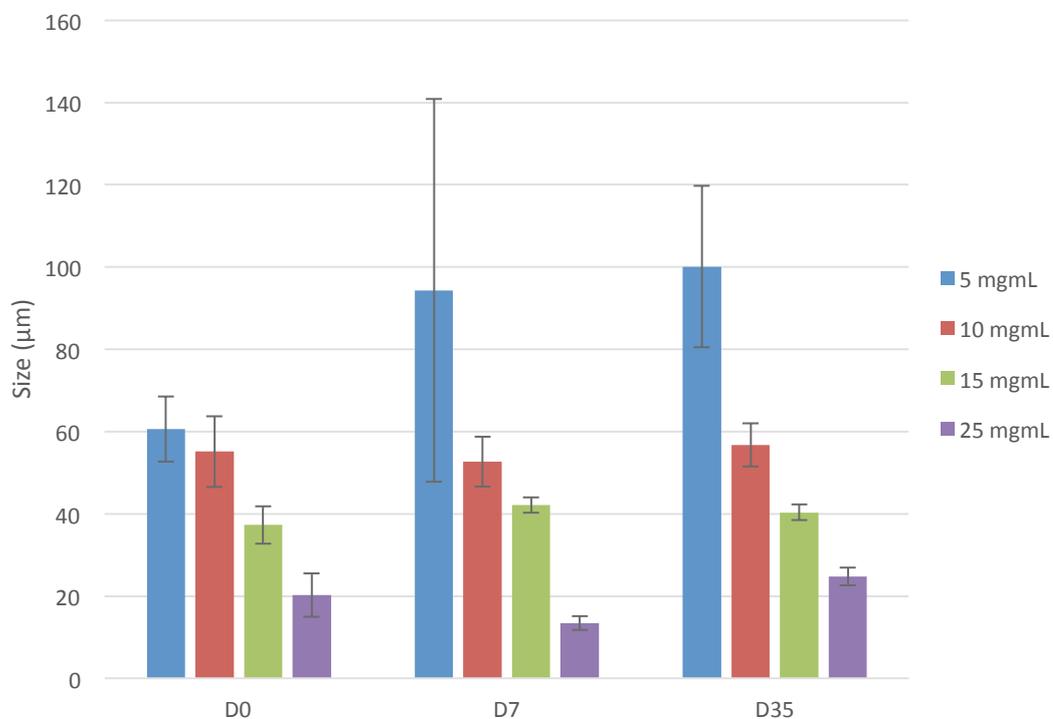


Figure 12: Drop size of the Pickering-emulsions at W/O ratio of 1/3 with different Nps concentrations at D0, D7 and D35.

## Influence of W/O ratio

In addition to studying the impact of Nps concentration, the effects of changing the W/O ratio while keeping the rest of formulation constant was also investigated. The concentration of 15 mg/mL was selected to perform this study. Four different W/O ratios were studied: 1/3, 1/2, 2/3, and 1/1. All the emulsions were successfully prepared with the desired W/O type. Microscopic images show similar aspects for emulsions 1/3, 1/2 and 2/3 whereas emulsion 1/1 exhibits more heterogeneous morphologies (Figure 13). Indeed, presence of some large droplets could be observed in solution. Inside these larger droplets, smaller droplets could be distinguished. This observation was confirmed by confocal microscopy (supporting information).

All emulsions were stable without phase separation for 24 hours according analysis by static light scattering (Supporting information).

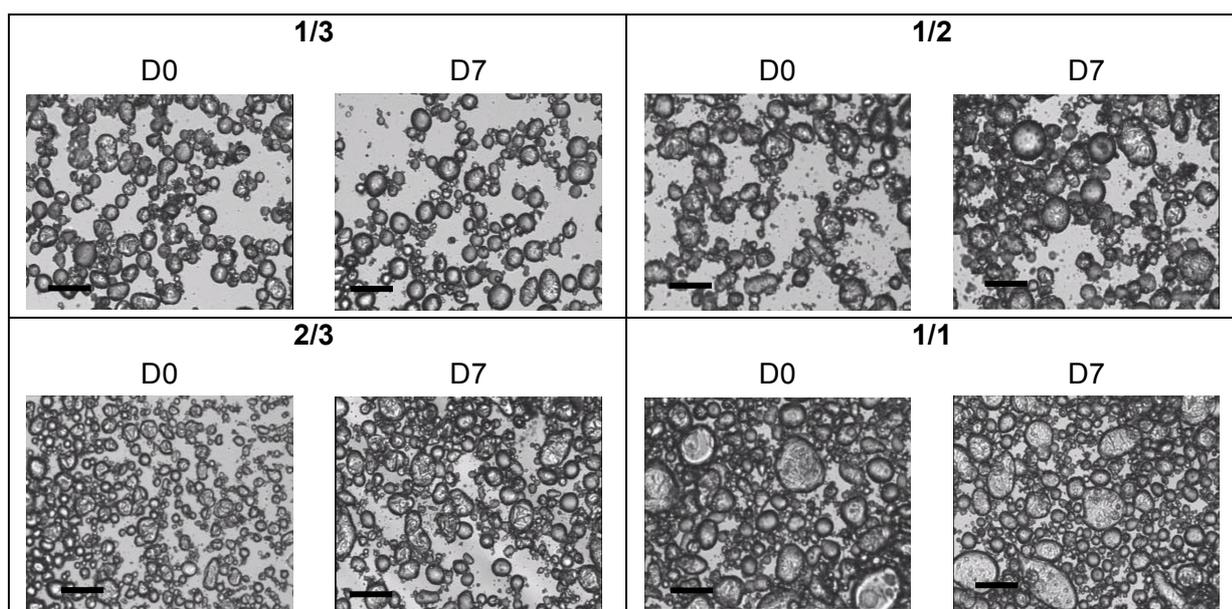


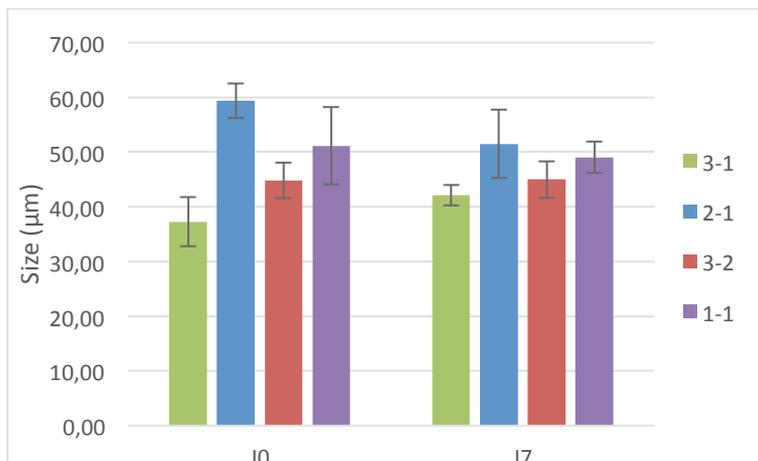
Figure 13: Optical microscopy of the W/O Pickering-emulsions at a Nps concentration of 15 mg/mL with different ratios at D0 and D7 (scale bar = 100  $\mu$ m).

For emulsions 1/3 and 1/2, a creaming phase was rapidly observed after their preparation which stabilized after 5-6h (Table 4). The relative creaming height was higher for emulsion 1/2 than the 1/3 (10% and 27% respectively). For the two other emulsions, 2/3 and 1/1, no creaming was depicted in the first 24 hours.

**Table 4:** Emulsion stability of the W/O Pickering-emulsions at a Nps concentration of 15mg/mL with different ratios at 24h.

	1/3	1/2	2/3	1/1
Creaming phase duration (h)	5.5 ± 0.5	6 ± 0.5	-	-
Relative height of creaming phase (%)	26.6 ± 1.1	9.2 ± 0.5	-	-
Image at 24h				

The average droplet size of the four emulsions does not lead to a clear effect of the ratio (Figure 14). Despite a significantly higher droplets size for the 1/2 emulsion, they were all in the 40-60 µm size range. At 7 days, no significant change was observed.



**Figure 14:** Drop size of the Pickering-emulsions at Nps concentration of 15 mg/mL with different W/O ratios at D0 and D7.

### **Doxorubicin loaded emulsion**

TACE typically involves injection of water-soluble chemotherapies such as doxorubicin, epirubicin, mitomycin and cisplatin emulsified with lipiodol. In particular,

doxorubicin hydrochloride is recognized as the standard first-line drug for the treatment of HCC.

Doxorubicin loaded emulsion was thus prepared from an aqueous solution of doxorubicin at 20 mg/mL using a W/O ratio of 3/1 and a nanoparticles concentration of 15 mg/mL. After pumping, a dark red emulsion in the desired W/O type was obtained. No specific difference could be observed with the bland emulsion prepared with saline by optical microscopy (Figure 15). In confocal images, the same type of morphology was also observed but with additional droplet red cores which correspond to the presence of doxorubicin inside the aqueous dispersed phase.

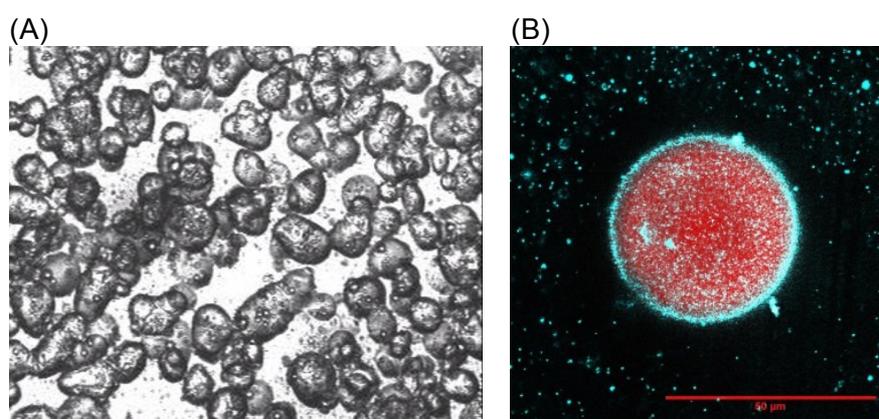


Figure 15: Doxorubicin loaded emulsion ( $W/O = 1/3$ ,  $[Nps] = 15 \text{ mg/mL}$ ,  $[doxorubicin] = 20 \text{ mg/mL}$ ), (A) Optical microscopy at  $D_0$  (scale bar =  $100 \mu\text{m}$ ) and (B) Confocal microscopy at  $D_0$  (scale bar =  $50 \mu\text{m}$ ).

The stability of the loaded emulsion followed by static light scattering over 24h shows similar behaviour than the bland emulsion. Rapidly, a creaming phase appeared but no phase separation was observed (supporting information).

In vitro doxorubicin release experiment from the emulsion was performed in TRIS solution as  $37^\circ\text{C}$  (Figure 16). A conventional doxorubicin emulsion was also assayed for comparative purpose. As control, pure doxorubicin solution was evaluated in the dialysis system to evaluate possible retention. After 4h of incubation in TRIS, only 5% was released from the Pickering-emulsion compared to 60% for the conventional emulsion.

After 24h, the released was complete for the Lipiodol-emulsion while around 30% was measured for the Pickering-emulsion. The release of doxorubicin from Pickering-emulsion was significantly sustained compared to the one from conventional Lipiodol-emulsion.

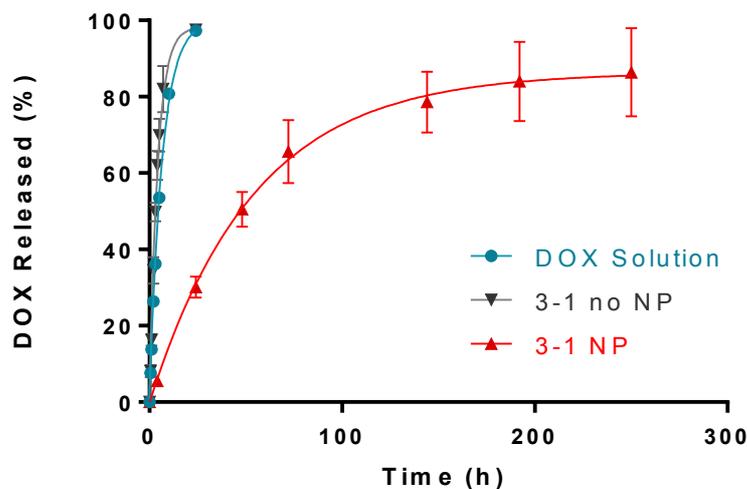


Figure 16: Doxorubicin release profiles from conventional Lipiodol-emulsion ( $W/O = 1/3$ ,  $[doxorubicin] = 20 \text{ mg/mL}$  and Pickering-emulsion ( $W/O = 1/3$ ,  $[Nps] = 15 \text{ mg/mL}$ ,  $[doxorubicin] = 20 \text{ mg/mL}$ ).

## Discussion

Solid nanoparticles are noticeable alternatives to synthetic surfactants for the stabilization of emulsions. Thanks to their strong adsorption at the interface of two immiscible liquids, highly stable emulsions also called Pickering-emulsions can be achieved. In many Pickering-emulsion studies, the oils have been relatively simple in structure (hydrocarbon or silicone) and with a lower density than water. In a study on the effect of oil type on particle stabilized emulsions, it was shown that the nature of the oil may strongly orientate the nature of the emulsion either W/O or O/W<sup>51</sup>.

Lipiodol has been used for TACE procedure for over 30 years because of its preferential tropism for the tumour compared to surrounding tissues<sup>52</sup>. This is an oily mixture of iodinated poppy-seed fatty acid esters. Due to the presence of numerous iodine atom, density of lipiodol is  $1.28 \text{ g/cm}^3$ . The high iodine content combined to the high

density contributes largely to difficulties encountered until now for the stabilisation of Lipiodol-emulsions.

In this study, results have demonstrated a novel strategy to stabilize Lipiodol-emulsion. The addition of PLGA-Nps into the aqueous-phase of the formulation has generated an inverse Pickering-emulsion by a simple emulsification process through two connected syringes. The resulting emulsion displayed remarkable stability against coalescence and maintained their morphology over several weeks.

Few publications address emulsion stabilization with degradable Nps. Laredj-Bourezg et al demonstrated that particles of poly(caprolactone)-block-poly(ethylene glycol)<sup>53</sup> and of poly(lactic acid)-block-poly(ethylene oxide)<sup>54</sup> were able to stabilize O/W emulsions using a triglyceride oil for topic applications. Earlier, Withby et al have reported the use of PLGA-PVA Nps as emulsifiers<sup>47</sup>. They showed that these particles preferentially stabilise oil-in-water emulsions from a wide range of oils (polar and non polar) even for equal volume of oil and aqueous-phase. Despite this trend, highly stable Lipiodol-emulsions were achieved over a large range of oil volume fraction and Nps concentrations. They all presented, as desired for TACE application, a water in oil type emulsion. As highlighted by microscopic analyses, the formed droplets were surrounded by PLGA-PVA Nps indicating that this kind of Nps was able to absorb at the water/lipiodol interface.

Although the droplet size was large and evolved over the first week for one emulsion, a decrease of droplets size was clearly observed for the three other emulsions when the Nps concentration was increased from 10 to 25 mg/mL. Stability of an emulsion stabilized by nanoparticles is mainly governed by a steric mechanism. Once the interfacial area is covered by nanoparticles, droplets coalescence stops and there is a direct relationship between the mass of Nps and the droplet size. In 1995, de Baere et al have demonstrated a higher efficacy of Lipiodol-emulsions when the aqueous droplets have a similar size as the tumour feeding arteries i.e. around 40  $\mu\text{m}$ <sup>25</sup>. Therefore, the Nps

concentration was set at 15 mg/mL corresponding to an average droplet size around 40  $\mu\text{m}$  for the preparation of stable Pickering-emulsions.

There is a strong interest to vary the W/O ratio for chemoembolization. Today, there is a consensus for the preparation of lipiodol-drug emulsion with a W/O ratio of 1/3 to form relatively stable emulsion<sup>20</sup>. But, for many drugs such as oxaliplatin, the amount of drug is limited by the concentration of the commercially available vial. Therefore, an increase of the aqueous-phase ratio may allow to incorporate more chemotherapies for TACE procedure. In this work, the Pickering-emulsions were stable over a large range of aqueous volume fraction to an equal volume of oil providing sufficient choice for the surgeon to load desired concentration of chemotherapies.

Doxorubicin hydrochloride is an antineoplastic agent widely used in chemoembolization for palliative treatment of HCC. Although doxorubicin is a potent antitumor agent, its clinical utility is hampered by its toxicities such as cardiac damage and myelosuppression. Its delivery through a W/O Lipiodol-emulsion system was expected to reduce/limit this toxicity. Despite a recent study that has shown a significant diminution of the plasmatic concentration using a Lipiodol-emulsion prepared with contrast medium rather than saline, this system still fails to show a sustained release and lowering the systemic exposure<sup>55</sup>.

Efficient encapsulation of the anticancer drug doxorubicin into the Pickering-emulsion was simply performed by mixing the doxorubicin aqueous-phase containing Nps with lipiodol. In spite a high doxorubicin concentration (20 mg/mL), the resulting emulsion was stable and no specific difference could be depicted with the bland emulsion of the same composition. The hydrophilic drug is retained in the internal phase due to its water solubility. The addition of PLGA-Nps not only improved the stability of the emulsion but also improved considerably the doxorubicin release. This sustained release might arise from the formation of a strong membrane created by the Nps layers surrounding aqueous droplets which slow down the diffusion process. Further experiments are required to

elucidate the release mechanism and investigate the influence of W/O ratios on the drug release.

## Conclusions

In this work, we successfully developed a new kind of Lipiodol-emulsion which remains stable over weeks. This emulsion answered the main requirements to allow optimum tumor uptake by TACE procedure: (1) stable at least 24h, (2) water-in-oil emulsion for higher tumor selectivity, (3) droplet sizes around 40  $\mu\text{m}$  since vessels close to the tumor are between 40 -60  $\mu\text{m}$  (4) loadable with a chemotherapeutic agent and ultimately (5) a sustained release of the chemotherapy to extend the tumor exposure. Results are very encouraging and this combination allows the assumption that the Pickering-emulsion will lead to better efficacy and safety than the conventional emulsion. Future work will consist in performing in vivo studies to confirm the potential of this system to be used as novel agent for TACE procedure.

## Pickering-emulsion: in vitro releases

The purpose of this study was to analyze the in vitro release' profiles of different chemotherapies from the Pickering-emulsion and to compare these releases to those obtained using other drug delivery platforms (Lipiodol or drug-eluding beads).

## Materials and methods

### *Loading of chemotherapy in drug delivery platforms*

The chemotherapies that we decided to evaluate in this experimental study are routinely used for liver intra-arterial therapies: Doxorubicin Hydrochloride (Adriablastine® 20 mg/mL , Pfizer, France), Irinotecan (Campto® 20 mg/mL , Pfizer, France) and Oxaliplatin (Eloxatine®, 5 mg/mL, Sanofi-Aventis, France). Chemotherapy were "loaded" in different drug delivery platforms in order to compare their in vitro' releases profiles.

For Lipiodol, the loading mechanism consisted in formulating a water-in-oil emulsion composed of Lipiodol (oily-phase) and chemotherapy (aqueous-phase). For this purpose, Lipiodol-emulsions were formulated by continuous injection of 1 volume of chemotherapy in 3 volumes of Lipiodol (ratio oily-/ aqueous-phase = 3/1) with an electric syringe-pump (flow-rate of 1 mL per minute) during repetitive pumping of two 10-mL syringes through a three-way stopcock.

For drug-eluding beads, the loading mechanism consisted in adding the chemotherapy directly in the vials of beads (DC-Beads® 300-500 µm, Biocompatibles, Farnham, United Kingdom) and gently agitating during 1 hour.

For Pickering-emulsion, the oily-phase was composed of Lipiodol and the aqueous-phase was composed of chemotherapy and Polylactic-co-glycolic acid (PLGA) nanoparticles (Nps). These Nps were directly added to the chemotherapy before the formulation of the emulsion at a concentration of 15 mg/mL. Then, the aqueous-phase (chemotherapy and Nps) was infused in the oily-phase (Lipiodol) with an electric syringe-pump (flow-rate of 1 mL per minute) during repetitive pumping of two 10-mL syringes

through a three-way stopcock. For Pickering-emulsion, different ratios of oily-/ aqueous-phase were formulated (3/1; 2/1; 1/1) and their in vitro releases profile compared.

### *In vitro comparison of 3 different drug delivery platforms.*

In vitro releases of chemotherapy from Lipiodol-emulsion, drug-eluding beads or Pickering-emulsion were performed twice for each drug delivery platform. Aliquots of Chemotherapy-loaded drug delivery platforms were loaded into mini GeBAflex tubes (molecular weight cut-off: 12–14 kDa; Gene Bio-Application Ltd., Kfar Hanagide, Israel). Those tubes were immersed in 40 mL of buffer solution and incubated in a shaking bath (37 °C) rotated at a speed of 150 rpm. At predetermined times (1h, 4h, 24 h, 48h, 72h, 1 week, 2 weeks and 1month), the aliquots (0.5 mL) were collected and replaced with equivalent volumes of fresh release media. The release profile reports the cumulative chemotherapy releases in percentage and in concentration of chemotherapy in the buffer solution. Comparisons between the 3 different drug delivery platforms were performed for doxorubicin, irinotecan and oxaliplatin. For oxaliplatin, only Lipiodol and Pickering-emulsion were compared since oxaliplatin cannot be loaded in drug-eluding beads.

The cumulative releases of 4 mg doxorubicin were compared according to the drug delivery platform used: Lipiodol-emulsion (ratio 3/1), drug-eluding beads or Pickering-emulsion (ratio 3/1). The released amounts of doxorubicin in the buffer solution (phosphate-buffered saline, pH 7.4) were determined using high performance liquid chromatography (HPLC).

The cumulative releases of 4 mg irinotecan were compared according to the drug delivery platform used: Lipiodol-emulsion (ratio 3/1), drug-eluding beads or Pickering-emulsion (ratio 3/1). The released amounts of irinotecan in the buffer solution (phosphate-buffered saline, pH 7.4) were determined using a UV-spectrophotometer at 370 nm.

The cumulative releases of 1 mg oxaliplatin were compared according to the drug delivery platform used: Lipiodol-emulsion (ratio 3/1) or Pickering-emulsion (ratio 3/1). The

released amounts of oxaliplatin in the buffer solution (acetate buffer 25 mM, pH 6.8) were determined using Atomic Absorption Spectrometry (AAS) (Platinum quantification served as our assay for oxaliplatin drug concentration: 1 mg of oxaliplatin corresponds to 0.49 mg of platinum).

### *In vitro comparison of different Pickering-emulsion' formulation*

For Pickering-emulsion, we compared the cumulative release of the chemotherapy using different ratios of oily-/ aqueous-phases (3/1 vs. 2/1 vs. 1/1). For each ratio, the same volumes of Pickering-emulsion (0.8 mL) were analyzed, For doxorubicin Pickering-emulsion, 0.8 mL of emulsion contains 4 mg, 5.33 mg or 8 mg of doxorubicin respectively for ratio 3/1, 2/1 and 1/1. For irinotecan Pickering-emulsion, 0.8 mL of emulsion contains 4 mg, 5.33 mg or 8 mg of irinotecan respectively for ratio 3/1, 2/1 and 1/1. For oxaliplatin Pickering-emulsion, 0.8 mL of emulsion contains 1 mg, 1.33 mg or 2 mg of oxaliplatin respectively for ratio 3/1, 2/1 and 1/1.

## **Results**

### *Doxorubicin:*

The doxorubicin release profiles from the three different drug delivery platforms are shown in the following Table 5 and in Figure 17. Briefly, we observed a very rapid and complete release of doxorubicin from Lipiodol-emulsion (97.5% at 24 hours). The release from drug-eluting beads was much slower (6.4% at 24 hours) but incomplete (54.8% at 250 hours). Pickering-emulsion demonstrated an intermediate speed of release (30.1% at 24 hours), almost complete (86.4% at 250 hours).

*Table 5: Release of 4 mg of doxorubicin (%) according to the three drug delivery platforms*

	<b>Lipiodol-emulsion</b>		<b>Pickering-emulsion</b>		<b>DC-Beads</b>	
	(ratio 3/1)		(ratio 3/1)		(300-500 $\mu$ m)	
<b>Time (h)</b>	<b>Release (%)</b>	<b>SD</b>	<b>Release (%)</b>	<b>SD</b>	<b>Release (%)</b>	<b>SD</b>
<b>4</b>	62,0	3,8	5,5	0,4	0,0	0,4
<b>24</b>	97,5	1,2	30,1	2,7	6,4	0,9
<b>48</b>	-	-	50,5	4,5	13,1	1,5
<b>72</b>	-	-	65,6	8,2	17,2	2,3
<b>142</b>	-	-	78,6	8,0	34,9	5,0
<b>190</b>	-	-	84,0	10,4	46,5	7,3
<b>250</b>	-	-	86,4	11,5	54,8	9,1

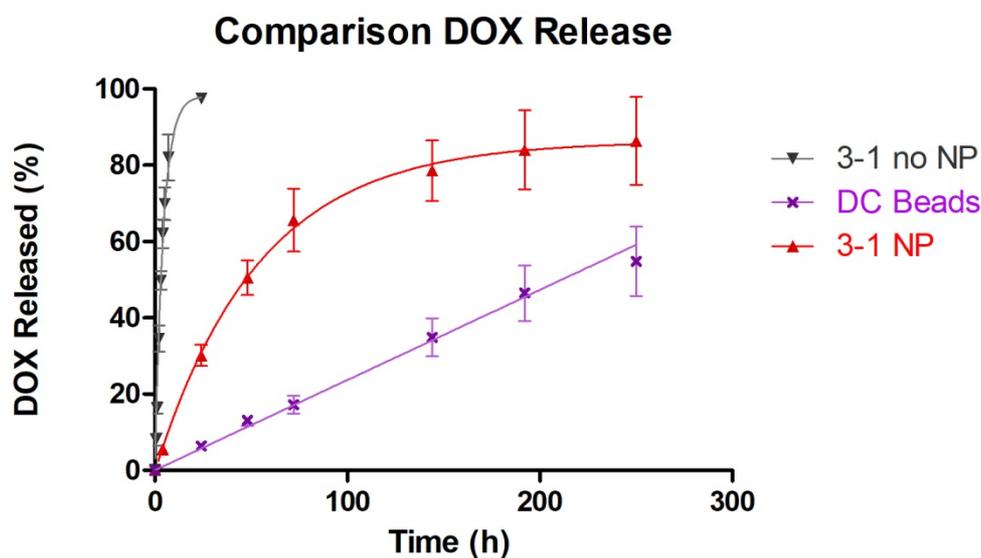


Figure 17: *In vitro* release of 4 mg doxorubicin from three different drug delivery platforms: Lipiodol-emulsion (emulsion 3/1 no NP), Pickering-emulsion (Emulsion 3/1 NP) and drug-eluting beads (DC Beads).

The doxorubicin release profiles from the three different Pickering-emulsions, are shown in the following Table 6 and in Figure 18. Briefly, we observed that, for similar volume of emulsion, the highest the ratio, the more doxorubicin released. For example, at 250 hours, the mass of doxorubicin released from Pickering-emulsion were 3.5, 4.4 and 5.8 mg for ratio 3/1, 2/1 and 1/1 respectively.

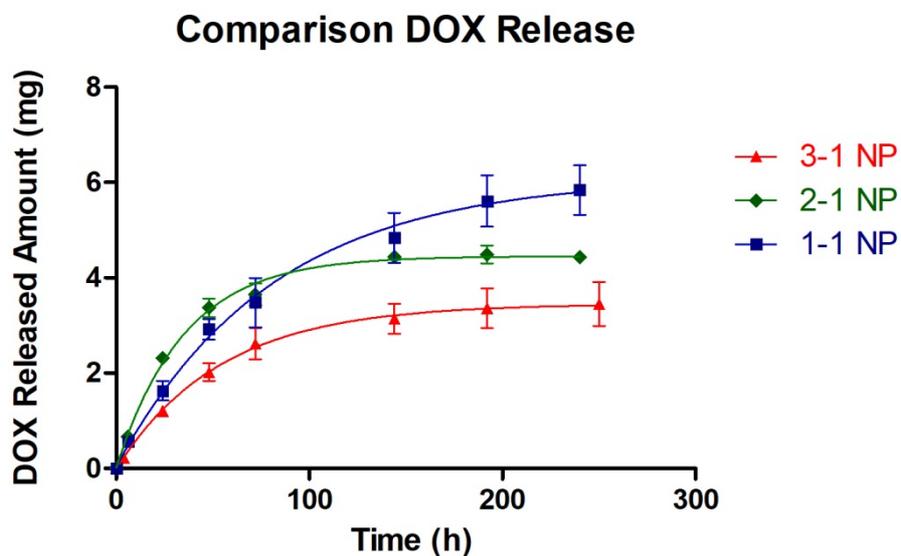


Figure 18: Release of doxorubicin (mg) from 0.8 mL of 3 different Pickering-emulsion' formulations.

Table 6: release of doxorubicin (mg) from 0.8 mL of 3 different Pickering-emulsion' formulations.

Time (h)	Pickering-emulsion (ratio 3/1)		Pickering-emulsion (ratio 2/1)		Pickering-emulsion (ratio 1/1)	
	Release (mg)	SD	Release (mg)	SD	Release (mg)	SD
4	0,2	0,0	0,7	0,1	0,6	0,1
24	1,2	0,1	2,3	0,1	1,6	0,2
48	2,0	0,2	3,4	0,2	2,9	0,2
72	2,6	0,3	3,6	0,2	3,5	0,5
142	3,1	0,3	4,4	0,1	4,8	0,5
190	3,4	0,4	4,5	0,2	5,6	0,5
250	3,5	0,5	4,4	0,1	5,8	0,5

#### Irinotecan:

The irinotecan release profiles from the three different drug delivery platforms are shown in Table 7 and in Figure 19. Briefly, we observed a very rapid and complete release of irinotecan from Lipiodol-emulsion (99.7% at 24 hours) and from drug-eluting beads

(57.4% at 24 hours). The release from Pickering-emulsion was much slower (13.6% at 24 hours) and almost complete (75% at 360 hours).

Table 7: Release of 4 mg of irinotecan (%) according to the three drug delivery platforms

	<b>Lipiodol-emulsion</b>		<b>Pickering-emulsion</b>		<b>DC-Beads</b>	
	(ratio 3/1)		(ratio 3/1)		(300-500 µm)	
<b>Time (h)</b>	<b>Release (%)</b>	<b>SD</b>	<b>Release (%)</b>	<b>SD</b>	<b>Release (%)</b>	<b>SD</b>
<b>4</b>	84,8	5,3	-	-	24,6	0,4
<b>24</b>	99,7	0,7	13,6	3,3	57,4	0,9
<b>48</b>	-	-	23,4	4,9	77,4	1,5
<b>72</b>	-	-	27,6	0,4	84,6	2,3
<b>142</b>	-	-	45,4	2,0	-	--
<b>190</b>	-	-	53,7	1,1	-	-
<b>250</b>	-	-	60,4	1,6	-	-
<b>360</b>	-	-	75,5	0,2	-	-

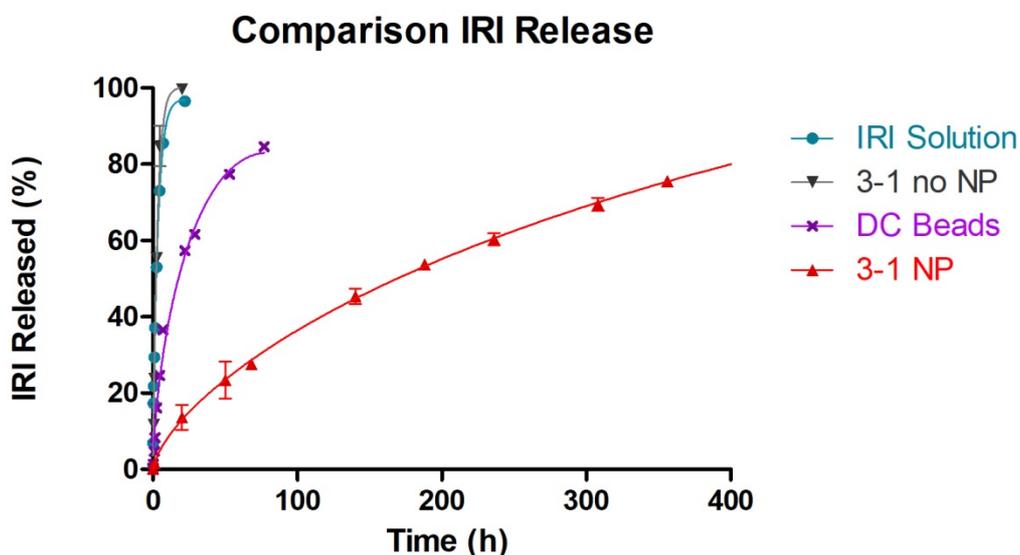


Figure 19: In vitro release of 4 mg irinotecan from three different drug delivery platforms: Lipiodol-emulsion (Emulsion 3/1 no NP), Pickering-emulsion (Emulsion 3/1 NP) and drug-encapsulating beads (DC Beads)

The irinotecan release profiles from the three different Pickering-emulsions, are shown in Table 8 and in Figure 20. Briefly, we observed that, for similar volume of emulsion, the highest the ratio, the more doxorubicin released. For example, at 360 hours, the masses of doxorubicin released from Pickering-emulsion were 3.0, 3.6 and 4.5 mg for ratio 3/1, 2/1 and 1/1 respectively.

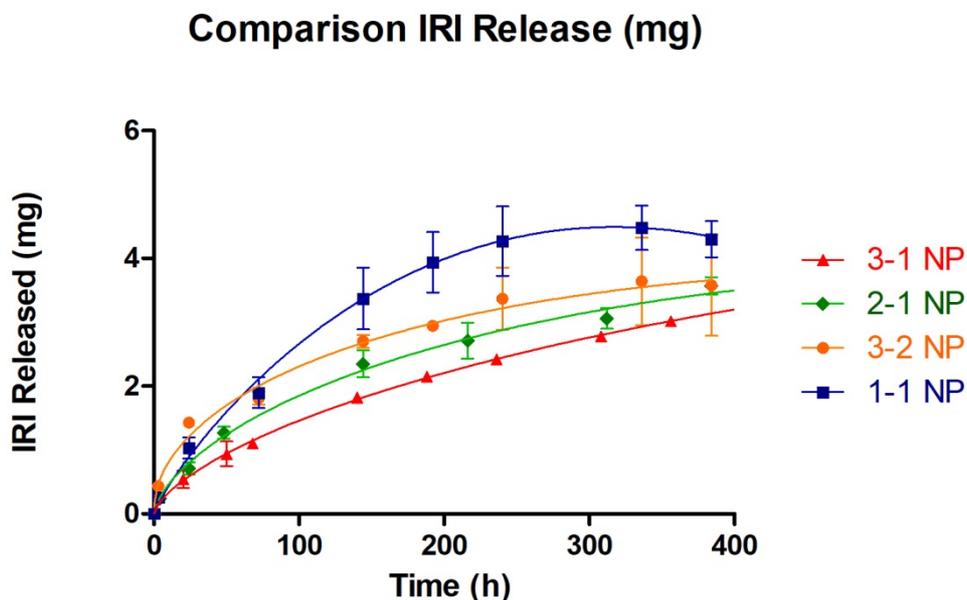


Figure 20: release of irinotecan (mg) from 0.8 mL of 3 different Pickering-emulsion' formulations.

Table 8 release of irinotecan (mg) from 0.8 mL of 3 different Pickering-emulsion' formulations.

	Pickering-emulsion <b>(ratio 3/1)</b>	Pickering-emulsion <b>(ratio 2/1)</b>	Pickering-emulsion <b>(ratio 1/1)</b>

<i>Time (h)</i>	<i>Release (mg)</i>	<i>SD</i>	<i>Release (mg)</i>	<i>SD</i>	<i>Release (mg)</i>	<i>SD</i>
<b>24</b>	0,5	0,1	0,7	0,1	1,0	0,2
<b>48</b>	0,9	0,2	1,3	0,1	-	-
<b>72</b>	1,1	0,0	-	-	1,9	0,2
<b>142</b>	1,8	0,1	2,4	0,2	3,4	0,5
<b>190</b>	2,1	0,0	2,7	0,3	3,9	0,5
<b>250</b>	2,4	0,1	-	-	4,3	0,5
<b>360</b>	3,0	0,0	3,6	0,1	4,5	0,3

### **Oxaliplatin**

The oxaliplatin release profiles from the Lipiodol-emulsions and from Pickering-emulsions are shown in Table 9 and in Figure 21. Briefly, we observed a very rapid and complete release of doxorubicin from Lipiodol-emulsion (85.3% at 24 hours) whereas the release from Pickering-emulsion was much slower (15.8% at 24 hours) and almost complete (66% at 600 hours).

*Table 9 : release (%) of 1mg of Oxaliplatin according to the two drug delivery platforms.*

	<b>Lipiodol-emulsion</b>		<b>Pickering-emulsion</b>	
	(ratio 3/1)		(ratio 3/1)	
<i>Time (h)</i>	<i>Release (%)</i>	<i>SD</i>	<i>Release (%)</i>	<i>SD</i>
<b>4</b>	50,0	13,8	4,7	0,9
<b>24</b>	85,3	3,3	15,8	3,0

<b>48</b>	91,6	0,1	21,7	4,0
<b>72</b>	95,7	2,4	26,1	5,3
<b>168</b>	-	-	37,7	3,9
<b>216</b>	-	-	43,3	3,1
<b>360</b>	-	-	59,1	2,3
<b>600</b>	-	-	65,8	2,8

### Comparison OXALI Release

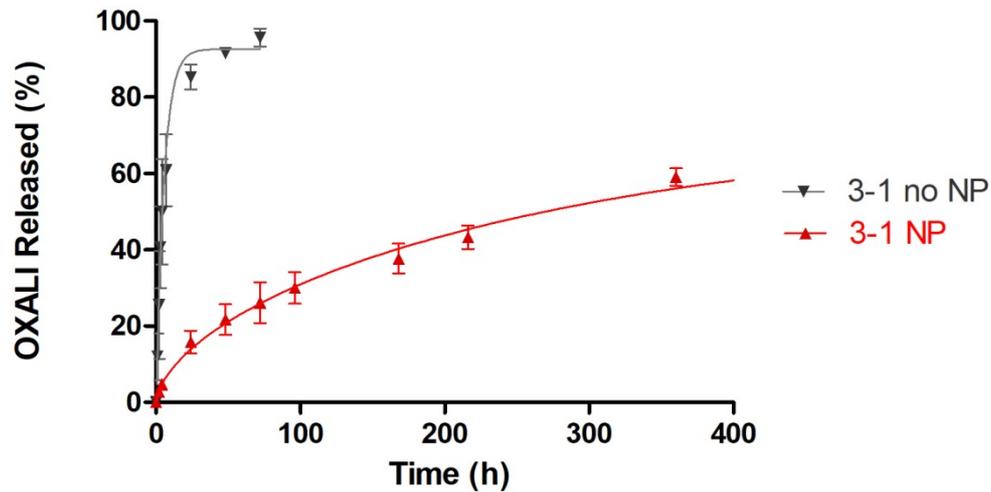


Figure 21 : *in vitro* release of 1 mg oxaliplatin from two different drug delivery platforms: Lipiodol-emulsion (Emulsion 3/1 no NP), and Pickering-emulsion (Emulsion 3/1 NP).

The oxaliplatin release profiles from the three different Pickering-emulsions, are shown in Table 10 and in Figure 22. Briefly, we observed that, for similar volume of emulsion, the highest the ratio, the more oxaliplatin released. For example, at 600 hours, the mass of doxorubicin released from Pickering-emulsion were 0.66, 0.85 and 1.5 mg for ratio 3/1, 2/1 and 1/1 respectively.

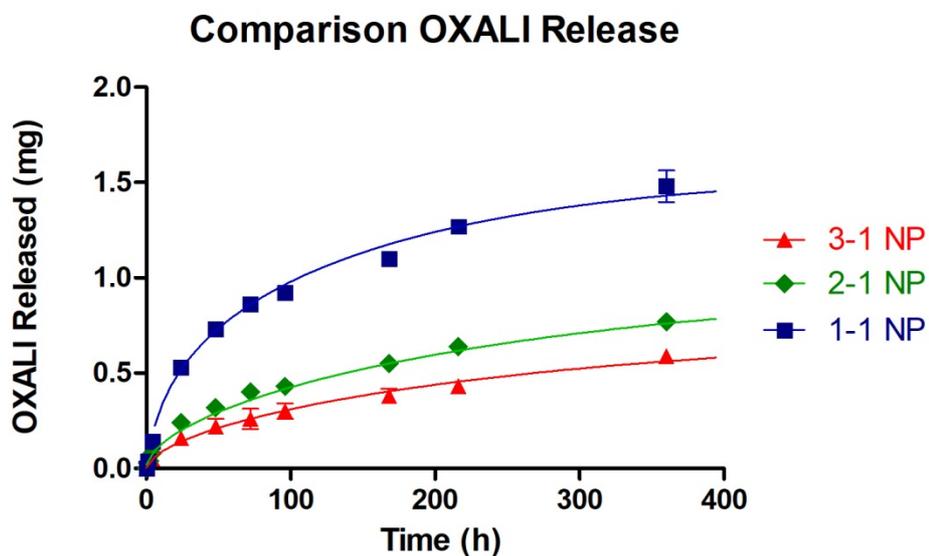


Figure 22: release of oxaliplatin (mg) from 0.8 mL of 3 different Pickering-emulsion' formulations.

Table 10 : release of oxaliplatin (mg) from 0.8 mL of 3 different Pickering-emulsion' formulations.

Time (h)	Pickering-emulsion (ratio 3/1)		Pickering-emulsion (ratio 2/1)		Pickering-emulsion (ratio 1/1)	
	Release (mg)	SD	Release (mg)	SD	Release (mg)	SD
<b>4</b>	0,05	0,01	0,08	0,01	0,14	0,00
<b>24</b>	0,16	0,03	0,24	0,01	0,53	0,02
<b>48</b>	0,22	0,04	0,32	0,00	0,73	0,03
<b>72</b>	0,26	0,05	0,40	0,01	0,86	0,00
<b>96</b>	0,30	0,04	0,43	0,01	0,92	0,02
<b>216</b>	0,43	0,03	0,64	0,03	1,27	0,04
<b>360</b>	0,59	0,02	0,77	0,01	1,48	0,08
<b>600</b>	0,66	0,03	0,85	0,04	1,50	0,02

## Discussion

Pickering-emulsion demonstrates a high polyvalence compared to drug-eluting beads that can load positively charged drugs only (Coulomb charge interactions). Doxorubicin, epirubicin or irinotecan are the only chemotherapies that can be readily used in clinical practice so far. The “loading” mechanism for Pickering-emulsion is not correlated with ionic interaction but with hydrophilic properties, offering a broad spectrum of “loadable” chemotherapies. In this study, we have demonstrated that doxorubicin, irinotecan and oxaliplatin can be emulsified and slowly released thank to the addition of PLGA-Nps. Theoretically, all hydrophilic therapeutic agents can be used to compose the aqueous-phase.

In addition, the ionic interaction' intensities between drug eluding-beads and the chemotherapy are highly variable according to the chemotherapy, resulting in variable releases: either incomplete and slow (doxorubicin demonstrates a strong drug-bead ionic interactions) or complete and fast (irinotecan demonstrates weaker ionic interactions, at physiological pH). In this study, we confirm the results of Jordan et al<sup>23</sup> who reported an incomplete doxorubicin release over 1 week (27% +/- 2 for DC-beads® and 18% +/- 7 for Hepaspheres®; p= 0.013) and a complete release for irinotecan, in a sustained manner over 2-3 hours for DC-Beads®, and in a significantly faster manner as a 7-minute burst for Hepaspheres®. Conversely, Lipiodol-emulsion demonstrates a rapid and complete release of chemotherapy within the first 24 hours, whatever the chemotherapy used. This can be explained by the instability of the Lipiodol-emulsion that results in a separation of the oily-phase and the aqueous-phase in few minutes/ hours. Interestingly, Pickering-emulsion demonstrates slow-release, whatever the chemotherapy used in this study. In addition, the total amount of chemotherapy released and the speed of release can be managed by modifying the ratio between the Lipiodol and the chemotherapy. In this study, we demonstrated that, for similar volumes of Pickering-emulsion, the total amount of chemotherapy released and the kinetic of released were higher when the volume ratio between Lipiodol and chemotherapy was 1/1, compared to 2/1 and compared to 3/1.

## Limitations

We acknowledge several limitations. The first one is that it is an in vitro study. Results must be confirmed by an in vivo study to evaluate and to compare the pharmacokinetic of Pickering-emulsion with Lipiodol-emulsion and drug eluding-beads. The second one is that the speed of release varies between chemotherapies. Thus, additional works must be done for better understanding of the release mechanism of the chemotherapy form the Pickering-emulsion.

## Conclusions

Compared to Lipiodol and drug eluding-beads, Pickering-emulsion allows controlled releases of a broad spectrum of hydrophilic chemotherapies. The total amount of chemotherapy released and the speed of release can be managed by variable volume' ratio between Lipiodol and chemotherapy.

## Pickering-emulsion for liver trans-arterial chemo-embolization

### Abstract<sup>3</sup>:

#### Purpose

Poly(lactic-co-glycolic acid) (PLGA) nanoparticles (Nps) can be adsorbed at the water/oil interface to stabilize the emulsion (forming Pickering-emulsion). The purpose of this study was to compare the release profiles of oxaliplatin from Pickering-emulsion and Lipiodol-emulsion.

#### Materials/methods

Pickering-emulsions and Lipiodol-emulsions were both formulated with oxaliplatin (5mg/mL) and Lipiodol (Water/oil ratio: 1/3). For Pickering-emulsion only, PLGA-Nps (15mg/mL) were dissolved into oxaliplatin before formulation. Oxaliplatin was injected in left hepatic arteries of 18 rabbits bearing VX2 liver tumors using either 0.5 mL Pickering-emulsion (n=10) or 0.5 mL Lipiodol-emulsion (n=8). In each group, half of the rabbits were sacrificed at 1h and half at 24h. ICP-MS mass-spectrometry was used to quantify drug pharmacokinetics in blood plasma and resulting tissues (tumors, right- and left-liver lobes) oxaliplatin concentrations.

#### Results

In this animal model studies, the plasmatic-peak and the area under the curve were significantly lower with Pickering-emulsion,  $0.49 \pm 0.14$  ng/mL vs.  $1.08 \pm 0.41$  ng/mL,  $p=0.01$  and  $19.8 \pm 5.9$  ng/mL vs.  $31.8 \pm 14.9$  ng/mL,  $p=0.03$ , respectively. This resulted in significantly lower oxaliplatin concentrations in tissues at 1h and higher ratio between tumors and left-liver lobes at 24h ( $44.1$  vs.  $11.8$   $p=0.02$ ) with Pickering-emulsion compared

---

<sup>3</sup> *Pickering-emulsion for liver Trans-Arterial Chemo-Embolization with oxaliplatin. Deschamps F, Harris KR, Li W, Moine L, Tselikas L, Isoardo T, Lewandowski RJ, Paci A, de Baere T, Salem R and Larson AC (Submitted to Radiology on Novembre, 27<sup>th</sup> 2017)*

to Lipiodol-emulsion.

### Conclusion

Slow release of oxaliplatin from Pickering-emulsion results in a significant decrease in systemic drug exposure and higher ratio between tumor and left-liver oxaliplatin at 24h.

## Introduction

We have developed a new concept for a drug delivery platform that allows a slow and controlled release of various types of chemotherapies while maintaining the added value of Lipiodol in terms of a radio-opacity, preferential tumor uptake<sup>26</sup> and transient vessels' occlusion<sup>25</sup>. The principle is based on Pickering technology<sup>45,44</sup> that uses solid particles to stabilize emulsions. The use of solid particles (Pickering technology) in the formulation of an emulsion avoids the use of toxic synthetic surfactants and results in very stable interfaces between the aqueous-phase and the oily-phase. The absence of synthetic surfactant makes Pickering-emulsion very attractive, especially for biomedical applications.

The purpose of this study was to compare the release profiles of platinum-based chemotherapy (oxaliplatin) from Pickering-emulsion in an *in vivo* setting. Oxaliplatin was the chemotherapy selected because hepatic arterial infusion of platinum-based chemotherapy has been reported effective in the treatment of unresectable colorectal liver metastases<sup>56</sup>, hepatocellular carcinoma<sup>57</sup> and intrahepatic cholangiocarcinoma<sup>58, 59</sup>, and because today, no intra-arterial drug delivery platform is available for platin-based chemotherapy in clinical practice. Indeed, platinum-based chemotherapies cannot be loaded in drug-eluting beads since these chemotherapies are not positively charged.

## Materials/ methods

VX2 rabbit liver tumor model were used to compare Pickering-emulsion and classical Lipiodol-emulsion for oxaliplatin pharmacokinetics including plasmatic and tissue concentration.

### **Formulation of the emulsions:**

Two different types of water-in-oil emulsions were formulated: Lipiodol-emulsion and Pickering-emulsion. For both emulsions, the oily-phase was composed of Lipiodol and

the aqueous-phase was composed of oxaliplatin (5 mg/mL). PLGA-Nps were added to the aqueous-phase during formulation of the Pickering-emulsion (15 mg/mL); latter component was the only difference between the 2 emulsions. These PLGA-Nps adsorb at the water/oil interface to stabilize the emulsion. Oxaliplatin was the chosen chemotherapy in this study for two main reasons. First, several studies have already demonstrated that platinum-based chemotherapy is effective for the treatment of HCC. Second, there is no appropriate drug delivery platform available for TACE with platinum-based chemotherapy so far.

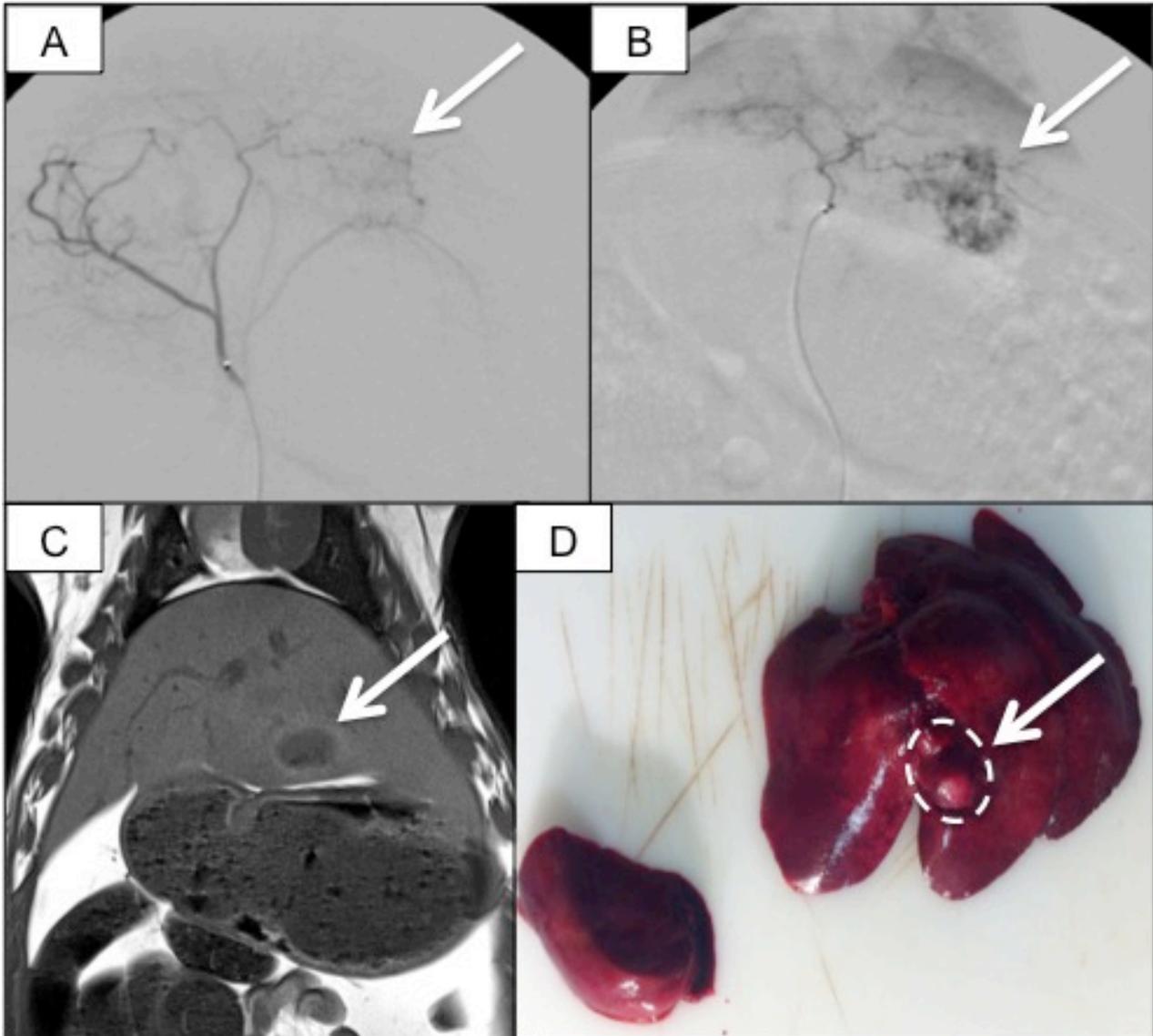
Water-in-oil emulsions were formulated by continuous injection of 1 volume of aqueous-phase in 3 volumes of oily-phase with an electric syringe-pump (flow-rate of 1 mL per minute) during repetitive pumping of two 10-mL syringes through a three-way stopcock<sup>60</sup>.

### *Animal study:*

This study was performed in accordance with the institutional Animal Care and Use Committee guidelines of Northwestern memorial University, Chicago, IL. VX2 liver tumors were percutaneously implanted in New Zealand White rabbits under general anesthesia (Intramuscular injections of Ketamine 20-40mg/kg and xylazine 3-5mg/kg, Isoflurane 3-5% for induction and 1.5-3% for maintenance mixed with O<sub>2</sub> at 1L/min). For this purpose, 1–2 mm freshly harvested tumor fragments, coming from the hind limb tumors of donor rabbits were implanted under ultrasound guidance through a 18G needle positioned at 3 different locations of the left-liver lobe. 1 week after the liver tumor implantations, MR checkup scans were performed to confirm tumor growth and locations within the liver.

Two weeks after liver tumor implantations, rabbits received general anesthesia, and TACE were performed by an experienced interventional radiologist (FD), under fluoroscopic monitoring (OEC 9800 Plus Mobile C-arm, Milwaukee, Wisconsin, USA). The femoral artery was surgically exposed and catheterized with a 4F vascular sheath using a Seldinger approach. A micro-catheter (2.4F Merit Maestro Merit medical®, South Jordan,

UT, USA) was used to selectively engage the left hepatic branch (Figure-15) and to inject 0.5 mL of emulsion corresponding to 0.625 mg of oxaliplatin. Attention was given to avoid any injection in the right hepatic artery and additional embolization with gelatin sponge was considered only in cases of persistent flow in the left hepatic branch after completion of the emulsion injection.



*Figure 23: Trans-Arterial Chemo-Embolization (TACE) with oxaliplatin in a New Zealand White rabbit: angiography in the celiac trunk (A) and in the left-hepatic artery (B), post-TACE MR scan (C) and resected liver (D) showing a 15 mM large VX2 liver tumor (white arrow).*

Heparinized systemic blood samples (2 mL) were collected from the vascular sheath 5, 10, 20, 30 and 60 minutes after the injection of the emulsion to measure the

plasmatic pharmacokinetics of oxaliplatin. The procedures were either non-recovery procedures (sacrifice at 1 hour, immediately after the last blood sample collection) or recovery procedures with sacrifice at 24 hours. Four different groups of rabbits were considered according to the type of emulsion each received and according to the delay before sacrifice:

- Group-1: rabbits received Lipiodol-emulsion and were sacrificed 1 hour after the injection.
- Group-2: rabbits received Pickering-emulsion and were sacrificed 1 hour after the injection.
- Group-3: rabbits received Lipiodol-emulsion and were sacrificed 24 hours after the injection.
- Group-4: rabbits received Pickering-emulsion and were sacrificed 24 hours after the injection.

Immediately after sacrifice, post-TACE MR scans (3T Magnetom Skyra; Siemens, Erlangen, Germany) using a 15 channels knee coil, were performed for each rabbit to measure and enumerate the liver tumors (Figure-2). Next, livers were resected. Tissues samples from the right- and left-liver lobes, as well as all the tumors were dissected separately and homogenized with a blender for oxaliplatin quantification. e tissues was used to evaluate the systemic exposure of the chemotherapy after TACE.

#### ***Oxaliplatin quantification:***

Quantification of oxaliplatin was accomplished using mass spectrometry ICP-MS of acid digested samples. Specifically, blood and tissue samples were digested in concentrated trace nitric acid (> 69%, Thermo Fisher Scientific, Waltham, MA, USA) and placed at 60 °C for 24 hours to allow for complete sample digestion. Ultra pure H<sub>2</sub>O (18.2 MΩ-cm) was then added to produce a final solution of 3.0% nitric acid (v/v) and 4.0% hydrochloric acid (v/v) in a total sample volume of 5 mL. Platinum quantification served as our assay for oxaliplatin drug concentration (1 mg of oxaliplatin corresponds to 0.49 mg of

platinum). Quantitative standards were made using individual 1000 µg/mL Pt elemental standards (Inorganic Ventures, Christiansburg, VA, USA) which were combined to create a 100 ng/mL mixed element standard in 2.0% nitric acid (v/v) and 4.0% hydrochloric acid (v/v) in a total sample volume of 50 mL. ICP-MS was performed on a computer-controlled (QTEGRA software) Thermo iCapQ ICP-MS (Thermo Fisher Scientific, Waltham, MA, USA) operating in STD mode and equipped with a ESI SC-2DX PrepFAST autosampler (Omaha, NE, USA). Internal standard was added inline using the prepFAST system. Online dilution was also carried out with the prepFAST system to generate calibration curves consisting of 100, 50, 20, 10, 5, 2, 1 ng/mL Pt. Each sample was acquired using 1 survey run (10 sweeps) and 3 main (peak jumping) runs (40 sweeps).

### ***Statistical Analysis***

Data were presented as the mean value  $\pm$  SD. Mann-Whitney test was used assess differences between means (t-test).  $P < 0.05$  was considered statistically significant.

### **Results**

A total of 18 rabbits ( $3.5 \pm 0.3$  kG) bearing a total of 33 VX2 liver tumors received selective left hepatic artery injection of 0.5 mL emulsion. No additional embolization with gelfoam was performed since forward flow was already completely stopped by the emulsion. Nine rabbits were sacrificed at 1 hour and 9 rabbits were sacrificed at 24 hours. Four rabbits bearing 6 tumors were included in Group-1, 5 rabbits bearing 9 tumors in Group-2, 4 rabbits bearing 9 tumors in Group-3 and 5 rabbits bearing 9 tumors in Group-4. The maximal tumor diameter was  $15.0 \pm 6.4$  mm without any statistically significant difference between the 4 groups.

### ***Plasmatic pharmacokinetics of oxaliplatin***

Plasmatic pharmacokinetics of oxaliplatin after injection of the two emulsions are shown in Table-11 and Figure-24. We observed a significantly lower peak oxaliplatin concentration in the blood (Cmax) after injection of Pickering-emulsion ( $0.49 \pm 0.14$  ng/mL) compared to Lipiodol-emulsion ( $1.08 \pm 0.41$  ng/mL,  $p < 0.01$ ). The AUC at 1 hour was significantly lower for the Pickering-emulsion ( $19.8 \pm 5.9$  vs.  $31.8 \pm 14.9$ ,  $p = 0.03$ ).

*Table 11: Mean oxaliplatin concentration (ng/mg) in blood and liver tissues according to the type of emulsion injected and according to the delay before sacrifice. \* significant difference.*

	Emulsions:		
	<u>Conventional</u>	<u>Pickering</u>	
• <b>Pharmacokinetic</b>	<i>n= 8 rabbits</i>	<i>n= 10 rabbits</i>	
C max (ng/mL)	$1.08 \pm 0.41$	$0.49 \pm 0.14$	$p < 0.01^*$
AUC	$31.8 \pm 14.9$	$19.8 \pm 5.9$	$p = 0.03^*$
• <b>Sacrifice at 1 hour</b>	<i>n= 4 rabbits (group-1)</i>	<i>n= 5 rabbits (group-2)</i>	
Oxaliplatin - Left-liver lobe	$2.3 \pm 1.4$	$0.6 \pm 0.2$	$p < 0.01^*$
- Right-liver lobe	$1.3 \pm 0.9$	$0.5 \pm 0.2$	$p < 0.01^*$
- ratio left-/ right-liver lobe	$1.8 \pm 0.7$	$1.1 \pm 0.2$	$p = 0.07$
• <b>Sacrifice at 24 hours</b>	<i>n= 4 rabbits (group-3)</i>	<i>n= 5 rabbits (group-5)</i>	
Oxaliplatin - Left-liver lobe	$0.4 \pm 0.1$	$0.3 \pm 0.1$	$p = 0.04^*$
- Right-liver lobe	$0.2 \pm 0.4$	$0.2 \pm 0.03$	$p = 0.5$
- ratio left-/ right-liver lobes	$1.7 \pm 0.4$	$1.4 \pm 1.0$	$p = 0.2$

## Pharmacokinetics

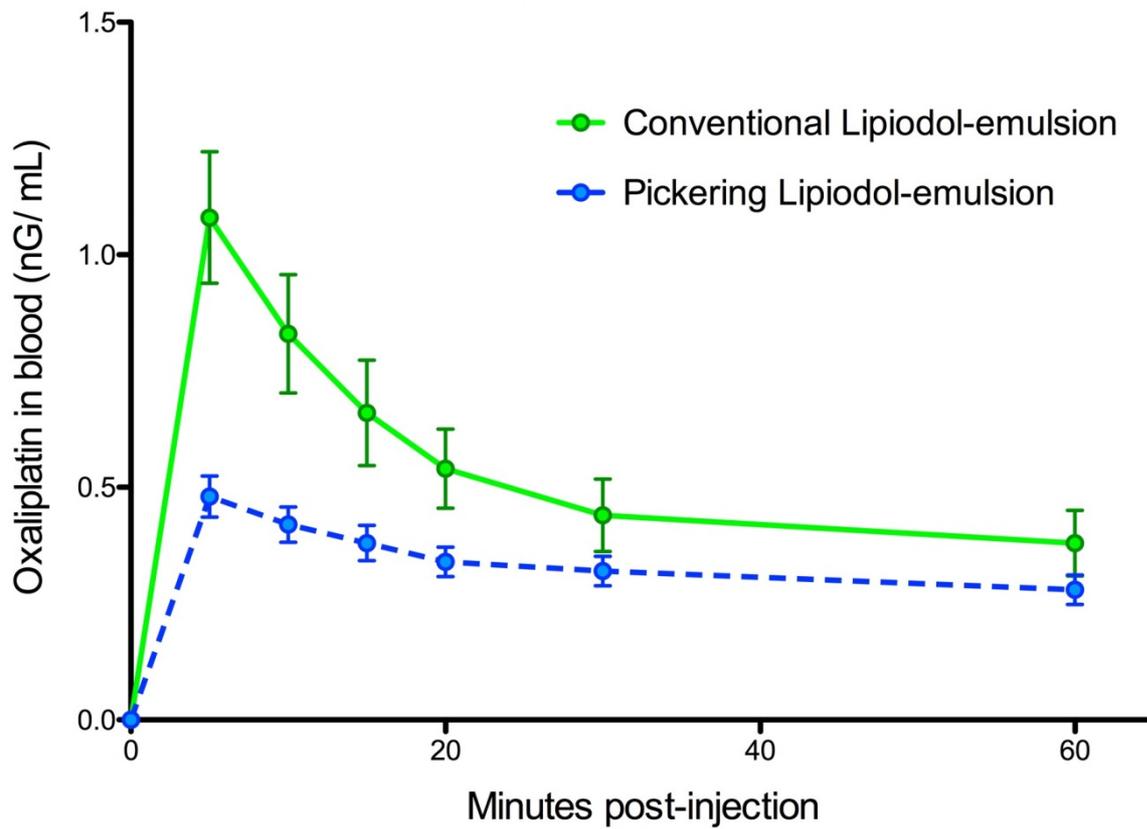


Figure 24: Oxaliplatin plasmatic pharmacokinetics after injection of the emulsions in the left hepatic arteries.

### *Oxaliplatin concentration in liver and tumor tissues*

Oxaliplatin concentrations in liver and tumor tissues are respectively presented in Table-11 and -12.

Table 12: Mean oxaliplatin concentration (ng/mg) in liver tumors according to the type of emulsion injected and according to the delay before sacrifice. \* significant difference.

	Emulsions:		
	<b><u>Conventional</u></b>	<b><u>Pickering</u></b>	
• <b><u>Sacrifice at 1 hour</u></b>	<i>n= 6 tumors (group-1)</i>	<i>n= 9 tumors (group-2)</i>	
Maximal tumor diameter (mm)	14.1 ± 11.1	16.0 ± 5.9	p= 0.6
Oxaliplatin :- Tumors	32.1 ± 21.2	17.6 ± 6.7	p= 0.02*
- ratio tumor/ left-liver lobe	18.0 ± 10.8	33.2 ± 14.9	p= 0.07
- ratio tumor/ right-liver lobe	29.1 ± 11.4	40.3 ± 19.3	p= 0.3
• <b><u>Sacrifice at 24 hours</u></b>	<i>n= 9 tumors (group-3)</i>	<i>n= 9 tumors (group-4)</i>	
Maximal tumor diameter (mm)	15.0 ± 2.9	14.5 ± 5.0	p= 0.4
Oxaliplatin: - Tumors	4.4 ± 3.8	10.4 ± 9.8	p= 0.08
- ratio tumor/ left-liver lobe	11.8 ± 8.5	44.1 ± 43.3	p= 0.02*
- ratio tumor/ right-liver lobe	18.6 ± 17.5	45.6 ± 38.5	p= 0.06

Oxaliplatin concentrations in tissues (tumors, right- and left-liver lobes) are respectively presented in Table-11 and in Table-12. At 1 hour, we observed lower concentration of oxaliplatin in livers and tumors tissues with Pickering-emulsion compared to Lipiodol-emulsion: right-liver lobes (0.5 +/- 0.2 vs. 1.3 +/- 0.9, p< 0.01), left-liver lobes (0.6 ± 0.2 vs 2.3 ± 1.4 ng/mg, p< 0.01) and tumors (17.6 ± 6.7 vs. 32.1 ± 21.2 ng/mg, p=0.02). The concentration of oxaliplatin in tumors significantly decreased from 1 hour to 24 hours for both, Pickering-emulsion (17.6 ± 6.7 vs. 10.4 ± 9.8 ng/mg, p=0.01) and Lipiodol-emulsion (32.1 ± 21.2 vs. 4.4 ± 4.8 ng/mg, p=0.001). The concentration of oxaliplatin in left-liver lobes significantly decreased from 1 hour to 24 hours for both, Pickering-emulsion (0.6 ± 0.2 vs. 0.3 ± 0.1 ng/mg, p< 0.01) and Lipiodol-emulsion (2.3 ± 1.4 vs. 0.4 ± 0.1 nn/mg, p=0.001). The oxaliplatin concentrations in tumors at 24 hours were higher with Pickering-emulsion compared to Lipiodol-emulsions but difference was significant (10.4 ± 9.8 vs. 4.4 ± 3.8, p=0.08).

At 24h, The oxaliplatin concentration ratio between tumor and the left-liver lobes was significantly higher with Pickering-emulsion compared to Lipiodol-emulsion ( $43.4 \pm 42.9$  vs.  $14.5 \pm 6.6$ ,  $p=0.04$ ) and there is a trend of significance for higher ratio between the oxaliplatin concentrations in tumors and right-liver lobes at 24 hours in favor of Pickering-emulsion compared to Lipiodol-emulsion ( $45.6 \pm 38.5$  vs.  $18.6 \pm 17.5$  ng/mg,  $p=0.06$ ).

## Discussion

The term “water-in-oil emulsion” refers to fine droplet dispersions of an aqueous-phase in a continuous oily-phase. The high interfacial energy of the dispersed droplets in the oily-phase contributes to thermodynamic instability and results in a coalescence process when droplets bump into each other and combine to form a larger droplet, so the average droplet size increases over time. However, a stable emulsion is critical for TACE, allowing slow release of the chemotherapeutic agent into the liver tumor feeders for prolonged exposure, while minimizing systemic exposure<sup>41,35,40</sup>. Stable emulsions can be obtained using substances known as emulsifiers that are able to decrease the interfacial tension between the two phases. Emulsifiers are commonly included in emulsion formulations. Synthetic surfactants have been used to stabilize Lipiodol-emulsions<sup>41</sup>. However, these surfactants can raise toxicity issues, either directly or indirectly. Alternatively, the Pickering-emulsion uses solid particles as emulsifiers. The adsorption of the solid particles at the water/oil interface leads to the formation of a protective film that resists droplet coalescence and phase separation. The low toxicity and high stability of Pickering-emulsions lend unique properties when compared against classical emulsions stabilized by synthetic surfactants and make them very attractive for biomedical applications such as TACE.

The herein report in vivo experiment demonstrates a low plasmatic peak and of the area under the curve of the oxaliplatin in accordance with our previous in vitro report.

Indeed, we have recently demonstrated in vitro that Pickering-emulsion with fully biodegradable and biocompatible solid PLGA-Nps results in a release of 15% of the oxaliplatin at 24 hours while at the same time point all the oxaliplatin was released from a classical Lipiodol-emulsion. The significantly higher concentrations of oxaliplatin in tumors and left-liver lobes at 1 hour are consistent with the “bolus” release of oxaliplatin from the Lipiodol-emulsion, previously demonstrated in vitro. Pickering-emulsion resulted in significantly lower systemic exposure and concentration in contralateral liver at 1 hour with a trend for higher tumor exposure at 24 hours. Pickering-emulsion demonstrated a higher ratio between tumors and the surrounding left-liver lobes at 24 hours, which is consistent with a stable water-in-oil emulsion and the theranostic property of Lipiodol demonstrated in one of our previous in vivo study. During TACE, these pharmacological advantages provide interesting opportunities, either to decrease the systemic toxicities for similar amount of chemotherapy or to increase the amount of chemotherapy during without additional systemic side effects.

Oxaliplatin is a valid option for chemotherapy in HCC and colorectal cancer. For HCC, a pooled analysis<sup>57</sup> of 13 studies with a total of 800 patients demonstrated a response rate was 16.8%. The median PFS and overall survival were 4.2 and 9.3 months, respectively, with a 1 year overall survival of 37%. In colorectal cancer, oxaliplatin is a pillar of many systemic regimens in combination with 5-FU. Moreover the drug has been used through Hepatic Arterial Infusion (HAI) of oxaliplatin and yielded overall response rate of 64% in second or more advanced line of IV chemotherapy<sup>58</sup> and 62% in patients who have progressed on IV oxaliplatin<sup>56</sup>. In rabbit VX2 liver tumor models<sup>61</sup>, HAI oxaliplatin has demonstrated a tumor to healthy liver ratio of 4.3 at 24h. Our study reports a ratio of 44.1 for Pickering-emulsions and 11.8 for Lipiodol-emulsions, clearly suggesting the potential benefit of using a drug delivery platform for HAI of oxaliplatin in order to increase concentration of drug in the tumor.

## Limitations

We acknowledge some limitations to current study. The first one was that we considered conventional Lipiodol-emulsion as a gold standard for evaluating the release of oxaliplatin from Pickering-emulsions. However, there is no other drug delivery system that we could have used for comparison since oxaliplatin cannot be loaded into clinically-approved drug eluting microspheres. A high ratio of Lipiodol/oxaliplatin (3/1) and a continuous and slow incorporation of oxaliplatin in the Lipiodol were considered in order to obtain the best stability of the Lipiodol-emulsion<sup>29,60</sup>. Another limitation was that no embolic agent was injected during the TACE procedures (as would commonly be performed for clinical TACE studies). However, hepatic artery antegrade flow was completely stopped at the conclusion of each emulsion injection thus additional embolization would have sub-optimally resulted in retrograde-flow within extra-hepatic arteries in our rabbit model. Additional embolizations would also introduce biases during the evaluation of oxaliplatin release from the emulsions.

## Pickering-emulsion for liver trans-arterial immunotherapy.

### Introduction

Immunotherapy is now changing the paradigm in the treatment of cancer. The most common strategy is to use antibodies to target checkpoint inhibitors against cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), programmed death 1 (PD-1) or its ligand, programmed death ligand 1 (PDL-1). Many clinical trials with systemic administration of checkpoint inhibitors are ongoing to treat HCCC with promising early results (Table below). CTLA-4 blockade has been evaluated using Tremelimumab <sup>62</sup>. In this study, the overall response rate was 17.6% and the disease control rate was 76.4%. The median time to tumor progression was 6.48 months. Toxicity was mainly hepatic. Many clinical trials are currently underway (table below) with the aim of reversing tumor immunotolerance and stimulating anti-tumor response. PD-1 blockade has also been evaluated and produced a response in 20% of patients with advanced hepatocellular carcinoma. The median duration of response was 9.9 months, with 67% of responses ongoing at data cutoff <sup>63</sup>.

<b>Immuno checkpoints inhibitors and HCC</b>	<b>On-going studies:</b>
Nivolumab (anti PD1)	NCT02576509
Nivolumab (anti PD-1) + Ipilimumab (anti CTLA-4)	NCT01658878
Tremelilumab (anti CTLA-4) + Loco regional treatments	NCT01853618
Tremelilumab (anti CTLA-4) + Durvalumab (anti PDL1)	NCT02519348
Tremelilumab (anti CTLA-4) + Durvalumab (anti PDL1) + Loco regional treatments	NCT02821754
Nivolumab (anti PD1 Ab) plus LRT (Yttrium 90Y glass microspheres)	NCT02837029

“In-situ immunization” is a new therapeutic strategy where immunostimulatory products are injected directly into one tumor site in order to prime the anti-tumor immune

response locally and generate a systemic (“abscopal”) anti-tumor immunity<sup>64</sup>. Interestingly, low doses of monoclonal antibodies are sufficient to generate a systemic anti-tumor immune response able to eradicate distant, not injected, tumor sites<sup>65, 66</sup>. In addition, local delivery of monoclonal antibodies is supported by the high rate of autoimmune and inflammatory toxicities observed after systemic administration. Thus, many “in-situ immunization” clinical trials are currently ongoing. However, the local delivery of monoclonal antibodies by percutaneous intra-tumoral injection has two major limitations. The first one is that, in clinical practice, only superficial tumors can be technically targeted to prime the systemic anti-tumor immune response. The second one is that the distribution of the monoclonal antibodies will be inappropriate, mostly within the low-pressure necrosis areas within the tumor. To solve these two limitations, intra-arterial injection of monoclonal antibodies could be an interesting alternative for local delivery. Thanks to interventional radiology techniques deep organs, such as the liver, can be targeted. In addition, contrary to a percutaneous approach, a selective intra-arterial injection within the arterial tumor supply will target, not only the viable part of the tumor, but also its microenvironment. Basically, the concept of a complex tumor ecosystem has emerged recently and supports that the tumor microenvironment must be targeted for initiating host anti-tumor immune responses<sup>67</sup>. To prevent rapid washout of monoclonal antibodies from the tumor after their arterial administration, we believe that monoclonal antibodies must be “loaded” in a dedicated drug delivery platform that allows its slow release.

The purpose of this study was to evaluate the feasibility of water-in-oil emulsion containing monoclonal antibodies by using first a Lipiodol-emulsion (because it is currently used in clinical practice) and second the Pickering-emulsion. Anti-CTLA-4 was the selected monoclonal antibody for this study because there is a strong rationale for its local administration. First, clinical studies have established a dose-efficacy relationship with better responses and higher survival rates at 10mg/kg than at 3mg/kg and second, higher dose level was also associated with a higher rate of immune-related adverse events<sup>68</sup>.

The release profiles of anti-CTLA4 from Pickering-emulsion and Lipiodol-emulsion will also be analyzed in an in vitro setting.

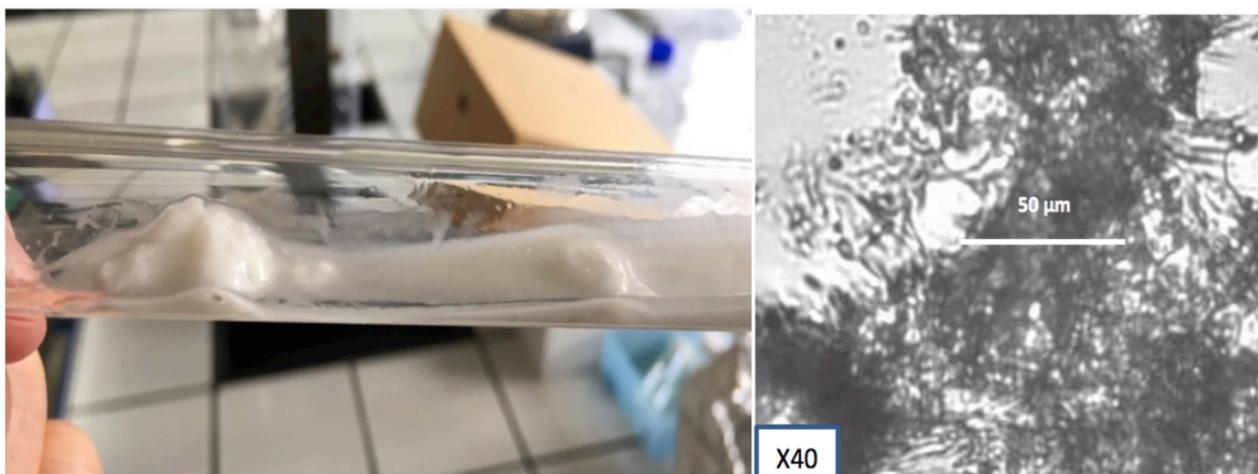
### **Materials and Methods:**

Two different types of water-in-oil emulsions were formulated: Lipiodol-emulsion and Pickering-emulsion. For both emulsions, the oily-phase was composed of Lipiodol and the aqueous-phase was composed of an anti-CTLA4 (Ipilimumab, 5 mg/mL). PLGA-Nps (15 mg/mL) addition to the aqueous-phase during formulation of the Pickering-emulsion was the only difference between the 2 emulsions. Emulsions were formulated by continuous injection of 1 volume of aqueous-phase in 3 volumes of oily-phase with an electric syringe-pump (flow-rate of 1 mL per minute) during repetitive pumping of two 10-mL syringes through a three-way stopcock<sup>10</sup>.

First the emulsions were microscopically analyzed to confirm the direction of the emulsion (water-in-oil or oil-in-water) and to evaluate the size of the internal phase aqueous droplets. Next, stability of the emulsion was evaluated twice for each emulsion. Finally, the release of the Ipilimumab from the emulsion was quantified by biochemical BCA assay (Bicinchoninic Acid).

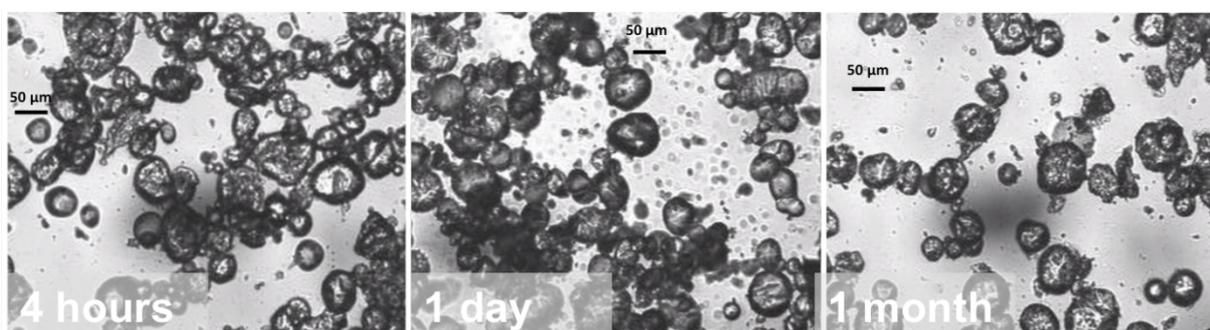
### **Results:**

Lipiodol-emulsions demonstrated an immediate coalescence with a flocculation process (Figure 25).



*Figure 25: immediate coalescence (flocculation) after formulation of Lipiodol-emulsion with Ipilimumab.*

Conversely, all Pickering-emulsions were water-in-oil with droplets' size around 50 micrometers. Pickering-emulsions demonstrated no coalescence process for at least 1 month (Figure 26).



*Figure 26: Microscopic analysis of Pickering-emulsion of Ipilimumab 4 hours, 1 day and 1 month after formulation*

Because of immediate coalescence, the release of Ipilimumab from Lipiodol-emulsion was not evaluated. The release profile of Ipilimumab from Pickering-emulsion is shown on Figure 27. The percentage of Ipilimumab released from Pickering-emulsions were respectively:  $4.9 \pm 4.1\%$  at 1 day,  $15.6 \pm 6.3\%$  at 3 days,  $28.8 \pm 36.6\%$  at 6 days,  $61.2 \pm 3.7\%$  at 16 days and  $70.7 \pm 1.0\%$  at 21 days.

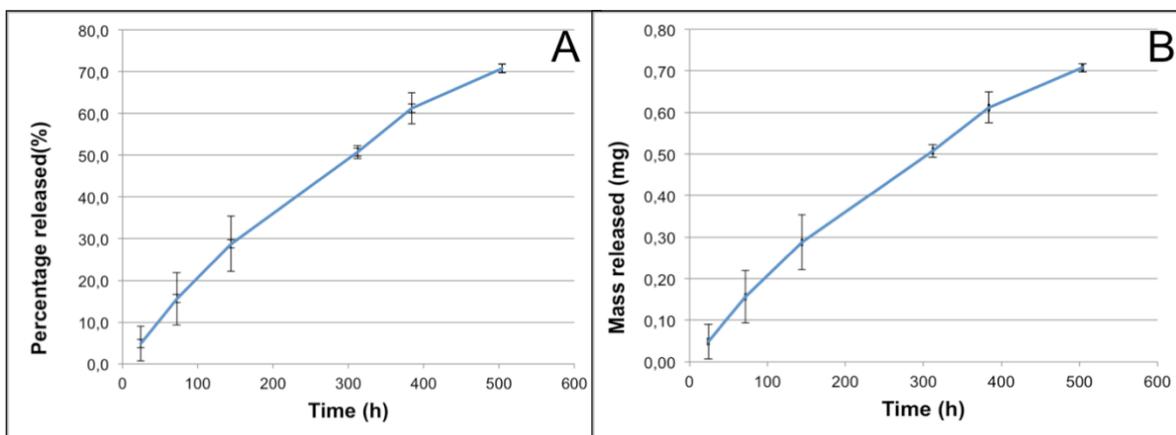


Figure 27: *In vitro* release of 1 mg Ipilimumab from Pickering-emulsion (ratio 3/1): percentage released (A) and mass released (B).

### Discussion:

The concept of a slow release of anti-CTLA4 delivered through a water-in-oil emulsion using an adjuvant (Montanid ISA51) has already been evaluated by Fransen et al<sup>65</sup>. By percutaneous injection of this slow release emulsion, they demonstrated that they were able to eradicate the local tumor and prevent the development of tumors at a distant non-injected site. In our study Pickering-emulsion allows slow release of anti-CTLA4 *in vitro*. We believe that the PLGA-Nps localized at the water/oil interface protect the proteins from the degradation induced by Lipiodol. Thus, Pickering-emulsion looks like an interesting drug-delivery platform for delivery of anti-CTLA4 into the tumor and the tumor microenvironment, in a slow and prolonged fashion. Intra-arterial route was our target option but direct puncture delivery could n-be another route of delivery. Further studies must be done to assess the antibodies activity after their release from the Pickering-emulsion. *In vivo* studies are also warranted to evaluate the benefice of slow and prolong delivery of anti-CTLA4 in liver tumors using Pickering-emulsion.

## Conclusions:

First, this work confirmed that stabilization of chemotherapy in a water-in-oil emulsion results in better theranostic properties: better Lipiodol tumor uptake, lower systemic exposure and higher concentration of chemotherapy in liver tumor model. Then, we analyzed technical parameters that formulate in vitro w/o emulsions with better stability without any additional emulsifier and found that progressive incorporation of chemotherapy in the Lipiodol results in much more predictable w/o type and higher stability and that higher ratio of Lipiodol/chemotherapy is also a critical parameter for stability. Finally, we develop a new concept of emulsion for TACE that uses PLGA-Nps to stabilize Lipiodol and chemotherapy (Pickering-emulsion). In vitro analysis demonstrated that Pickering-emulsion is the only drug delivery system (versus Lipiodol-emulsion and drug eluding beads) that allows slow and complete release of various chemotherapies (doxorubicin, irinotecan, oxaliplatin) but also of antibodies (anti-CTLA4), making Pickering-emulsion an universal carrier, not only for TACE but also for trans-arterial immunotherapy.

There are several perspectives of this work. The first one is to demonstrate the biocompatibility of Pickering-emulsion and then to use it for TACE with oxaliplatin in a phase 1 study for patients suffering from liver tumors. Another perspective is to use Pickering-emulsion for local delivery of immunotherapy. For this purpose, we need to confirm that antibodies released from the emulsion are still active and that a local slow release of antibodies is more effective than a systemic administration.

## Publications/ Communications related to this work

- Articles:
  - Deschamps F, Moine L, Isoardo T, Tselikas L, Paci A, Mir LM, Huang N, Fattal E, de Baère T. Parameters for Stable Water-in-Oil Lipiodol Emulsion for Liver Trans-Arterial Chemo-Embolization. *CardiovascIntervent Radiol*. 2017 Dec;40(12):1927-1932.
  - Deschamps F, Farouil G, Gonzalez W, Robic C, Paci A, Mir LM, Tselikas L, de Baère T. Stabilization Improves Theranostic Properties of Lipiodol®-Based Emulsion During Liver Trans-arterial Chemo-embolization in a VX2 Rabbit Model. *Cardiovasc Intervent Radiol*. 2017 Jun;40(6):907-913. doi: 10.1007/s00270-017-1616-2.
  - Pickering-emulsion for liver Trans-Arterial Chemo-Embolization with oxaliplatin. Deschamps F, Harris KR, Li W, Moine L, Tselikas L, Isoardo T, Lewandowski RJ, Paci A, de Baere T, Salem R and Larson AC (SubmLitted to Radiology on Novembre, 27<sup>th</sup>)
  
- Electronic Posters:
  - Deschamps F, Moine L, Isoardo T, Tselikas L, Paci A, Mir LM, Huang N, Fattal E, de Baère T. Parameters for Stable Water-in-Oil Lipiodol Emulsion for Liver Trans-Arterial Chemo-Embolization. CIRSE 2017
  - Deschamps F, Farouil G, Gonzalez W, Robic C, Paci A, Mir LM, Tselikas L, de Baère T. Stabilization Improves Theranostic Properties of Lipiodol®-Based Emulsion During Liver Trans-arterial Chemo-embolization in a VX2 Rabbit Model. CIRSE 2015 **(Cum Laode)**
  
- Brevet déposé en juin 2017 (Pickering pour injection thérapeutique d'émulsion).

## Bibliography

1. Pons F, Varela M, Llovet JM. Staging systems in hepatocellular carcinoma. *HPB (Oxford)* 2005; 7: 35-41.
2. Llovet JM, Ricci S, Mazzaferro V et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; 359: 378-390.
3. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; 56: 908-943.
4. Yamada R, Sato M, Kawabata M et al. Hepatic artery embolization in 120 patients with unresectable hepatoma. *Radiology* 1983; 148: 397-401.
5. Breedis C, Young G. The blood supply of neoplasms in the liver. *Am J Pathol* 1954; 30: 969-977.
6. Llovet JM, Real MI, Montana X et al. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 2002; 359: 1734-1739.
7. Bruix J, Llovet JM, Castells A et al. Transarterial embolization versus symptomatic treatment in patients with advanced hepatocellular carcinoma: results of a randomized, controlled trial in a single institution. *Hepatology* 1998; 27: 1578-1583.
8. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. *Hepatology* 2003; 37: 429-442.
9. Marelli L, Stigliano R, Triantos C et al. Transarterial therapy for hepatocellular carcinoma: which technique is more effective? A systematic review of cohort and randomized studies. *Cardiovasc Intervent Radiol* 2007; 30: 6-25.
10. Majno PE, Adam R, Bismuth H et al. Influence of preoperative transarterial lipiodol chemoembolization on resection and transplantation for hepatocellular carcinoma in patients with cirrhosis. *Ann Surg* 1997; 226: 688-701; discussion 701-683.
11. Chapman WC, Majella Doyle MB, Stuart JE et al. Outcomes of neoadjuvant transarterial chemoembolization to downstage hepatocellular carcinoma before liver transplantation. *Ann Surg* 2008; 248: 617-625.
12. Yao FY, Kerlan RK, Jr., Hirose R et al. Excellent outcome following downstaging of hepatocellular carcinoma prior to liver transplantation: an intention-to-treat analysis. *Hepatology* 2008; 48: 819-827.
13. Kan Z. Dynamic study of iodized oil in the liver and blood supply to hepatic tumors. An experimental investigation in several animal species. *Acta Radiol Suppl* 1996; 408: 1-25.
14. Terayama N, Matsui O, Gabata T et al. Accumulation of iodized oil within the nonneoplastic liver adjacent to hepatocellular carcinoma via the drainage routes of the tumor after transcatheter arterial embolization. *Cardiovasc Intervent Radiol* 2001; 24: 383-387.
15. de Baere T, Denys A, Briquet R et al. Modification of arterial and portal hemodynamics after injection of iodized oils and different emulsions of iodized oils in the hepatic artery: an experimental study. *J Vasc Interv Radiol* 1998; 9: 305-310.
16. Takayasu K, Arii S, Matsuo N et al. Comparison of CT findings with resected specimens after chemoembolization with iodized oil for hepatocellular carcinoma. *AJR Am J Roentgenol* 2000; 175: 699-704.
17. El Khaddari S, Gaudin JL, Abidi H et al. [Chemoembolization in hepatocellular carcinoma: multivariate analysis of survival prognostic factors after the first session]. *Gastroenterol Clin Biol* 2002; 26: 728-734.

18. Kim DY, Ryu HJ, Choi JY et al. Radiological response predicts survival following transarterial chemoembolisation in patients with unresectable hepatocellular carcinoma. *Aliment Pharmacol Ther* 2012; 35: 1343-1350.
19. Gaba RC, Baumgarten S, Omene BO et al. Ethiodized oil uptake does not predict doxorubicin drug delivery after chemoembolization in VX2 liver tumors. *J Vasc Interv Radiol* 2012; 23: 265-273.
20. Masada T, Tanaka T, Nishiofuku H et al. Techniques to Form a Suitable Lipiodol-Epirubicin Emulsion by Using 3-Way Stopcock Methods in Transarterial Chemoembolization for Liver Tumor. *J Vasc Interv Radiol* 2017.
21. Hong K, Khwaja A, Liapi E et al. New intra-arterial drug delivery system for the treatment of liver cancer: preclinical assessment in a rabbit model of liver cancer. *Clin Cancer Res* 2006; 12: 2563-2567.
22. Kettenbach J, Stadler A, Katzler IV et al. Drug-loaded microspheres for the treatment of liver cancer: review of current results. *Cardiovasc Intervent Radiol* 2008; 31: 468-476.
23. Jordan O, Denys A, De Baere T et al. Comparative study of chemoembolization loadable beads: in vitro drug release and physical properties of DC bead and hepasphere loaded with doxorubicin and irinotecan. *J Vasc Interv Radiol* 2010; 21: 1084-1090.
24. Becker S, Lepareur N, Cadeillan V et al. Optimization of hepatocarcinoma uptake with radiolabeled lipiodol: development of new lipiodol formulations with increased viscosity. *Cancer Biother Radiopharm* 2012; 27: 149-155.
25. de Baere T, Dufaux J, Roche A et al. Circulatory alterations induced by intra-arterial injection of iodized oil and emulsions of iodized oil and doxorubicin: experimental study. *Radiology* 1995; 194: 165-170.
26. de Baere T, Zhang X, Aubert B et al. Quantification of tumor uptake of iodized oils and emulsions of iodized oils: experimental study. *Radiology* 1996; 201: 731-735.
27. Demachi H, Matsui O, Abo H, Tatsu H. Simulation model based on non-newtonian fluid mechanics applied to the evaluation of the embolic effect of emulsions of iodized oil and anticancer drug. *Cardiovasc Intervent Radiol* 2000; 23: 285-290.
28. Baumgarten S, Gaba RC, van Breemen RB. Confirmation of drug delivery after liver chemoembolization: direct tissue doxorubicin measurement by UHPLC-MS-MS. *Biomed Chromatogr* 2012; 26: 1529-1533.
29. de Baere T, Arai Y, Lencioni R et al. Treatment of Liver Tumors with Lipiodol TACE: Technical Recommendations from Experts Opinion. *Cardiovasc Intervent Radiol* 2015.
30. Lencioni R, de Baere T, Soulen MC et al. Lipiodol transarterial chemoembolization for hepatocellular carcinoma: A systematic review of efficacy and safety data. *Hepatology* 2016; 64: 106-116.
31. Miyayama S, Matsui O, Yamashiro M et al. Ultraselective transcatheter arterial chemoembolization with a 2-f tip microcatheter for small hepatocellular carcinomas: relationship between local tumor recurrence and visualization of the portal vein with iodized oil. *J Vasc Interv Radiol* 2007; 18: 365-376.
32. Bouvier A, Ozenne V, Aube C et al. Transarterial chemoembolisation: effect of selectivity on tolerance, tumour response and survival. *Eur Radiol* 2011; 21: 1719-1726.
33. Lee IJ, Chung JW, Yin YH et al. Cone-Beam Computed Tomography (CBCT) Hepatic Arteriography in Chemoembolization for Hepatocellular Carcinoma: Performance Depicting Tumors and Tumor Feeders. *Cardiovasc Intervent Radiol* 2015; 38: 1218-1230.
34. Ronot M, Abdel-Rehim M, Hakime A et al. Cone-Beam CT Angiography for Determination of Tumor-Feeding Vessels During Chemoembolization of Liver

Tumors: Comparison of Conventional and Dedicated-Software Analysis. *J Vasc Interv Radiol* 2015.

35. Choi JW, Cho HJ, Park JH et al. Comparison of drug release and pharmacokinetics after transarterial chemoembolization using diverse lipiodol emulsions and drug-eluting beads. *PLoS One* 2014; 9: e115898.

36. Mondazzi L, Bottelli R, Brambilla G et al. Transarterial oily chemoembolization for the treatment of hepatocellular carcinoma: a multivariate analysis of prognostic factors. *Hepatology* 1994; 19: 1115-1123.

37. Vogl TJ, Trapp M, Schroeder H et al. Transarterial chemoembolization for hepatocellular carcinoma: volumetric and morphologic CT criteria for assessment of prognosis and therapeutic success-results from a liver transplantation center. *Radiology* 2000; 214: 349-357.

38. Wang Z, Chen R, Duran R et al. Intraprocedural 3D Quantification of Lipiodol Deposition on Cone-Beam CT Predicts Tumor Response After Transarterial Chemoembolization in Patients with Hepatocellular Carcinoma. *Cardiovasc Intervent Radiol* 2015; 38: 1548-1556.

39. Yin X, Guo Y, Li W et al. Chemical shift MR imaging methods for the quantification of transcatheter lipiodol delivery to the liver: preclinical feasibility studies in a rodent model. *Radiology* 2012; 263: 714-722.

40. Boulin M, Schmitt A, Delhom E et al. Improved stability of lipiodol-drug emulsion for transarterial chemoembolisation of hepatocellular carcinoma results in improved pharmacokinetic profile: Proof of concept using idarubicin. *Eur Radiol* 2016; 26: 601-609.

41. Deschamps F, Farouil G, Gonzalez W et al. Stabilization Improves Theranostic Properties of Lipiodol(R)-Based Emulsion During Liver Trans-arterial Chemo-embolization in a VX2 Rabbit Model. *Cardiovasc Intervent Radiol* 2017; 40: 907-913.

42. Jahanzad F JD, Mansourian A and Sajjadi S. Dynamics of Transitional Phase Inversion Emulsification: Effect of Addition Time on the Type of Inversion and Drop Size. *Ind. Eng. Chem. Res.* 2019; 49: 7631-7637.

43. Idee JM, Guiu B. Use of Lipiodol as a drug-delivery system for transcatheter arterial chemoembolization of hepatocellular carcinoma: a review. *Crit Rev Oncol Hematol* 2013; 88: 530-549.

44. Ramsden. Separation of Solids in the Surface-layers of Solutions and 'Suspension' (Observations on surface-membranes, Bubbles, Emulsions, and Mechanical Coagulation). Preliminary Account. *Proc. R. Soc. Lond.* 1903; 72: 156-164.

45. Pickering EC. Emulsion. *J Chem Soc* 1907; 91.

46. Chevalier Y BM-A. Emulsions stabilized with solid nanoparticles: Pickering emulsions. *Coll. Surf. A* 2013; 439: 23-34.

47. Whitby CP, Lim LH, Eskandar NG et al. Poly(lactic-co-glycolic acid) as a particulate emulsifier. *J Colloid Interface Sci* 2012; 375: 142-147.

48. Guzman LA LV, Song C, Jang Y, Lincoff AM, Levy R, Topol EJ. Local intraluminal infusion of biodegradable polymeric nanoparticles. A novel approach for prolonged drug delivery after balloon angioplasty. *Circulation* 1996; 94: 1441-1448.

49. Astete CE SC. Synthesis and characterization of PLGA nanoparticles. *J Biomater Sci Polym Ed.* 2006; 17: 247-289.

50. Bala I HS, Kumar MN. PLGA nanoparticles in drug delivery: the state of the art. *Crit Rev Ther Drug Carrier Syst.* 2004; 21: 387-422.

51. Binks BP, Fletcher PD, Holt BL et al. Phase inversion of particle-stabilised perfume oil-water emulsions: experiment and theory. *Phys Chem Chem Phys* 2010; 12: 11954-11966.

52. Okayasu I, Hatakeyama S, Yoshida T et al. Selective and persistent deposition and gradual drainage of iodized oil, Lipiodol in the hepatocellular carcinoma after injection into the feeding hepatic artery. *Am J Clin Pathol* 1988; 90: 536-544.
53. Laredj-Bourezg F CY, Boyron O, Bolzinger M-A. . Emulsions stabilized with organic solid particles. *Coll. Surf. A* 2012; 413: 252-259.
54. Laredj-Bourezg F BM-A, Pelletier J, Rovere M-R, Smatti B, Chevalier Y. . Pickering Emulsions Stabilised by Biodegradable Particles Offer a Double Level of Controlled Delivery of Hydrophobic Drugs. . *Adv Dermatological Sci* 2014; 20: 143-156.
55. Lilienberg E, Dubbelboer IR, Karalli A et al. In Vivo Drug Delivery Performance of Lipiodol-Based Emulsion or Drug-Eluting Beads in Patients with Hepatocellular Carcinoma. *Mol Pharm* 2017; 14: 448-458.
56. Boige V, Malka D, Elias D et al. Hepatic arterial infusion of oxaliplatin and intravenous LV5FU2 in unresectable liver metastases from colorectal cancer after systemic chemotherapy failure. *Ann Surg Oncol* 2008; 15: 219-226.
57. Petrelli F, Coinu A, Borgonovo K et al. Oxaliplatin-based chemotherapy: a new option in advanced hepatocellular carcinoma. a systematic review and pooled analysis. *Clin Oncol (R Coll Radiol)* 2014; 26: 488-496.
58. Ducreux M, Ychou M, Laplanche A et al. Hepatic arterial oxaliplatin infusion plus intravenous chemotherapy in colorectal cancer with inoperable hepatic metastases: a trial of the gastrointestinal group of the Federation Nationale des Centres de Lutte Contre le Cancer. *J Clin Oncol* 2005; 23: 4881-4887.
59. Kim JH, Yoon HK, Sung KB et al. Transcatheter arterial chemoembolization or chemoinfusion for unresectable intrahepatic cholangiocarcinoma: clinical efficacy and factors influencing outcomes. *Cancer* 2008; 113: 1614-1622.
60. Deschamps F, Moine L, Isoardo T et al. Parameters for Stable Water-in-Oil Lipiodol Emulsion for Liver Trans-Arterial Chemo-Eembolization. *Cardiovasc Intervent Radiol* 2017.
61. Dzodic R, Gomez-Abuin G, Rougier P et al. Pharmacokinetic advantage of intra-arterial hepatic oxaliplatin administration: comparative results with cisplatin using a rabbit VX2 tumor model. *Anticancer Drugs* 2004; 15: 647-650.
62. Sangro B, Gomez-Martin C, de la Mata M et al. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J Hepatol* 2013; 59: 81-88.
63. El-Khoueiry AB, Sangro B, Yau T et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; 389: 2492-2502.
64. Marabelle A, Kohrt H, Caux C, Levy R. Intratumoral immunization: a new paradigm for cancer therapy. *Clin Cancer Res* 2014; 20: 1747-1756.
65. Franssen MF, van der Sluis TC, Ossendorp F et al. Controlled local delivery of CTLA-4 blocking antibody induces CD8+ T-cell-dependent tumor eradication and decreases risk of toxic side effects. *Clin Cancer Res* 2013; 19: 5381-5389.
66. Marabelle A, Kohrt H, Sagiv-Barfi I et al. Depleting tumor-specific Tregs at a single site eradicates disseminated tumors. *J Clin Invest* 2013; 123: 2447-2463.
67. Pitt JM, Marabelle A, Eggermont A et al. Targeting the tumor microenvironment: removing obstruction to anticancer immune responses and immunotherapy. *Ann Oncol* 2016; 27: 1482-1492.
68. Wolchok JD, Neyns B, Linette G et al. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol* 2010; 11: 155-164.