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Flavien Sciortino

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Flavien Sciortino. Nouveau procédé d'élaboration de nanocapsules inorganiques et hybrides (Hybridosomes®) : des films multifonctionnels aux applications biomédicales. Chimie inorganique. Université de Rennes, 2017. Français. NNT : 2017REN1S169 . tel-01884176

HAL Id: tel-01884176

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THÈSE / UNIVERSITÉ DE RENNES 1
sous le sceau de l'Université Bretagne Loire

pour le grade de

DOCTEUR DE L'UNIVERSITÉ DE RENNES 1

Mention : Chimie

Ecole doctorale Matière, Molécules et Matériaux

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Préparée à l'Institut des Sciences Chimiques de Rennes
(UMR CNRS 6226)
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Nouveau procédé d'élaboration de nanocapsules inorganiques et hybrides (Hybridosomes®): des films multifonctionnels aux applications biomédicales

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Remerciements

Je tiens tout d'abord à remercier les membres du jury Stéphane Roux, Jean-Paul Chapel, Sébastien Mériaux et Nicolas Noiret qui me font l'honneur de juger ces trois années de recherche.

Ces travaux de thèse ont été réalisés dans les laboratoires de l'Institut des Sciences Chimiques de Rennes, UMR CNRS 6226, au sein de l'équipe ICMV initiallement ensuite regroupée au sein de léquipe CORINT. Une partie a également été initiée au National Institue for Material Science à Tsukuba au Japon.

Je tiens tout d'abord à exprimer toute ma reconnaissanec à Fabienne et Soizic, mes directrices de thèse, pour leur suivi, leurs conseils, leur disponibilité et leur patiente. Merci à vous, pour votre implication durant ces trois ans. Merci de m'avoir ouvert les portes de la recherche académique et surtout merci pour m'avoir laissé cette liberté dans ma recherche. Notre réunion a permis de faire naître les Hybridosomes au cours ce projet et a permis d'en ouvrir tant d'autres. Quelques un sur le papier, la plupart dans nos esprits et qui vont murir jusqu'à être exaucés un jour peut être. Merci de m'avoir laissé poser tant de questions et d'avoir pu ainsi nourrir ma curiosité scientifique, mais pas seulement. Merci également de m'avoir permis d'étendre mon expérience, de vie et de recherche vers de nouveaux horizons au Japon. Cette expérience tant attendue et très particulière a été plus qu'enrichissante en de très nombreux points. Merci, pour cette expérience de vie scientifique et humaine avant tout, durant ces trois années avec vous. Merci infiniement !

Je remercie les nombreux collaborateurs de ces recherches, avec qui j'ai eu très grand plaisir à travailler et qui ont contribué de façon considérable à ce projet interdisciplinaire.

Je remercie les collègues Toulousains Myrtil Kahn et Gérald Casterou, dont j'ai repris le flambeau, la chaise et le bureau, pour leur expertise en nanochimie et la synthèse des nanoparticules qui alimentent ce projet depuis le début et même bien avant. Merci à vous pour vos conseils précieux dès les premiers instants et encore aujourdhui.

Evidemment je remercie nos collègues biologistes Marie Bérengère Troadec et Hélène Jakobczyk avec qui nous avons eu d'intenses échanges et grace à qui nous avons pu mener avec succès les études *in vitro* et surtout *in vivo* qui ont été des expériences très enrichssantes. Ces expériences n'auraient pas non plus été possibles sans le savoir faire et la disponibilité de Pierre Antoine Elliat et de l'IRM petit animal de la plateforme PRISM. Un grand merci également à Agnès Burel pour ta grande disponibilité et ton expertise, mais aussi pour la formation que tu m'as donné sur le TEM.

Merci de m'avoir permis de m'évader pendant de nombreuses heures dans l'observation des quelques centaines de grilles et permis d'admirer les milliers de photos qui ont fait bondir Fabienne un certain nombre de fois. Merci aussi à Vincent Dorcet pour ta formation aussi sur l'autre TEM, merci pour ta disponibilité pour répondre à mes interrogations et m'accompagner dans les observations.

Merci aux collègues et amis de l'étage qui ont été là au quotidien. Merci pour vos conseils, vos soutiens, pour ces moments de partage et de rires, au labo ou en dehors qui ont fait passé ces trois années à une vitesse incroyable ! Un merci particulier à Solène et Poussin pour m'avoir offert refuge et survie et sans qui je n'aurais très certainement pas fini d'écrire cette thèse aujourd'hui. Je remercie aussi les jeunes qui sont restés quelques semaines, quelques mois dans l'équipe et qui ont su dynamiser le labo et couloir. Je souhaite aussi remercier particulièrement Maxime pour son investissement dans ce projet au cours de son stage dans notre groupe. Merci à toi pour ton implication dans ce travail, les quelques mois où nous avons pu être ensemble au labo ont été riches, décisifs et notre collaboration va continuer à porter ses fruits.

J'adresse un grand merci également à Fabien-san qui, en plus d'avoir éveillé ma curiosité envers les clusters, m'a aussi soutenu dans le projet de mobilité au Japon. Il y a beaucoup trop de mots pour exprimer ce qui résulte de cette expérience, un peu comme les dizaines de sens que l'on pourrait donner à un seul Kanji à 42 traits, malheureusement puisque je ne les connais pas encore どうもありがとうございます. Un immense merci à Gaulthier pour ce premier beau projet que nous avons mené ensemble au NIMS, merci évidemment pour cette expérience japonaise innoubliable, pour tes très précieux conseils, pour ton énergie et la motivation que tu me donnes pour avancer chaque jour, tel un mentor. Je souhaite aussi remercier le Pr. Ariga et les membres de l'équipe à Tsukuba pour leur accueil au sein de l'équipe et les discussions scientifiques en réunion de groupe, aux labos. Sans oublier celles au balcon, au konbini, ainsi qu'à HS au soleil levant. J'en profite ici pour remercier le groupe également David, Helen, Steve, Geri, Thien, Isa, Sengo, Phong, Atsuko, Andrei, Matthew, Jihui, Kevin, Victor pour les moments forts de cette expérience.

Je souhaite également remercier les membres de l'équipe CSM grâce à qui j'ai pu initier ce projet autour des clusters. Merci pour les connaissances et compétences que vous m'avez apportées dans de nombreux domaines que je n'avais encore jamais expérimentés. Je remercie Stéphane pour le temps accordé à m'expliquer la chimie des clusters. Merci beaucoup à toi Pierric pour le temps que tu as volontiers consacré pour me faire des cours de cristallo, sur ton nanotableau ou devant le diffracto. Merci pour ton aide indispensable dans les mesures de DRX et pour ton aide précieuse. Ce projet n'a pas seulement été en collaboration avec CSM puisqu'il a soulevé de nombreuses questions

et éveillé la curiosité de certains. Cette curiosité a été très motrice et m'a conduit vers vos différentes expertises.

Je souhaite remercier Alain pour tout le temps que tu as consacré pour les analyses Raman, sans oublier Bertrand avec qui nous avons passé de nombreuses heures dans l'obscurité. J'ai pris beaucoup de plaisir à travailler avec vous dans la découverte de cette technique et de ces nouveaux cristaux. Ce projet m'a aussi emmené vers l'électrochimie et je remercie aussi chaleureusement Corinne. Merci pour ta bonne humeur, pour le temps consacré dans l'exploration des signaux de ces capsules et pour les connaissances que tu m'as transmises. Merci aux électrochimistes Gabriel et Yann qui ont su me venir en aide et répondre à mes questions.

Avant de revenir ves les supports bretons je tiens aussi à remercier ceux qui m'ont emmené jusqu'ici. Je souhaite remercier sincèrement l'équipe des chimistes qui m'est chère. C'est avec cette équipe que les portes de la science sont apparues au fils des ans à la fac au Bourget du lac. Je remercie les enseignants qui ont su éveiller notre curiosité scientifique. Un grand merci à Richard, Jean Marc, Nicolas, Laurence pour leurs cours passionnants et captivants. Merci donc aux chimistes pour leur soutien depuis ces années, un énorme merci à Antho, CamCam, Julien, Alex, Kim, Marine, Vincent, Julie, Tosh, Clem, Micky, Tina, Armel, Coco qui avez cru en moi, merci pour m'avoir redonné confiance dans les moments durs de cette période, merci à tous !

Ensuite, je souhaite remercier la seconde équipe de chimiste de Montpellier. Celle qui a tissé les liens entres apprentis chimistes il y a quelques années, qui a fait rédiger ces quelques lignes à certains récemment et qui m'amenne à le faire aujourd'hui, à mon tour. Je remercie aussi profondément Jomachin, Jordy, Atho, Joris, Alex, Emilio, Manu, Gui, Fanny, Mel, Maeva. Merci à vous aussi pour m'avoir apporté votre soutien lors de nos entrevues rapides aux quatre coins de la France durant ces trois ans. C'est aussi à l'IBMM que tout à commencé à la paillasse mais pas nimporte quelle paillasse ! Celle d'un de mes mentor, Dr. Jebors Said que je remercie très sincèrement pour les compétences qu'il m'a transmises et surtout pour le soin qu'il a pris (non pas pour notre paillasse) dans cette transmission du savoir et de cette flame. Sans oublier Céline qui m'a aussi mis sur la voie et conforter dans mes choix.

Par la suite j'ai trouvé paillasse chez Biomérieux au sein de l'équipe du CR&AT à qui j'aimerais exprimer ma gratitude également. En particulier à mes proches collègues en commencant par Alain, Arnaud, Béné, Raph avec qui j'ai beaucoup aimé travailler et énormément appris. Merci à vous tous et particulièrement à Alain pour ce cadre que tu as défini et qui m'a conduit jusque là. Merci de m'avoir offert cette vision de la recherche et du développement durant cette année passé avec vous. Merci de m'avoir fait confiance dans certains moments clés du projet et d'avoir su entretenir cette

flame grandissante. Je me rappelle de la fois où tu m'as confié qu'il te semblait évident que mon chemin irait ou devais aller vers celui de la thèse. Tu m'as laissé me questionner quant à cette direction et c'est à cette époque que j'étais rentré en contact avec bien des chercheurs dans l'espoir de trouver des réponses. Stéphane Roux faisait déjà parti des gens avec qui je m'étais entretenu pour une thèse et aujourd'hui il me fait l'honneur de rapporter la mienne. Merci Alain de m'avoir ouvert ce chemin, de m'avoir ouvert ou monde des nano, cette expérience Grenobloise a été un tremplin dans mon expérience de la recherche et m'a permis de franchir bien des étapes dans l'accomplissement de cette thèse.

Je tiens à remercier mes amis Rennais qui ont été présents dans cette épreuve, des premiers instants aux derniers en ce jour, à commencer par mes collocs. Les premiers, Thom & Armelle. J'ai beaucoup appris de vos deux expériences de la gestion des RH. J'ai pu grâce à celles-ci mieux cerner et gérer les verrous dans la gestion de mon projet de recherche. Merci pour l'énergie que vous avez pu me transmettre, la confiance en moi que vous m'avez souvent redonné. Merci à vous deux pour avoir fait de la rue de Toulouse une des meilleures rue de Rennes. Aussi, Thom, どうもありがとうございます . Je n'oublie pas mes plus récents collocs post Japon, Lo, Ced, et Cyrioulle accompagnées de Maylis et Ju, merci pour ces moments de fous rires et moments dans la cuisine qui m'ont permis de beaucoup décompréssér ces derniers mois, les plus rudes dans ces trois ans, il m'en faudrait encore bien plus et sans ces moments je n'y serais jamais arrivé.

Je n'oublie pas non plus tous mes soutiens locaux de l'institut de chimie, de physique, de géosciences, de l'inria de l'école de chimie, de l'insa et du campus. Merci énormément à TamTam et Julia pour ces chouettes moments de detentes souvent musciales ou culinaires en dehors de nos labos respectifs. Merci aussi et surtout à Benoît, Lorène, Val, Nico, Pierre et Isa pour les instants de respirations intermanips ou interchapitre le temps d'un verre, d'un repas ou d'un jeu pour bien décompréssér.

Bien évidemment, je souhaite remercier mes amis lointains du pays des montagnes qu'il fut douloureux de quitter et compliquer de voir le temps d'un weekend durant ces trois dernières années. Merci les gars, merci les nards pour votre soutien, encouragements et l'énergie que vous m'avez transmis lors de mes passages éclairs qui étaient synonymes d'intenses bols d'air purs.

Je souhaite aussi remercier toutes les personnes que j'ai pu rencontrer lors de congrès, séminaires de conférences, doctorants, postdocs, scientifiques passionnés mais avant tout humains et qui dans des périodes de doutes ont su trouver des mots forts pour me redonner espoir et force pour avancer dans ma vie.

Enfin je n’oublierais pas ma famille en leur exprimant ma profonde gratitude. Tous mes oncles et tantes, cousins et cousines, ma filleule, mes petits cousins et mes petites cousines qui par de petites attentions m’ont touché et encouragé au court de ces années. Bien sur mes deux mini sœurs qui aussi jusqu’au bout m’ont soutenu, encouragé et pour lesquelles je n’ai rien lâché, merci les filles je vous aime. Sans t’oublier maman. Merci mam de m’avoir soutenu du début à la fin de cette thèse dans les meilleurs moments et surtout dans les plus difficiles.s Merci de m’avoir donné le privilège d’étudier et d’avoir pu en arriver jusqu’ici. Merci infiniement, pour avoir toujours trouvé les mots justes et continué de me faire avancer. Merci aussi de m’avoir encouragé et toujours soutenu dans mes choix d’orientation, je t’aime.

Pour finir je tiens à remercier chacun d’entre vous qui faites celui que je suis aujourd’hui et qui m’avez porté jusqu’ici, Merci beaucoup !

Liste des abréviations

AFM: Atomic force microscopy
ALL: Acute lymphoblastic leukemia
Ag: Silver
ATR-FTIR: Spectroscopie infrarouge à transformée de Fourier en mode réflectance totale atténuée
Au: Gold
BCR: B cell receptor
B-ALL: B cell acute lymphoblastic leukemia
BHT: Butylhydroxytoluène
CAR: Chimeric antigen receptor
CL: Constant Light chain
CH: Constant Heavy chain
Chol: Choline
CLL: Chronic lymphocytic leukemia
CTAB: Bromure de céthyltriméthylammonium
CV : Voltammétrie cyclique
DLS: Diffusion de la lumière
DOTAP: 1,2-dioleoyl-3-trimethylammoniumpropane
DOPE: Dioleoylphosphatidylethanolamine
DSPE: Distearoylphosphatidylethanolamine
EC: European Commission
EDC: (1-ethyl-3-(3- dimethyl-aminopropyl)carbodiimide hydrochloride
EDX: Energy dispersive X-ray spectroscopy
EggPC: Egg yolk phosphatidylcholine
EPR: Enhanced Permeation and Retention
Fab: Variable region
Fc region: Constant region
FDA: US Food-and-Drug-Administration
FTO: Oxide d'étain fluoré
HES: Hémalun eosine safran
IFNγ: Interferon gamma
IL6: Interleukin 6
IONP: Nanoparticule d'oxyde de fer
HD37-CCH: Hybridomas HD37-c-myc-Cys-His5 scFv
HSPC: Hydrogenated soy phosphatidylcholine
LBL: Couche par couche
LNP: Liposomal nanoparticle
mAb: Monoclonal antibody
MGITC: Malachite Green Isothiocyanate
MHC-Ig: Major Histocompatibility Complex-Immunoglobulin
MPS: Mononuclear phagocyte system
MRI: Magnetic Resonance Imaging
MRI/CT: Magnetic resonance imaging/ Computerized tomography
MRI/PET: Magnetic resonance imaging/ Positron emission tomography
NHS: N-hydroxysuccinimide

NP:Nanoparticle
NTA: Nanoparticle tracking analysis
PA : Photoacoustique
PAA: polyacrylique
PBLG-b-HA: poly(g-benzyl-L-glutamate)-block-hyaluronan
PCL: poly(cprolactone)
PDT : Therapie photodynamique
PDADMAC: poly(diallyl dimethylammoniumchloride)
PEG: Polyethylene glycol
PEG-DEG: poly(ethyleneglycol) diglycidyl ether
PEI: Poly(ethylèneimine)
PET : Positron emission tomography
PLA: Acide polylactique
PLGA: Poly(lactic-co-glycolic acid)
PMMA: Poly(methacrylate de méthyle)
PPG-DEG: poly(propylèneglycol) diglycidyl ether
PS: Polystyrène
PSS : Polystyrène sulfonate
PTT: Therapie photothermale
PVP: Polyvinylpyrrolidone
QD: Quantum dots
RES: Reticuloendothelial system
SEM: Scanning electron microscopy
SERS: Surface Enhanced Raman Scattering
SiO2: Silicon dioxide
siRNA: Small interference RNA
SMCC: N-succinimidyl 4-(N maleimidomethyl)cyclohexane-1-carboxylate
SPDP: N-succinimidyl 3-(2-pyridylthio)propionate
SPECT: Single photon emission computed tomography
scFv: Single-chain variable fragment
SYK: Spleen Tyrosine Kinase
TCR: T cell receptor
TEM: Transmission electron microscopy
THF: Tétrahydrofurane
TPM: Methacryloxypropyltrimethoxysilane
UCNPs: Up-converting nanoparticles
US: Ultrasons
VL: Variable Light chain
VH: Variable Heavy chain

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Chapitre 1

Introduction aux assemblages de nanoparticules sous la forme de nanocapsules

1. Capsules de particules solides

Dans ce premier chapitre nous allons nous intéresser aux capsules dont la paroi est constituée de particules solides. Il existe une multitude de capsules de ce type de différentes tailles, composées de micro ou nano particules de polymères, de nanoparticules inorganiques, coassemblées parfois avec des polymères. Ces capsules sont très souvent nommées colloïdosomes et nous détaillerons les trois principales voies d'obtention de ce genre d'assemblages reconstitués en 3D Figure 1a. Nous mentionnerons les principaux domaines dans lesquels ils trouvent des applications. Ensuite nous reviendrons plus particulièrement sur les colloïdosomes basés sur les émulsions.

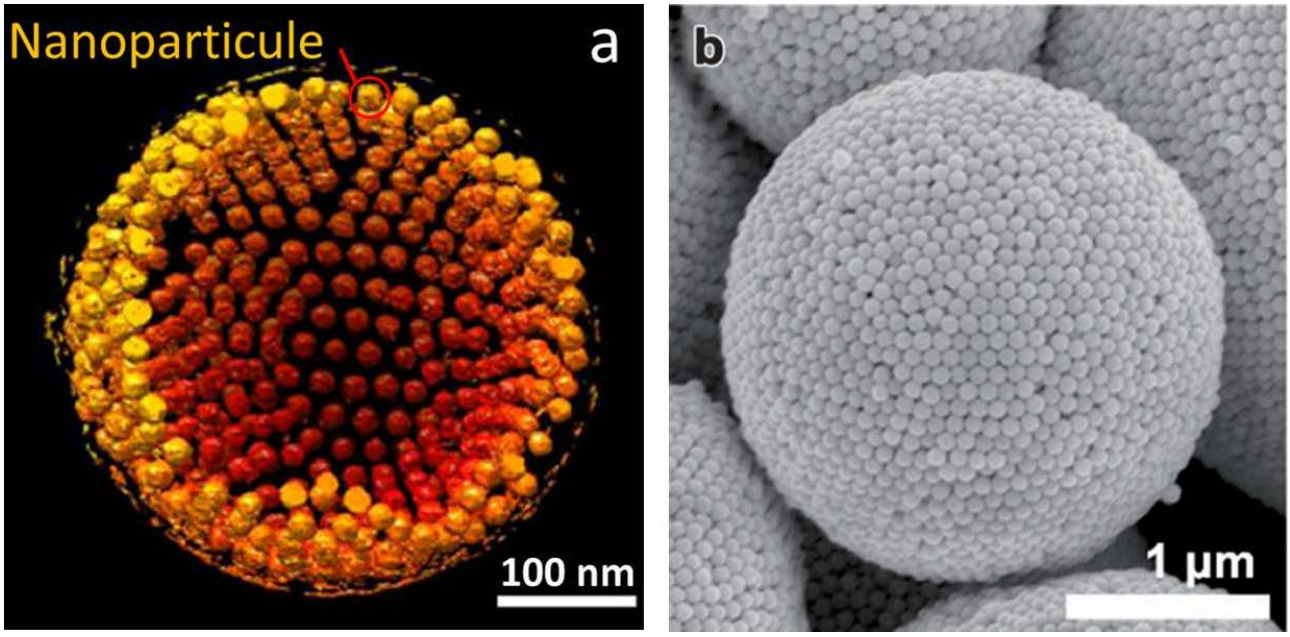

Figure 1: (a) Reconstruction 3D par tomographie d'un colloïdosome de nanoparticules d'oxyde de fer et (b) Micrographie électronique à balayage d'un colloïdosome nanoparticules de silice. Adaptée de [1,2].

1.1 Stratégie copolymères à blocs: nanoprécipitation et polymersomes

Les polymères à blocs sont des copolymères amphiphiles c'est-à-dire qu'ils comportent un bloc hydrophile ainsi qu'un bloc hydrophobe. La stratégie d'assemblage consiste à faire varier la balance solvophobe/solvophile au sein de la solution contraignant ainsi le polymère à s'agréger.

La première voie d'obtention de capsules de nanoparticules fait l'usage de copolymères à blocs. Il s'agit de polymersomes contenant des nanoparticules. Initialement les polymersomes sont des capsules uniquement composées de copolymères à blocs[3] mais leur développement a conduit à l'encapsulation en leur cœur de principes actifs[4] ou de nanoparticules [5] et plus récemment à l'incorporation de nanoparticules au sein de leur paroi.[6]

Le développement des polymères à blocks a conduit à des assemblages sous différentes formes sphérique principalement, parfois en chaîne[7], mais aussi en formes cylindriques[8], sous forme de disques[9,10] ou encore d'hélices .[11]

Lorsque des particules sont présentes dans le milieu lors de l'assemblage on parle de coprécipitation. Cette méthode de est devenue une des principales utilisées grâce aux méthodes de fonctionnalisation de surface de plus en plus maîtrisées, et des particules de plusieurs nature (oxydes de fer, or etc) ont été assemblées. Le contrôle poussé de la balance hydrophile/hydrophobe de la particule en ajustant la nature et le ratio des ligands, a permis de contrôler la position des particules au sein de l'assemblage[12] (Figure 2).

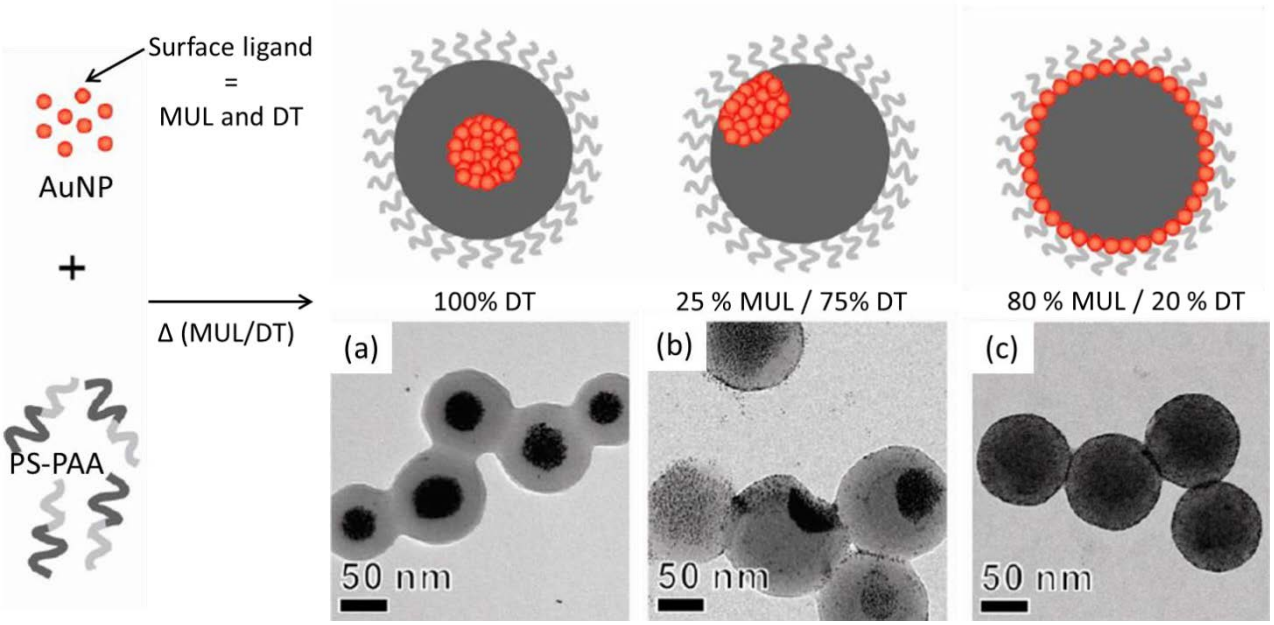

Figure 2: Variété d'assemblages de nanoparticules d'or selon le ratio de ligands hydrophiles/hydrophobes (mercaptoundecanol=MUL/dodecanethiol=DT) en surface. (a) 0/100% (b) 25%/75% (c) 80%/20%. Adaptée de [12].

L'autre méthode très utilisée consiste à fonctionnaliser la particule à l'aide de polymères à blocs. Dans ce cas la particule est aussi considérée comme amphiphile et l'on parle d'auto-assemblage. Les principales particules utilisées sont d'origine inorganiques et de taille de l'ordre de 5 nm à quelques dizaines de nanomètres. Plusieurs natures de particules ont été assemblées comme des oxydes de métaux, de particules d'or (AuNP)[13], mais aussi de différentes formes, principalement sphériques mais également des nano bâtonnets (Figure 3). En effet, les propriétés plasmoniques de l'or, permettent de corréler les variations d'absorbance aux différents états d'agrégation.[13]

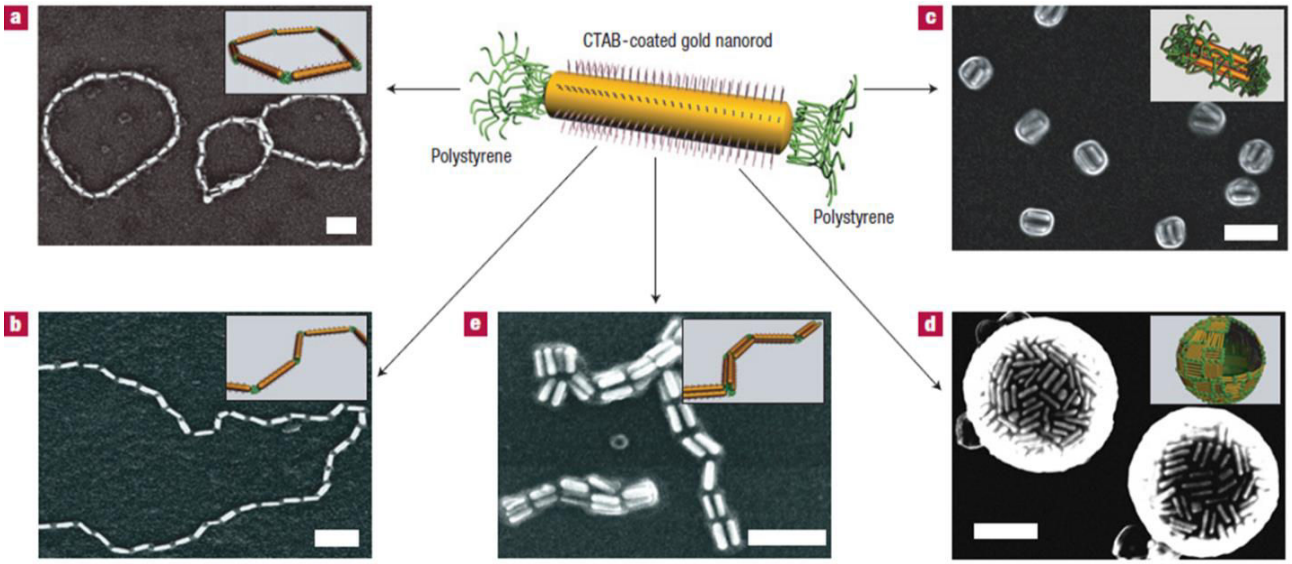

Figure 3: Images SEM d'auto-assemblage de nanobâtonnets d'or en forme (a) d'anneau et (b) de chaînes assemblées dans un mélange diméthylformamide/eau 6 et 20% en ratios volumiques respectivement, (c,d) de nanosphères assemblées dans un mélange tétrahydrofurane/eau 6/20% en ratios volumiques respectivement et (e) de ballots assemblés dans un mélange ternaire DMF/THF/Eau 42.5/42.5/15 % en ratios volumiques. Barres d'échelle 100nm, adaptée de [7].

Dans cet exemple, Nie *et al.* ont en rendu des nanobâtonnets d'or amphiphiles en fonctionnalisant les faces basales par un polymère hydrophobe de type polystyrène (PS). Les faces latérales sont quant à elles hydrophiles et recouvertes d'un tensioactif, le CTAB (Bromure de cétyltriméthylammonium). Par cette technique ils ont pu considérer ces nanobâtonnets comme une sorte de « tribloc » amphiphile et ont fait varier les formes d'assemblage selon les compositions des mélanges. De plus, en changeant la taille des blocs ainsi que la taille des particules, He et. al ont aussi pu contrôler les distances interparticules au sein de capsule.[14]

Enfin plus récemment, la forme des assemblages et la densité des particules sont aussi contrôlées. En utilisant des particules au caractère amphiphile et en présence de copolymères à blocs, des structures de vésicules à patchs ont été obtenues. C'est-à-dire des vésicules dont la paroi comprend différentes zones plus ou moins riches en particules. Liu *et al.* ont ainsi créer des vésicules hémisphères ainsi que des disques et qui confère des caractères Janus aux assemblages.[15]

Un des points importants à propos des assemblages formés est leur taille. Très dépendant de la taille des particules utilisées, certains auteurs ont réussi à contrôler le nombre de particules assemblées et donc la taille des objets formés. Ainsi il est possible d'obtenir des structures « core@shell » avec une, deux, ou davantage de particules au sein du cœur.[16] La plupart des assemblages obtenus sont de l'ordre de quelques centaines de nanomètres.

1.2 Stratégie « template »

La seconde stratégie d'élaboration de nanocapsules de particules repose sur une stratégie « template », qui peut être solide ou bien liquide. Dans le cas du template liquide on retrouve les émulsions de Pickering qui sont par définition des émulsions stabilisées par des particules solides.

Nous détaillerons ce cas dans la partie 2.2. Dans le cas du template solide, la construction de l'assemblage se fait à la surface d'un gabarit. La plupart des « templates » sont des particules sphériques de polymères ou bien de silice.[17] Dans cette stratégie, les interactions qui régissent l'auto-assemblage sont essentiellement des interactions électrostatiques entre le « template » et les particules. La formation des capsules est également soumise aux forces répulsives et attractives inter-particules.

On peut discerner deux types de cas dans l'obtention des capsules nanoparticules par « template » solide:

- Soit le « template » est une capsule et l'assemblage formé est déjà une capsule qui peut être hybride selon le choix des matériaux.
- Soit le « template » est une sphère pleine et une autre étape est nécessaire pour le retirer ou le rendre creux. La plupart du temps il s'agit d'un traitement thermique (calcination), physique ou chimique (Figure 4a) qui vient dissoudre le « template ». C'est aussi ce genre de traitement qui conduit à la formation de capsules « hochets » appelées « yolk shell », produits intermédiaires ou finaux (Figure 4c).

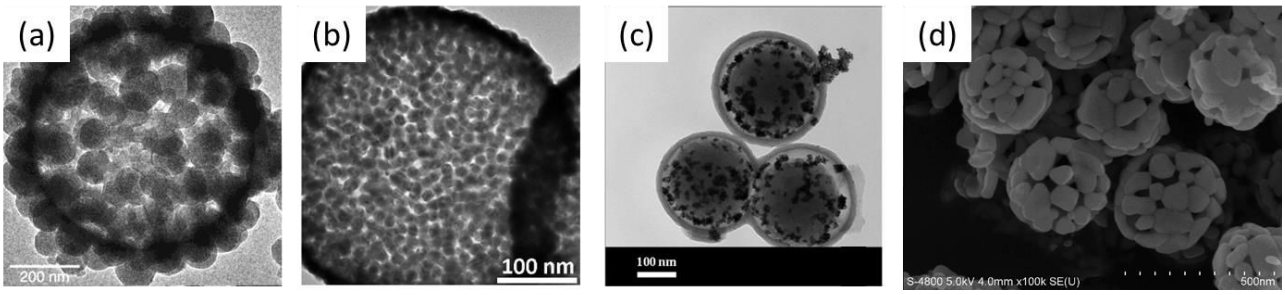

Figure 4: Différents assemblages obtenus par stratégie « template » solide à base de particule (a) de Silice[18], (b) d'or[19], (c) de platine@silice[20], (d) d'oxyde de Fer[21].

Il est aussi possible d'envisager plusieurs couches de particules en alternant les superpositions de particules chargées positivement et négativement par la méthode de « layer-by-layer ».[22]

Grâce à cette stratégie, de nombreux travaux montrent aussi la croissance de particules en surface du « template ». Des espèces en solution peuvent aussi être adsorbées en surface du « template » par interactions électrostatiques et liaisons hydrogène. Il s'agit souvent de précurseurs ou intermédiaires de nanocristaux, comme des hydroxydes de métaux par exemple.

C'est ensuite le procédé hydrothermal qui entraine la nucléation et la croissance des particules en surface, tout en retirant parfois le cœur pour aboutir *in fine* à une capsule. Les traitements thermiques permettent également un contrôle de la porosité de la capsule (Figure 4d).[21] Dans d'autres travaux, la synthèse du « template » est modifiée pour y inclure ces précurseurs, qui formeront des cristaux ou clusters ensuite durant l'étape de chauffage.[23] Grâce à cette stratégie la taille des capsules est très modulable et des assemblages de l'ordre de quelques dizaines de nanomètres[24] à plusieurs microns ont été préparées.[25]

1.3 Propriétés et applications des nanocapsules de particules

Les colloidosomes trouvent de nombreux domaines d'application puisqu'intrinsèquement ils peuvent être constitués de nanoparticules, clusters et nanoédifices d'une très grande variété d'éléments chimiques. Ainsi ils trouvent des applications dans les domaines du stockage de l'énergie avec des assemblages à base d'oxydes d'étain, de titane, ou de cobalt qui montrent des activités intéressantes dans le développement de batteries lithium-ion.[26,27] Dans le cas de l'étain, Deng et Lee ont comparé les capacités de charge/décharge de capsules de SnO_2 et de sphères de SnO_2. Les nanocapsules présentent de meilleures capacités grâce à leur microporosité et permettent une meilleure réactivité du lithium.

Des assemblages à base de métaux nobles comme le platine, l'or ou le palladium, ont été utilisés comme nanoréacteurs et engagés dans des réactions de catalyse hétérogène classiques de type Suzuki[28] ou encore d'oxydation et réduction d'alcools.[20,29,30] Lorsque les capsules sont comparés aux sphères, c'est le caractère poreux qui explique la meilleure réactivité des capsules, notamment grâce à une meilleure diffusion des espèces ou un phénomène de multiréflection d'un rayonnement lumineux dans le cas d'une réaction photocatalytique.

L'étude des interactions électriques et magnétiques inter-particules au sein d'assemblages de particules plasmoniques, intéresse beaucoup le domaine de l'optique et de nouvelles propriétés sont observées dans l'études des métamatériaux.[31] Dans ce cas, lorsque les assemblages sont soumis à une irradiation lumineuse, des phénomènes de résonnance plasmonique permettent de générer une réponse magnétique.

Enfin les applications biomédicales figurent parmi les plus nombreuses et les particules magnétiques, plasmoniques, luminescentes suscitent un grand intérêt dans le développement de la nanomédecine. En effet, les propriétés intrinsèques des nanoparticules ont déjà montrés des résultats prometteurs dans les diverses méthodes d'imagerie préclinique et clinique, des méthodes de détection, mais aussi des applications thérapeutiques. Dans ce contexte, l'avantage des nanocapsules de particules peut être considéré comme multiple.

Premièrement il permet d'assembler plusieurs types de particules au sein d'une même capsule. Liu *et al.* ont par exemple crée des capsules hybrides et mixtes d'oxydes de fer et d'or et ont démontré leur efficacité en imagerie duale. En effet, les capsules présentent un effet d'agent de contraste en imagerie par résonnance magnétique (IRM) ainsi qu'en imagerie photoacoustique (PA) (Figure 5).[32]

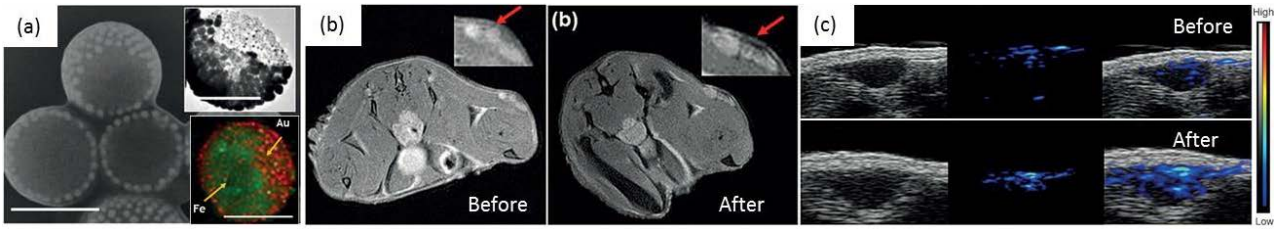

Figure 5: Assemblages hybrides et mixtes d'oxydes de fer et d'or pour l'imagerie duale IRM/Photoacoustique. (a) Micrographie SEM, TEM, analyse EDS, barres d'échelle 500nm (b) Images MR *in vivo* avant et après injection, (c) Images *in vivo* photoacoustique ultrasonique avant et après injection. Adaptée de [32].

D'autres combinaisons ont déjà montré leur efficacité *in vivo* pour les techniques duales comme l'imagerie par émission de positrons (PET) couplée à la PA,[33] l'imagerie par ultrason (US) couplée à l'IRM,[34] ou encore l'IRM et l'imagerie scanner (CT)[35].

Le second avantage conféré par leur structure de capsules et donc de véhicule, a poussé leur développement comme « conteneur ». Il est parfois envisagé d'encapsuler ou de piéger des molécules actives et de déclencher par divers stimuli leur relargage. Par exemple l'utilisation de polymères photosensibles permet le déclenchement de la destruction de la capsule et la libération de son contenant et ses constituants [36]. Les structures hybrides de certaines capsules permettent aussi de faire varier la nature de ces stimuli qui peuvent être endogènes. Ainsi Song *et al.* ont encapsulé une molécule anticancéreuse (doxorubicine) dans des capsules de particules d'or auxquelles est couplé un anticorps, dans le but de cibler des cellules tumorales. L'assemblage possède une signature Raman très spécifique qui disparait lorsque la capsule est détruite par un stimulus de pH au sein de la cellule. En suivant cette signature, ils sont capables de tracer *in vitro* le relargage de la drogue.[6]

Enfin, d'autres stimuli peuvent entraîner des réponses chimiques ou thermiques et à la fois jouer le rôle de destruction des capsules mais aussi de thérapie. C'est notamment le cas de l'hyperthermie ou de la thérapie photothermale (PTT). Dans le premier cas, la hausse de chaleur est induite par la réponse de particules magnétiques à un champ magnétique. Cette hausse de température est suffisamment importante pour induire la mort cellulaire. Sanson *et .al* ont démontré la triple efficacité *in vitro* de polymersomes à base d'oxydes de fer. Les capsules ont un effet d'agent de contraste en IRM, une efficacité en hyperthermie qui a également pour effet de déclencher le relargage de la drogue qui peut alors produire son effet.[6]

Dans le second cas, Song *et al.* ont également démontré les potentialités en théranostic *in vivo* de capsules de nanobâtonnets d'or qui combinent la bimodalité en imagerie PET et PA ainsi que la propriété thérapeutique en PTT.[33]

Dans ce cas, ou la combinaison de la thérapie à l'imagerie à lieu au sein du même nanomatériau, on parle de théranostic. Ces récents développements présentent la triple fonction des nanocapsules et sont prometteurs pour la nanomédecine.

2. Les différentes émulsions

Une des stratégies importantes dans la construction de nanocapsules de nanoparticules repose sur les « templates » liquides, c'est-à-dire les émulsions. Nous allons donner dans cette partie quelques notions à propos de l'émulsion standard ainsi qu'au sujet des émulsions de Pickering qui donnent aussi naissance aux colloidosomes.

2.1 Emulsions standards

Une émulsion est définie comme un milieu hétérogène composé de deux liquides non miscibles, en général eau et huile, qui constituent une phase dispersée dans une autre phase continue. L'interface est stabilisée par un tensioactif. Les tensioactifs sont des molécules amphiphiles c'est-à-dire composées d'une partie hydrophile (tête polaire) et d'une partie lipophile (queue apolaire). Cette structure leur permet de s'adsorber au en monocouche à l'interface huile/eau et de stabiliser l'émulsion. Lorsque la phase aqueuse constitue la phase continue, l'émulsion est dite de type huile dans eau (H/E). A l'inverse, lorsque l'eau constitue la phase dispersée, on parle d'émulsion eau dans huile (E/H) ou d'émulsion inverse (Figure 6).

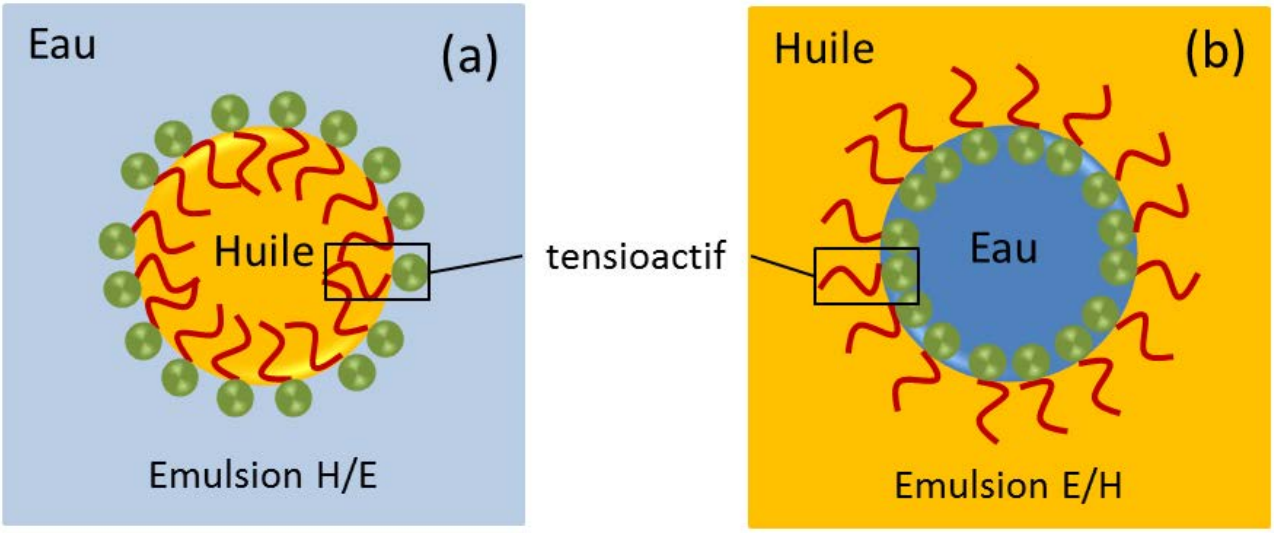

Figure 6: Représentation schématique des émulsions (a) huile dans eau et (b) eau dans huile.

Les émulsions sont souvent classées selon leur stabilité, taille des gouttes, composition qui sont évidemment des critères intrinsèquement liés ce qui peut parfois porter à confusion.

2.1.1. Microémulsions et Nanoémulsions

Dans le cas des microémulsions, l'énergie libre de la dispersion colloïdale à l'état d'émulsion est plus faible que l'énergie libre des deux phases séparées, c'est pourquoi on dit que les microémulsions sont thermodynamiquement stables. Cependant il existe toujours une barrière d'énergie d'activation (ΔG^*) séparant ces deux états, qui détermine l'énergie nécessaire au système pour former l'émulsion (Figure 7).

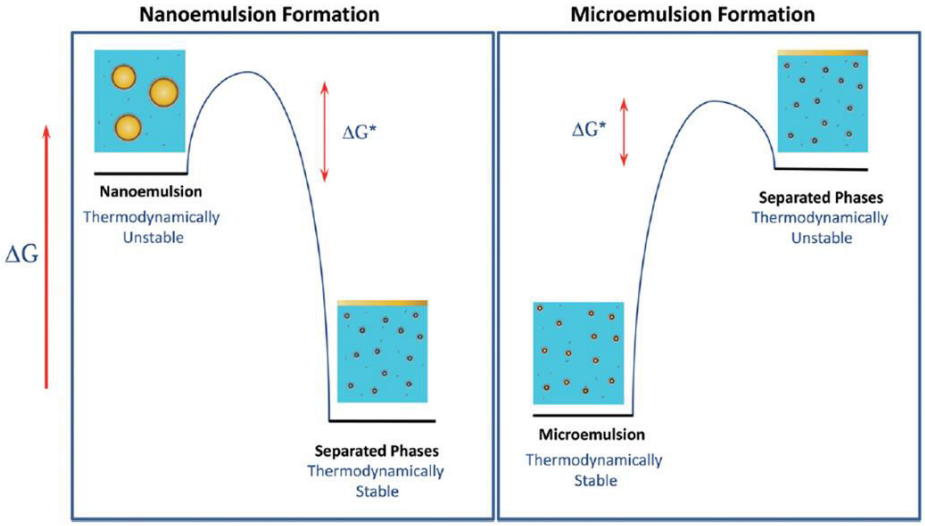

Figure 7: Schéma du diagramme d'énergie libre de microémulsions et nanoémulsions comparées aux systèmes des phases séparées avec ΔG* l'énergie d'activation. Adaptée de [37].

En effet, même si les microémulsions peuvent en principe être formées de manière spontanée simplement en amenant les trois composés ensemble, en pratique il est souvent nécessaire d'apporter de l'énergie externe (sous forme d'agitation ou de chauffage) afin de franchir les barrières d'activation cinétique.

Les nanoémulsions sont constituées, comme leur nom l'indique, de gouttes de taille nanométrique (20-500 nm). On retrouve dans la littérature plusieurs autres dénominations comme les émulsions sub-microniques, émulsions ultrafines ou encore les miniémulsions.[38] A l'inverse des microémulsions, elles sont thermodynamiquement instables (Figure 7).

Les microémulsions peuvent également comporter des gouttes de tailles nanométriques et donc les préfixes utilisés portent à confusion. Cette confusion résulte du développement historique dans la science des colloïdes puisque le terme microémulsion est employé depuis 1961[39] alors que le terme nanoémulsion n'a fait son apparition que bien plus tard, en 1996[40] avec le développement croissant du domaine des nanotechnologies.

La composition des deux types d'émulsions est identique, cependant quelques différences sont à noter à propos de la concentration en surfactants qui est de l'ordre de (2 - 10 % wt) pour les nanoémulsions alors qu'elle dépasse les 20 % wt pour les microémulsions.[41] On retrouve parfois aussi l'utilisation de co-surfactants.

En principe les microémulsions peuvent être obtenues de manière spontanée alors que les nanoémulsions requièrent des processus de fabrication plus énergétiques. Les nanoémulsions, contrairement aux microémulsions, requièrent toujours un apport d'énergie extérieure puisque elles sont thermodynamiquement instables.

2.1.2. Applications des émulsions

Les émulsions ont stimulé l'innovation dans l'industrie alimentaire, cosmétique et pharmaceutique et dans les applications de « delivery ».[42] Dans l'industrie alimentaire, les émulsions ont permis l'encapsulation de composés hydrophobes comme des vitamines, des couleurs, des antioxidants. Dans ces cas l'encapsulation de molécules hydrophobes peut apporter plusieurs avantages : elle peut faciliter son incorporation dans un produit, améliorer sa biodisponibilité ou encore le protéger d'une dégradation chimique.[44,45] Par exemple, dans les cas des vitamines de la famille des caroténoïdes qui sont des composés sensibles à la lumière, à l'oxygène et à la chaleur, leur encapsulation permet une meilleure administration et adsorption par l'organisme.[45,46] Ainsi de nombreux travaux ont portés sur l'amélioration de la stabilité des émulsions dans les conditions de pH et de température physiologiques.[47]

Les émulsions ont également permis la diversification des méthodes de synthèse de nanomatériaux. De nombreux procédés reposent sur les nano et microémulsions pour le contrôle de la taille, dispersion, porosité des nanoparticules.[48–50] En s'appuyant sur une microémulsion inverse de type cyclohexane/eau en présence d'un surfactant (Triton X-100 : polyoxyéthylène tert-octylphényléther) et d'un co-surfactant (hexanol) Lin *et al.* ont réussi à synthétiser des nanoparticules de silice sphériques, des nanocapsules et des particules type hochet en changeant simplement les quantités de précurseurs.[50]

D'autres applications importantes reposent sur les propriétés dont nous n'avons pas encore parlé qui sont les propriétés rhéologiques de ces émulsions. Certains travaux ont démontrés des propriétés similaires à certains gels[51] et de la thermoréversibilité[52] qui pourraient trouver des applications dans les matériaux adhésifs, ou l'impression 3D.

Nous avons vu que les émulsions sont stabilisées par des tensioactifs, mais d'autres composés peuvent être utilisés pour stabiliser ces interfaces tels que des polymères synthétiques ou encore des protéines globulaires,[53,54] des virus,[55] des spores,[56] des bactéries,[57] et jouent le rôle de stabilisant.

Cependant dans ces exemples, l'agent stabilisant de l'interface est de taille bien supérieure à un tensioactif moléculaire et esy parfois considéré comme une particule. Ces émulsions constituent la classe des émulsions de Pickering que nous allons détailler dans la partie suivante.

2.2 Emulsions de Pickering

Une émulsion de Pickering comporte des particules solides colloïdales comme stabilisant à la place de surfactants. Ce phénomène a d'abord été observé par W. Ramsdem[58] , puis rapporté par S.U. Pickering.[59] De la même manière que dans le cas des émulsions classiques, les particules solides s'adsorbent à l'interface huile/eau et l'on parle également d'émulsions huile dans eau (H/E) ainsi qu'eau dans huile (E/H) (Figure 8).

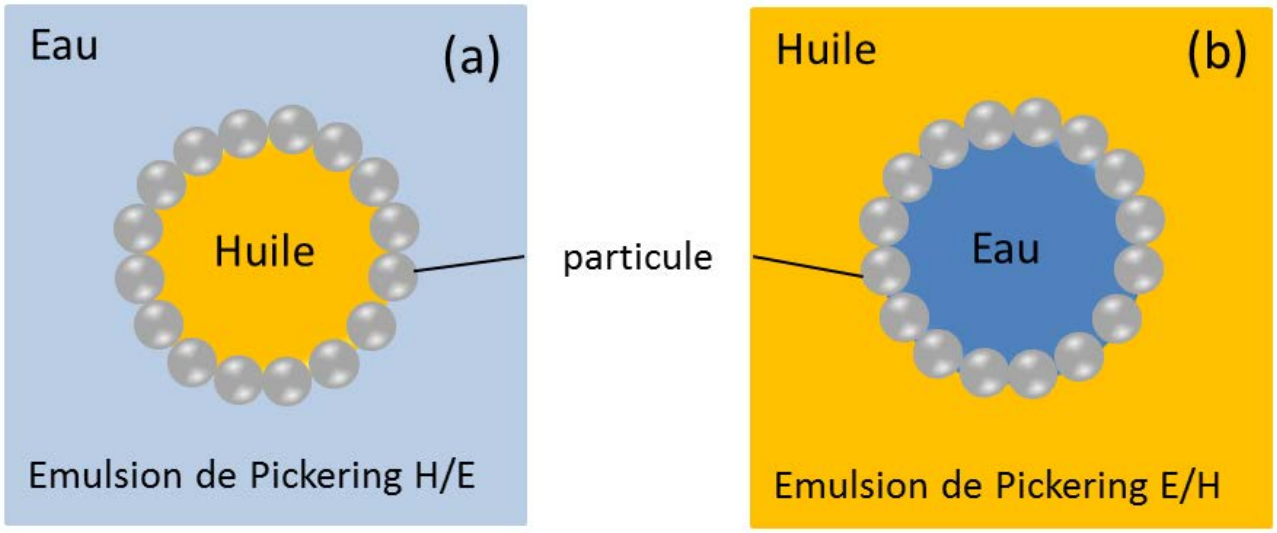

Figure 8: Représentation schématique des émulsions de Pickering (a) huile dans eau et (b) eau dans huile.

Les particules s'organisent à l'interface et l'on peut définir un taux de couverture exprimé en % qui définit la proportion de la surface de goutte recouverte par des particules. Beaucoup de cas d'arrangements de particules sous formes de chaînes ou autres structures pontées ont été reportées pour des taux de couverture entre 5 et 35 %.[60–63]

De nombreux systèmes peuvent être obtenus grâce à ce type d'émulsions qui peuvent également être doubles, voire multiples. Les mêmes phénomènes de Pickering sont aussi étudiés dans le cas d'interfaces eau/gaz [64] et de nouveaux matériaux ont été créés.

On trouve parmi ces matériaux les « bijels » (biscontinuous interfacially jammed emulsion gel),[65] les gouttes sèches,[66–68] ou encore des matériaux poreux comme les « polyHIPEs » (polymerized High Internal Phase Emulsions).[69,70] Ces émulsions trouvent aussi de nombreuses applications en cosmétique, en agroalimentaire, et dans le domaine des mousses.[71]

L'adsorption des particules à l'interface huile/eau est caractérisé par différentes grandeurs que nous allons décrire dans la partie suivante. La compréhension de ces systèmes repose sur les interactions inter-particules en solution ainsi que les comportements aux interfaces. Dans cette partie nous allons traiter des forces et interactions mises en jeu lors de l'adsorption de particules au sein des émulsions de Pickering.

2.2.1. Physicochimie des particules solides à l'interface liquide/liquid

D'après les travaux de Binks, Levine et Bowen, la stabilisation des émulsions par des particules dans un système non coalescent se fait en quatre étapes[72–75] :

1. Formation des gouttes par fracture
2. Approche des particules de l'interface et collision
3. Adsorption des particules
4. Formation du réseau de particules et stabilisation

Nous allons nous intéresser plus particulièrement principales forces qui s'appliquent lors de l'étape 3 c'est-à-dire lors de l'adsoprtion des particules à l'interface liquide/liquide.

2.2.2. Tension de surface

Dans un système où coexistent deux phases (liquide/liquide ou liquide/gaz), il existe une énergie liée à l'interface entre ces deux phases. L'énergie interne Fs de cette interface, selon Gibbs [76] est définie par :

$$Fs = \frac{\partial F}{\partial A} A = \gamma_{int} A \text{ (Eq. 1)}$$

La tension interfaciale, notée γ_{int}, caractérise la variation d'énergie interne Fs associée à une variation de surface entre les deux phases. Elle est donc définie comme une énergie par unité de surface et s'exprime en N/m. Les nanoparticules s'adsorbent de manière spontanée à l'interface, car ainsi elles diminuent l'énergie interfaciale du système.

2.2.3. Angle de contact

Dans le cas d'émulsions de Pickering les particules sphériques adsorbées à l'interface eau/huile sont caractérisées par un angle de contact θ qui décrit la mouillabilité de la particule. La loi de Young relie cette grandeur aux trois tensions interfaciales du système (Figure 9).

$$\cos\theta = \frac{\gamma_{P/H} - \gamma_{P/E}}{\gamma_{H/E}} \text{ (Eq. 2)}$$

Avec $\gamma_{P/H}$,$\gamma_{P/E}$ et $\gamma_{H/E}$ les tensions interfaciales particule/huile, particule/eau, et huile/eau respectivement.

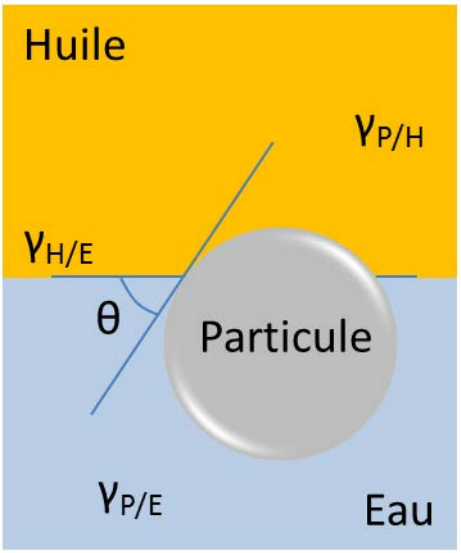

Figure 9: Représentation schématique d'une particule à l'interface huile/eau, des tensions interfaciales et de l'angle de contact θ.

L'angle de contact et la mouillabilité de la particule sont reliés à sa nature chimique ainsi qu'à son état de surface. Considérons une émulsion à volumes égaux huile/eau. Dans le cas d'une particule hydrophile, la majorité de sa surface est mouillée par la phase aqueuse et l'angle de contact est inférieur à 90° (Figure 10a). Inversement, pour une particule hydrophobe, la phase huileuse mouille majoritairement la surface de la particule et l'angle formé est supérieur à 90° (Figure 10c). Dans ces deux cas la phase qui mouille d'avantage la particule est la phase continue de l'émulsion. Par conséquent, les particules hydrophobes conduisent à des émulsions de type E/H et inversement pour des particules hydrophiles (émulsions eau/huile).

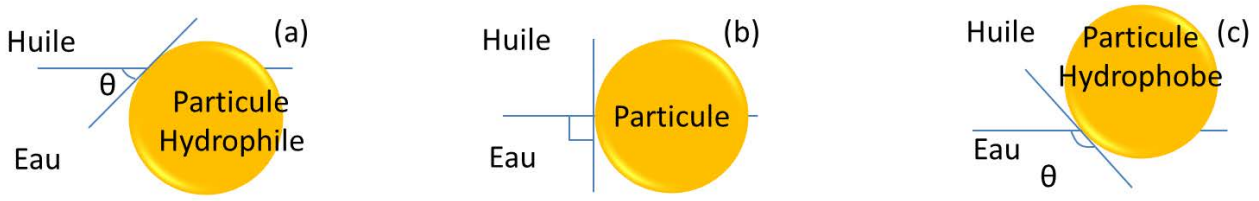

Figure 10: Configuration d'une particule adsorbée à l'interface huile/eau pour un angle de contact θ (a) de 90°, (b) inférieur à 90° et (c) supérieur à 90°.

Les études de Kaptay suggèrent que pour stabiliser une émulsion H/E l'angle de contact θ idéal se situe entre 70° et 86° et entre 94° et 110° pour une émulsion E/H.[77]

Dans le cas particulier où l'angle de contact est égal à 90°, le système est dans état intermédiaire et les particules ne se trouvent pas préférentiellement mouillées par une phase ou l'autre mais sont dispersées. Il s'agit du cas particulier où l'on observe une inversion de phase et où l'émulsion n'est pas stable ni proprement définie[72] puisque l'énergie du système est maximale (Figure 11a).

2.2.4. Energie d'ancrage et de désorption

Les particules adsorbées aux interfaces huile/eau le sont très souvent de manière irréversible et l'énergie à apporter au système pour déstabiliser l'émulsion représente une barrière importante. Si l'on considère une particule de rayon r à l'interface Huile/Eau, formant un angle de contact θ, alors son énergie d'adsorption est:

$$\Delta E = \gamma_{H/E}\, \pi r^2 (1 - \cos\theta)^2 \text{ (Eq. 3)}$$

On remarque que cette énergie est toujours positive et varie en fonction du carré du rayon de la particule. Les Figure 11a et Figure 11b représentent respectivement l'énergie d'absorption en fonction de l'angle de contact et en fonction du rayon pour $\theta = 90°$.[72]

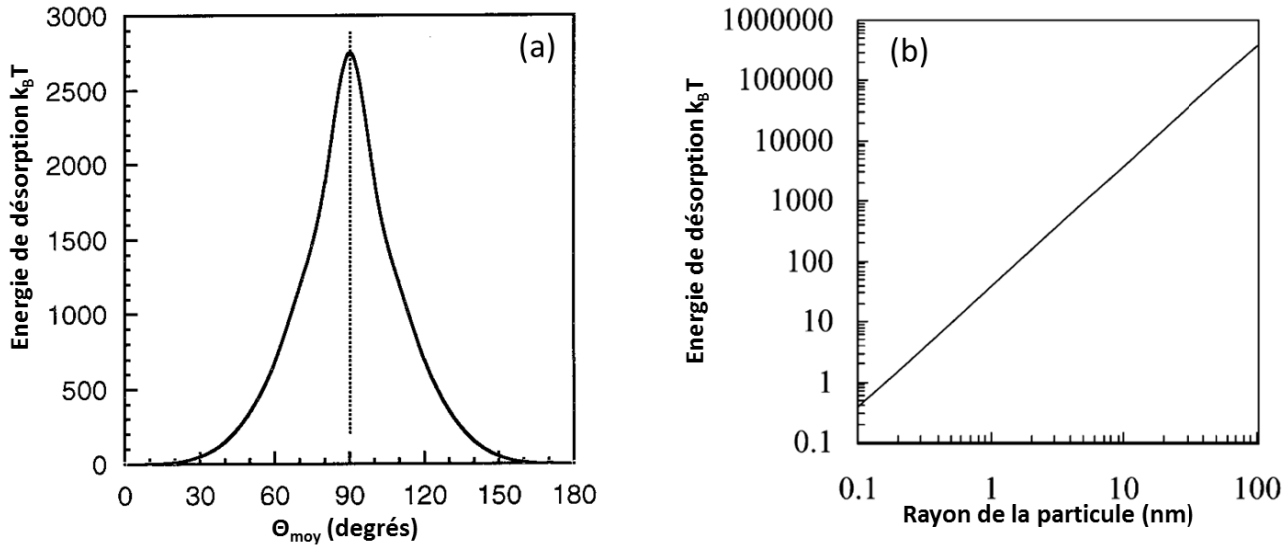

Figure 11: (a) Variation de l'énergie de désorption pour une particule à l'interface H/E en fonction de l'angle de contact θ. (b) avec rayon r= 10 nm, $\gamma_{H/E} = 36\ mN.m^{-1}$, à 298 K. (b) Représentation graphique de l'évolution de l'énergie de désorption en fonction du rayon de la particule avec $\theta = 90°$ et $\gamma_{H/E} = 50\ mN.m^{-1}$. Adaptée de [72].

Cette relation montre que pour une particule de 10 nm, l'énergie nécessaire pour sa désorption de l'interface H/E est de $1000\ k_B\,T$ ce qui traduit l'irréversibilité de l'adsorption des particules contrairement aux émulsions classiques stabilisées par des surfactants moléculaires.[72] En revanche, dans le cas de très petites particules (rayon inférieur au nanomètre) leur comportement pourrait tendre vers celui des surfactants et un équilibre d'échange entre la surface des gouttes et le milieu pourrait exister.

2.3 Stabilité des émulsions de Pickering

Nous venons de voir que la taille de la particule est un paramètre critique dans la stabilisation des émulsions de Pickering. Cependant, beaucoup de travaux se sont également intéressés aux autres paramètres du mélange. Dans cette partie nous verrons le comportement des émulsions de Pickering selon les différentes propriétés des particules puisque l'état de dispersion de particules à l'interface dépend également de leur forme, de leur état de surface (caractère hydrophobe/hydrophile) qui définit leur mouillabilité, de leur état de dispersion, de leur concentration et de leur localisation initiale dans le mélange. Nous parlerons également de l'influence des propriétés des deux phases.

2.3.1. Caractéristiques de la particule

2.3.1.1 Taille, rapports d'aspects et rugosité

Depuis la découverte des émulsions de Pickering, un grand nombre de modèles de particules a été utilisé. Les premiers travaux publiés par Binks *et al.* décrivent l'influence de la taille de particules de polystyrène sur la taille des gouttes de l'émulsion et ont observé une évolution d'abord une croissante puis atteignant un plateau pour des particules d'environ 1 µm.[78]

La majeure partie des études mentionne des particules sphériques. Quelques auteurs ont utilisés des particules ellipsoïdales de différents rapports d'aspects[79], cubiques et en forme de cacahuètes[80], des disques d'argile (Figure 12).[81] Une étude de modélisation s'est concentrée sur des émulsions de Pickering stabilisées par des nanobâtonnets de taille, rapports d'aspects (longueur / diamètre) et nombre croissants.[82] Tarimala *et al.* ont étudié le co-assemblage de particules de taille 1 et 4 µm pour mettre en avant les interactions électrostatiques, destructrices dans ce cas.[83]

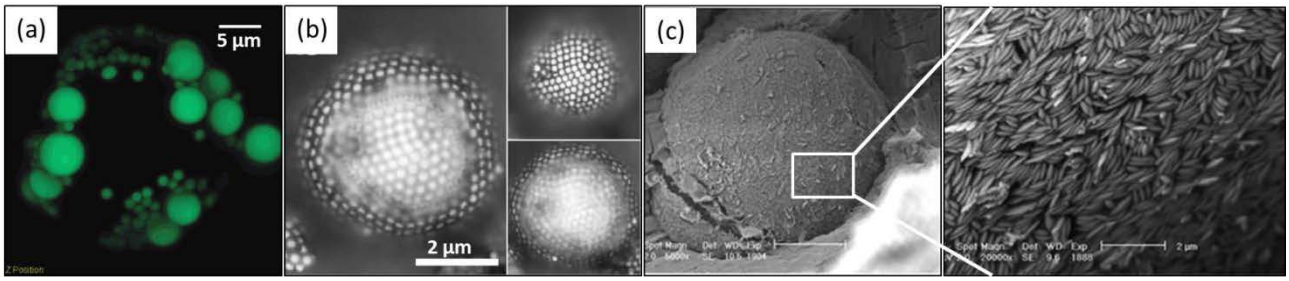

Figure 12 : Différents colloidosomes (a) composés de deux tailles de particules de silice 1 µm et 4 µm, (b) de particules cubiques de (c) de particules ellipsoïdales d'hématite. Adaptée de [79,80,83].

Une seule étude théorique a également pris en compte différentes formes de nanobâtonnets[82] mais il n'y a pas à ce jour beaucoup d'études à proprement parler en fonction de la forme des particules. Récemment Katepalli *et al.* ont rapporté un étude comparative entre de la silice colloïdale et de la « silice fumée » qui s'apparente à des agglomérats fractals, de structure non contrôlée de particules d'une dizaine de nanomètres.[84] Ils ont observé des gouttes de plus petites tailles, ainsi que des réseaux emboîtés à l'interface grâce à la structure fractale de la silice fumée créant ainsi une émulsion plus stable.

D'autres études se sont également intéressées à des paramètres qui sont moins pertinents et moins accessibles dans le cas de nanoparticules. Dans le cas de microparticules, l'aspect de surface et de rugosité de la particule peuvent être plus facilement modifié. Ainsi, Behrens *et al.* ont modifié la surface des particules de silice par un post-traitement et ont ainsi fait varier l'état de rugosité. Ils ont donc fait varier le paramètre de mouillabilité des particules sans en affecter la nature chimique. Deux régimes sont décrits pour le mouillage, un régime homogène (régime de Wenzel) et un hétérogène (régime de Cassie-Baxter). Les auteurs ont identifié une valeur maximale de rugosité améliorant la stabilité de l'émulsion tant que le mouillage se fait en régime homogène.

2.3.1.2 Nature chimique, mouillabilité

Comme expliqué au paragraphe précédent, la mouillabilité est relative à l'état de surface de la particule et est donc modifiable physiquement ou chimiquement. Les premières études rapportées par Binks *et al.* ont permis de mettre en évidence la diminution de la taille des gouttes ainsi que l'affinement de ces distributions lorsque l'hydrophilie de particules de silice augmente.[85] Plus tard ils ont également considérer une particule Janus et donc plusieurs angles de contact, définis à partir des trois domaines de l'interface huile/particule/eau.[85–88] Plusieurs exemples montrent des modifications chimiques afin de faire varier le caractère hydrophile/hydrophobe et ainsi directement modifier l'angle de contact à l'interface.[89]

Plus, récemment Noguchi *et al.* ont étudié le comportement de particules amphiphiles Janus en faisant varier les ratios volumiques de l'émulsion. Ils ont observé l'évolution de la structure des assemblages selon le ratio volumique eau / particules. A très faible quantité d'eau 9%, des agrégats de quelques particules se forment. Puis les agrégats prennent une forme de bâtonnets pour une quantité d'eau légèrement supérieure 27%. Enfin à partir de 60% d'eau les assemblages prennent la forme de gouttes sphériques. Ils ont déduits de ces trois observations que les particules Janus avaient des caractéristiques à la fois proches des surfactants et des colloides.[90]

2.3.1.3 Etat de dispersion, concentration

L'état de dispersion ou encore d'agrégation des particules peut être relié à la salinité de la phase aqueuse, son pH et les interactions interparticules ont une importance non négligeable dans la stabilisation des émulsions de Pickering. Plusieurs travaux semblent dire que les particules ont besoin d'être dans un état naissant de floculation pour stabiliser au mieux l'émulsion.[91]

L'état de dispersion des particules, c'est-à-dire leur dispersion initiale dans la phase huile ou bien eau avant l'étape d'émulsification, a aussi été étudiée. Binks *et al.* ont étudié les phénomènes d'inversion de phase dans un système eau/tiglycérides/particule de silice d'hydrophobicité variable. Ils ont constaté que le type d'émulsion formé dépend de la phase dans laquelle sont dispersées les particules. Ainsi, si les particules sont dans la phase aqueuse, alors l'émulsion H/E sera inversée en émulsion E/H lorsque la quantité d'eau est augmentée. A l'inverse, si les particules sont initialement dans l'huile, les auteurs ont observé pour la première fois lors de l'inversion de l'émulsion E/H, la présence de double émulsion E/H/E.[92]

Nous avons mentionné plus haut que la concentration avait un impact sur le taux de recouvrement de l'interface.[92,93] La plupart de ces études présentent des émulsions de Pickering où la quantité de particules représente entre 0,5 et 5 % de la masse totale. Cette grandeur est rarement uniformisée à cause de la nature des particules utilisées. Il est parfois compliqué de l'appréhender puisqu'il est possible de l'exprimer sous la forme d'une concentration molaire d'un seul élément, d'un motif d'un polymère ou du polymère entier, ou encore sous la forme d'une surface spécifique lorsque les particules sont poreuses. Dans la suite de ces travaux de thèse, les informations de concentration seront le plus souvent exprimées en mole de l'élément d'intérêt ou bien en masse de particules.

2.3.2. Caractéristiques des phases liquides

2.3.2.1 Phase aqueuse

Masliyah *et al.* ont étudié l'effet du pH de la phase aqueuse de particules d'argile sur la taille des émulsions. Il semble qu'à pH 6 le taux de recouvrement soit maximal. La taille de goutte croit également avec le pH[94]. Les études de Binks *et al.* complètent les précédents résultats avec la variation de la quantité de sels afin de rendre la surface des particules plus ou moins chargée et donc influencer la stabilité de l'émulsion.[87,95,96] Dans le cas de particules de silice hydrophile, l'ajout d'agents cationiques améliore considérablement la stabilité de l'émulsion. L'adsorption des molécules à la surface des particules rend les interactions électrostatiques entre gouttes plus importantes. Dans ce cas, les particules peuvent créer une sorte de réseau interconnecté entre les gouttes améliorant considérablement les propriétés rhéologiques de l'émulsion. Les interactions inter-particules conduisent également à l'augmentation de la taille des gouttes.[95]

2.3.2.2 Phase huileuse

Plusieurs travaux ont aussi confronté la polarité de la phase huileuse à la stabilité de l'émulsion.[97,98] Ils ont corrélé les angles de contact liquide/liquide et la mouillabilité de particules hydrophiles dans ces mélanges. Dans les phases huiles polaires la mouillabilité est accrue et la stabilité étendue.[86,87]

Enfin Fournier *et al.* ont étudié des huiles de différentes viscosités pour déterminer son rôle dans la stabilité de l'émulsion. Ils ont mesuré la quantité de volume émulsionné par rapport au volume total des deux phases, selon un procédé d'émulsification précis. Ils ont conclu que plus la viscosité augmente, plus le volume réellement émulsifié diminue et donc que la stabilité de l'émulsion est fortement impactée.[99]

3. Colloidosomes

Nous allons ici nous intéresser plus particulièrement aux capsules nommées colloïdosomes. Terme introduit en 2002, il s'agit de capsules dont la paroi est composée de particules et formé par les stratégies template.[100] La taille des capsules peut varier de quelques centaines de nanomètres à plusieurs millimètres, en fonction de la taille initiale des particules et des gouttes de l'émulsion.

Les premiers colloidosomes ont été développés à partir de particules de polymère type polystyrène (PS), polyméthacrylate de méthyle (PMMA)[100] ou à partir de sols de silice (latex).[101] Dans chacun de ces cas les particules viennent se placer à l'interface huile / eau pour former une capsule.

Velev *et. al* ont été parmi les premiers à rapporter en 1996 l'assemblage de particules en utilisant les gouttes d'émulsions comme « template ».[102–104] Par un procédé en cinq étapes ils ont formé des assemblages de particules de polystyrène sulfate (PSS). Une première étape consiste à rendre partiellement hydrophobe les particules, les « sensibiliser » afin de les adsorber à l'interface. Initialement les particules présentent en surface des fonctions sulfates chargés négativement à pH 6. Par l'adsorption d'une lysine positivement chargée à pH 6, les auteurs ont modifié la charge des latex. Ensuite la phase huileuse est émulsifiée et les particules viennent stabiliser l'émulsion H/E. Une première étape de stabilisation est rendue possible par l'ajout de caséine afin d'éviter un effet de crémage et permettre l'observation de l'émulsion au microscope. La caséine joue le rôle de coagulant partiel et semble créer un faible lien interparticule qui maintient partiellement la structure. C'est pourquoi une étape de plus forte coagulation est nécessaire pour figer l'assemblage. L'ajout d'HCl et $CaCl_2$ induit l'agrégation des particules de latex au sein de la monocouche formée autour de l'émulsion. Cette étape rend possible ensuite le transfert des assemblages dans une phase uniquement aqueuse. L'étape de coagulation (agglomération, frittage) est nécessaire pour figer et maintenir la structure de capsule.

Il existe plusieurs procédés permettant de stabiliser la structure, conduisant ainsi à une grande variété de colloidosomes. Nous allons détailler dans la suite de cette partie les principales stratégies présentées dans la Figure 13.

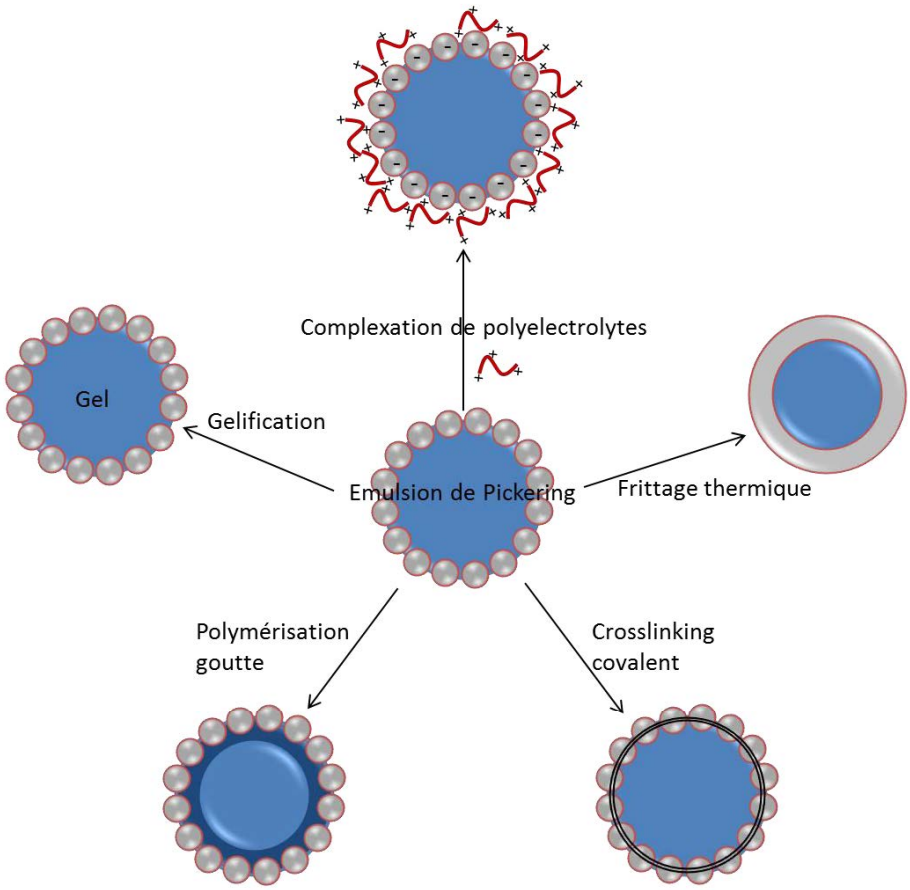

Figure 13: Résumé des différentes voies de stabilisation de colloidosomes par auto-assemblage à l'interface huile/eau. Adaptée de [105]

3.1 Recuit thermique

L'assemblage de billes de polystyrène est maintenu à l'interface de l'émulsion par une étape de frittage (chauffage à 105°C). Les auteurs ont observé des interstices inter-particules et montrent donc une certaine porosité avec une taille d'interstices autour de 150 nm (Figure 14).[100]

En faisant varier la durée de chauffage lors de cette étape de recuit certains auteurs ont suggéré la possibilité de contrôler la porosité de colloidosomes jusqu'à la rendre nulle en formant une membrane continue de polystyrène.[106] Cependant, le choix de matériaux polymères comme le polystyrène, contraint l'étape de frittage à des températures relativement élevée (105°C) et rend impossible l'encapsulation de substances thermosensibles ou de volatilité inférieure. C'est pourquoi Salari *et al.* ont eu recours à d'autres polymères dont la transition vitreuse se situe autour de 50°C. Lors de cette étape de recuit, le problème de fusion inter-colloidosomes a pu également être surmontée grâce à l'utilisation de copolymères à block de type poly(styrene-block-ethylene-co-propylene), la partie hydrophile venant jouer un rôle de stabilisant.[107]

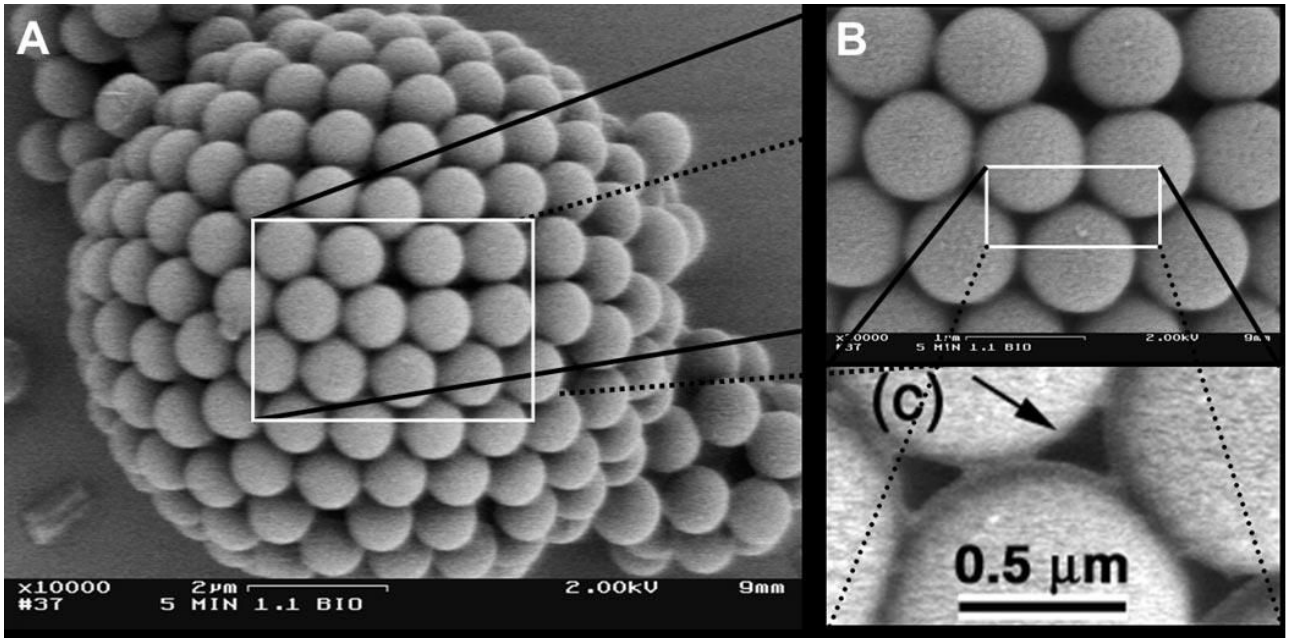

Figure 14: Images de microscopie électronique à balayage de colloidosomes de polystyrène, frittés 5minutes à 105°C à différents grandissements.[100]barres d'échelles (a) 2µm, (b) 1µm et (c) 0.5µm.

3.2 « Layer By Layer » (LBL)

D'autres polyélectrolytes peuvent apporter plus de robustesse à l'assemblage et peuvent être ajoutés au sein des deux phases de l'émulsion: plusieurs couches peuvent être envisagées par adsorption couche par couche (Layer-by-Layer). Li et Stover ont d'abord rendu ationique la surface de particules de silice en adsorbant du PSS. Ils ont ensuite formé les colloidosomes avec les particules de silice fonctionnalisées. Enfin, en venant superposer une autre couche cationique de poly(diallyl dimethylammoniumchloride) (PDADMAC), ils stabilisent et renforcent la structure du colloidosome.[108]

Rossier-Miranda *et al.* ont également adopté le LBL en intercalant des couches de protéines issues du lactosérum (cationiques), de pectine metoxylée (anionique) et de silice, créant ainsi une multitude de combinaisons pour maintenir la structure du colloidosome de silice présenté Figure 15.[109]

Plus récemment des études de relargage d'ibuprofène encapsulé au sein de colloidosomes à base de particules de laponite ont été réalisées par Liu *et al.* Ils ont démontré le contrôle de la porosité des colloidosomes en fonction du nombre de couche de polyelectrolytes présentes.[110]

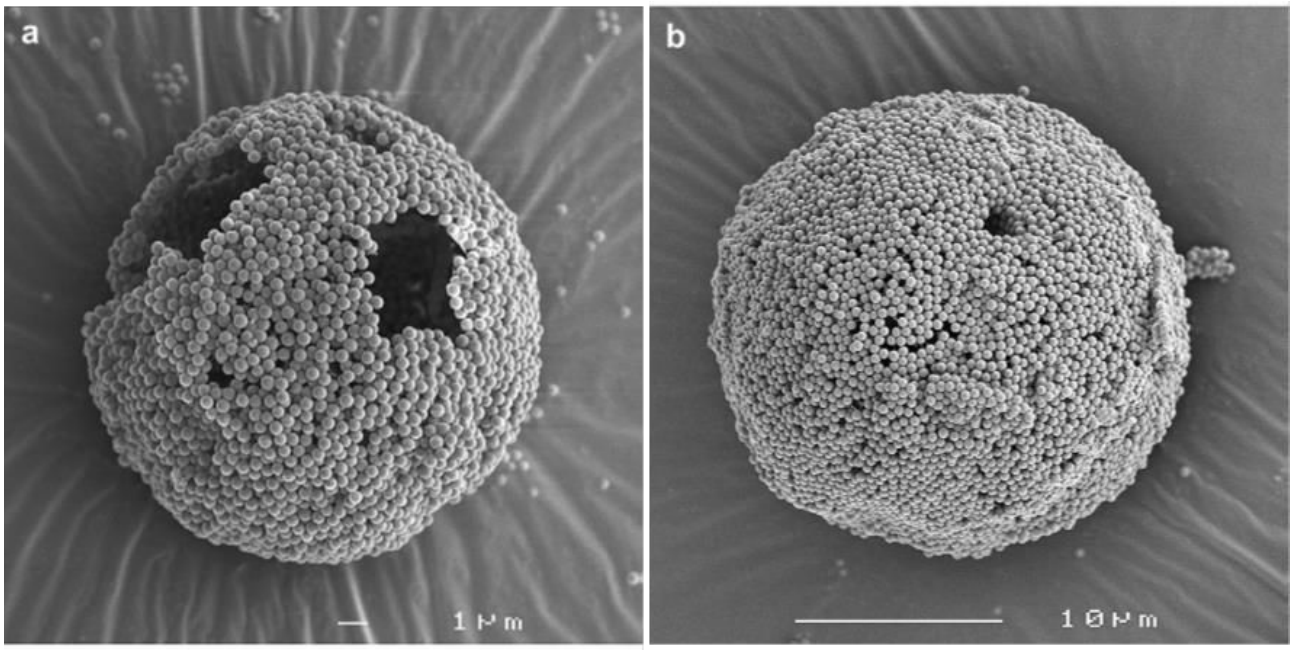

Figure 15: Micrographie SEM de colloïdosomes formés par LBL à partir de particules de silice-NH_2 adsorbées sur (a) une seule séquence de dépôt de protéine cationique/protéine anionique et (b) une séquence de dépôt de protéine cationique/protéine anionique. Adaptée de [109].

3.3 « Gel Trapping »

Une autre technique de stabilisation des colloidosomes consiste à gélifier le cœur dans le cas d'une émulsion eau dans huile. La gélification d'agarose, ou encore d'alginate contenu dans le cœur aqueux, conduit à la rigidification de la structure et permet d'accroître la stabilité de la structure et donc le transfert ensuite vers une phase pure. Pour cela, Cayre *et al.* ont solubilisé de l'agarose à 75°C dans la phase aqueuse avant de créer l'émulsion. Après l'ajout de particules de polystyrène, la gélification a lieu lors du refroidissement de l'émulsion à 25°C figeant ainsi le cœur.[106]

De la même manière, dans le cas d'émulsions huile dans eau, les propriétés de la phase huileuse ont été exploitées afin de cristalliser le cœur huileux en cire.[89,111] Cette méthode permet l'utilisation d'un grand nombre de particules, sans étape de fonctionnalisation au préalable puisque la stabilisation est de nature physique. De plus, cette voie permet l'observation par technique de microscopie électronique de la phase constituant le cœur, une fois figée, et permet donc la caractérisation de grandeur telle que l'angle de contact vu précédemment. En faisant varier le caractère hydrophobe/hydrophile de particules de silice à l'aide d'un surfactant cationique, l'observation des trous laissés par les particules sur la cire ont permis à Granick *et al.* de calculer les angles de contact (Figure 16).[89]

Il est important ici de signaler que ces études sont possibles dans le cas de colloidosomes de grande taille. Dans les exemples présentés dans cette partie, la taille des particules solides est de l'ordre du micron et les colloidosomes formés présentent des tailles de l'ordre de la centaine de microns.

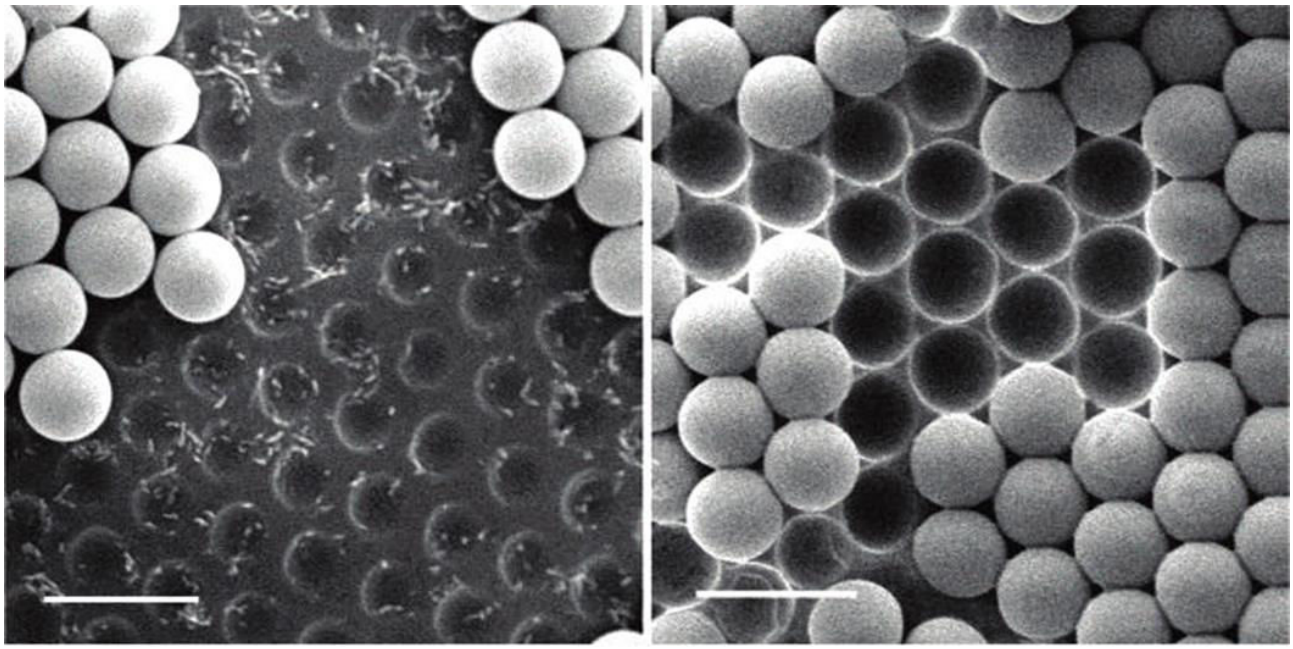

Figure 16: Clichés SEM de particules de silice adsorbées à l'interface cire/eau laissant apparaitre des creux plus ou moins importants selon le caractère hydrophobe croissant des particules. Barres d'échelle 5 µm. Adaptée de [89].

3.4 Polymerisation et réticulation

Les colloidosomes peuvent aussi servir de réacteurs et certaines modifications chimiques peuvent être initiées à partir d'un des constituants de l'émulsion de Pickering : de la phase continue, de la phase discontinue ou de l'interface par l'intermédiaire des particules. Par exemple, dans le cas d'émulsions huile dans eau, la modification de surface des particules par un initiateur permet une polymérisation d'hydroxy-méthacrylate en surface du colloidosome.[112]

Une autre stratégie consiste à solubiliser l'initiateur dans la phase huileuse et ainsi initier la polymérisation par le cœur du colloidosome.[113] Enfin, toujours dans le cas d'émulsion huile dans eau, en solubilisant un polymère de type polyméthylacrylate (PMMA) au sein d'un cœur dichlorométhane/hexadécane, la polymérisation ou plutôt la précipitation a lieu lors de de l'évaporation partielle du cœur. En effet le dichlorométhane s'évapore dans un premier temps laissant le cœur essentiellement composé d'hexadécane, mauvais solvant pour le PMMA. Le polymère précipite alors et se retrouve piégé à l'interface par la couronne de particules.[114]

Cette technique est aussi décrite comme procédé de réticulation puisqu'il y a réaction chimique au sein de l'émulsion de Pickering. Très souvent il s'agit de polymérisation et les principales stratégies (radicalaire, ouverture de cycle) ont été mises en œuvre.[115] Ces stratégies suscitent beaucoup d'intérêts puisqu'elles permettent d'apporter une nouvelle fonctionnalité à l'assemblage grâce à des polymères thermosensibles,[116] pH sensibles,[117] ou photoactivables.[118,119]

Dans la continuité de cette démarche le cas de colloidosomes multiples ont été préparés. La technique développée permet à partir de particules de silice fumée fonctionalisées par un polymère de type poly(ethylèneimine) (PEI) et grâce à deux agents réticulants de type poly(propylèneglycol) et poly(ethyleneglycol) (PPG-DEG et PEG-DEG), de créer des colloidosomes multicompartiments au sein d'émulsions doubles représentés en Figure 17. En modifiant simplement les rapports de masses silice/PEI, le contrôle de la mouillabilité des particules permet de basculer d'une émulsion H/E à E/H.

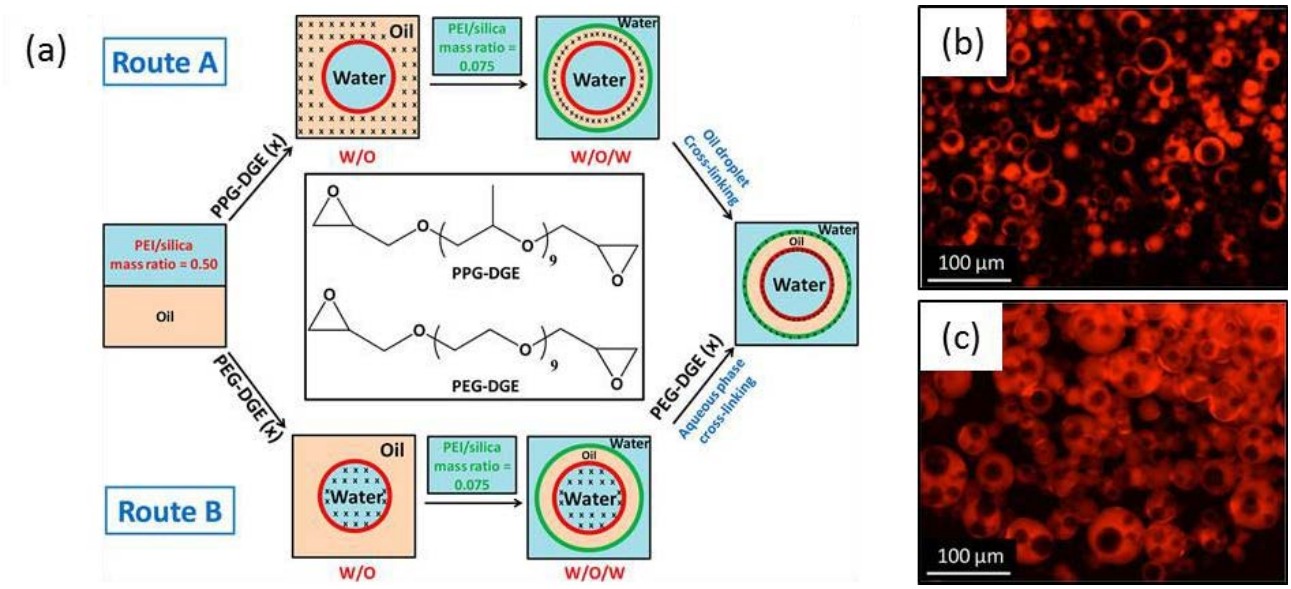

Figure 17: (a) Schématisation des deux voies de synthèses de colloidosomes multicompartiments à partir de particules de silice/PEI. La route A montre la formation de colloidosomes-dans-colloidosomes en solubilisant l'agent réticulant PPG-DEG dans la phase huileuse avant homogenisation. La route B présente la formation de colloidosomes-dans-colloidosomes par l'ajout en deux fois de l'agent réticulant (PEG-DEG) dans la phase aqueuse. (b) Image de microscopie confocale obtenues avec le fluorophore Rouge Nile dans la phase (b) huileuse au sein de l'émulsion double E/H/E et (c) aqueuse. Adaptée de [120]

Par cette méthode des doubles émulsions de type E/H/E peuvent être obtenues. Ensuite la réticulation peut être effectuée à la fois dans les phases huileuse ou aqueuse en utilisant l'agent réticulant approprié, PPG-DEG et PEG-DEG respectivement.[120]

En résumé, une grande variété de colloidosomes peut être préparée à partir des émulsions de Pickering selon plusieurs voies. Certaines de ces méthodes requièrent un traitement thermique, ce qui peut être dommageable pour l'espèce encapsulée. Cependant lorsque la stabilisation se fait par voie chimique,

la nature et localisation de l'agent réticulant doivent être choisis avec précaution afin de minimiser les interactions avec l'espèce encapsulée. D'une manière générale, le grand choix de particules et de polymères permettent d'obtenir des colloidosomes fonctionnels pour des applications particulières. Par exemple, des colloidosomes thermosensibles[116] ou magnétosensibles[121], ou pH sensibles[117] ont été obtenus. Les colloidosomes offrent de nombreuses potentialités pour l'encapsulation, mais qui restent cependant limités à quelques heures dans le cas de petites molécules[122].

Cela semble orienter vers le de dépôt d'une nouvelle couche protectrice, ou vers l'encapsulation de macromolécules qui diffuseront plus lentement vers l'extérieur.

4. Effet ouzo

4.1 Principes et définitions

Parfois appelé nanoprécipitation, déplacement de solvant, émulsification spontannée[123], l'effet Ouzo tire son nom d'une célèbre boisson alcoolisée grecque (l'Ouzo) et l'on retrouve ce phénomène dans d'autres breuvages comme le Pastis en France ou encore le Raki en Turquie. Ce phénomène prend place dans un mélange ternaire composé de deux liquides miscibles et d'un soluté hydrophobe en très petite quantité.

Dans l'exemple du pastis, il s'agit d'un mélange d'eau, d'alcool ainsi que du trans-anéthol (0.2 %).[124] Cet arôme est soluble dans l'alcool avec qui il constitue la phase solvant. Cependant le soluté n'est pas soluble dans l'eau et lorsque ce mélange est dilué avec de l'eau, un phénomène de sursaturation a lieu, conduisant à la nanoprécipitation du soluté. Cette précipitation conduit à la formation de gouttes de soluté qui donne un aspect turbide à la solution. A basse température la cristallisation des gouttes peut être observée.

Considérons un mélange ternaire composé de :

- un soluté : molécule pouvant s'apparenter à une phase huile, un polymère, parfois liquide ou solide
- un solvant dans lequel le soluté est soluble
- un second solvant, très souvent de l'eau, dans lequel le soluté n'est pas soluble, mais totalement miscible au premier solvant

L'émulsification spontannée du soluté au sein du mélange se fait dans une zone métastable (zone Ouzo) située entre les courbes binodales et spinodales (Figure 18). La courbe binodale représente la limite de solubilité tandis que la courbe spinodale correspond à la limite de la stabilité thermodynamique du système. La binodale sépare donc le domaine homogène à une seule phase du domaine métastable ou

deux phases apparaissent. La spinodale délimite quant à elle le domaine dans lequel la séparation de phase a lieu selon un procédé de nucléation croissance, du domaine ou les phases apparaissent spontanément (Figure 18). La zone Ouzo se trouve entre ces deux courbes et pour de faibles quantités de soluté. Elle est caractérisée par une émulsification spontanée en gouttes de taille homogène dans le cas d'un soluté liquide. Dans le cas d'un soluté solide, des nanoparticules solides de taille homogènes sont obtenues. [124–127]

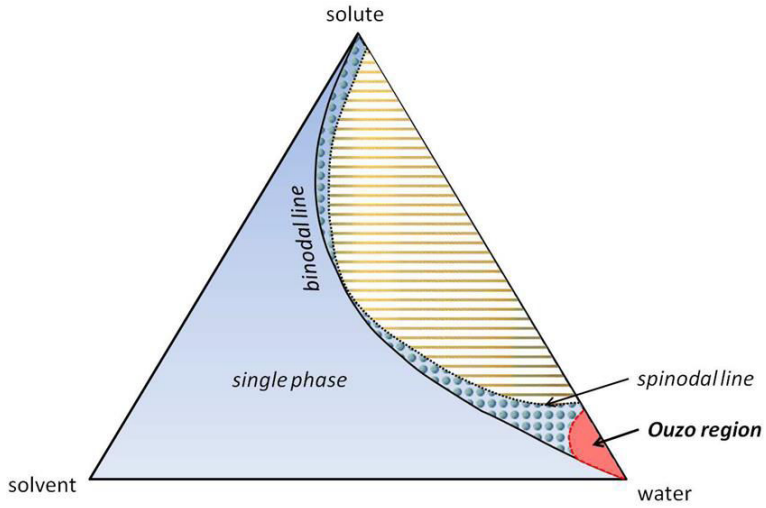

Figure 18: Diagramme de phase d'un système ternaire soluté/solvant/eau. Adaptée de [125].

Depuis quelques années le procédé n'apparait plus comme un cas métastable accidentel et des efforts sont faits pour comprendre ce phénomène particulier que l'on peut aussi considérer comme une émulsion sans surfactant. Plusieurs systèmes ternaires ont été étudiés dans le domaine Ouzo afin de comprendre et d'expliquer ce phénomène. Les tailles de gouttes peuvent varier de l'ordre d'une centaine de nanomètre[128] à plusieurs micromètres, dépendant essentiellement de l'excès de soluté.[129] Cependant le phénomène Ouzo forme des gouttes remarquablement monodisperses.[130]

Dans le domaine Ouzo, des gouttes ont d'abord été observé dans des mélanges de type divinylbenzene/éthanol/eau ou trans-anéthol/éthanol/eau.[128,130–133] La taille de goutte a d'abord été reliée à l'excès de concentration du soluté dans la phase solvant, c'est-à-dire à l'excès de concentration par rapport à la valeur de concentration à saturation dans la phase continue solvant/eau : plus cet excès est important, plus le diamètre moyen des gouttes est élevé. [134]

Plus tard les techniques de RMN ont permis de proposer un mécanisme par la formation initialement de petits agrégats d'environ 2 nm, puis une rapide coalescence en des gouttes micrométriques observables alors par les techniques de diffusion de la lumière ou de neutrons.[128] Cette croissance rapide s'explique d'après le mécanisme d'agrégation limité par la diffusion dans lequel les petits agrégats croissent plus rapidement que les gros. D'autres études ont également pu montrer l'existence de gouttes d'environ 200 nm en utilisant une plus grande quantité en soluté.

Dans des mélanges tertiaires de type eau/solvant/soluté Bouchemal *et al.* ont montré que la taille des gouttes diminues lorsque la viscosité du solvant est élevée.[129] Il également important de noter que dans cette étude les solutés sont des molécules amphiphiles de type acide gras (tocophérol, Myritol®, hexyl laurate, etc).

La température est un facteur qui joue également un rôle sur la taille et la distribution des gouttes obtenues puisque la solubilité du soluté est aussi fonction de la température. A plus haute température, la solubilité augmente et la quantité de soluté en excès est alors plus faible, ce qui conduit à des gouttes de plus petites taille et à une distribution plus fine.[126,134,135]

4.2 Nanoprécipitation par effet Ouzo

Beaucoup de travaux ont été menés dans le développement de particules polymériques et les phénomènes de précipitation sont bien connus. Cependant, le phénomène d'émulsification spontanée dans la région métastable a probablement mal été interprété dans certains cas.[126] Plusieurs observations ont été décrites dans le cas de solutés solides tels que des polymères, biopolymères, copolymères ou terpènes. Dans ces travaux les mécanismes sont identifiées comme émulsification spontanée, précipitation spontanée, polymérisation spontanée ou encore polycondensation spontanée. Ganachaud *et al.* ont proposé une réinterprétation de ces phénomènes par l'effet Ouzo.[126] Nous allons voir dans cette partie plusieurs exemples qui peuvent être associés à l'effet Ouzo et qui conduisent à des nanoparticules ou nanocapsules.

Stainmess *et al.* ont montré dans le cas de poly(cprolactone) (PCL) que la présence d'un surfactant n'était pas nécessaire. Ils ont observé la nanoprécipitation de la PCL en particules d'environ 120 nm lorsque la PCL se trouve solubilisée en faible quantité dans l'acétone et en présence d'une phase aqueuse.[136] Dans ce cas, les auteurs établissent la relation entre la quantité de PCL et la constante diélectrique du mélange final pour prédire la taille des particules. Par la suite, de nombreux travaux ont mis en évidence la nanoprécipitation d'autres polymères [137] et de copolymères à blocs[138] parfois en présence de surfactants.

Certains travaux ont utilisés des solutés solides et de taille plus petite que les polymères, par exemple des composés de la famille des terpénoïdes. Couvreur *et al.* ont envisagé une approche pro-drogue en couplant le squalène à différents principes actifs.[139,140] Les composés amphiphiles résultants possèdent la particularité de s'assembler dans un mélange éthanol / eau, en des particules d'environ 150 nm (Figure 19).

Dans certains cas l'eau ou le solvant peuvent déclencher la polymérisation du soluté. Par exemple dans le cas des poly(cyanoacrylates), la polymérisation anionique peut être initiée en présence d'une petite quantité d'OH^-.[141] Dans ce cas on parle de polymérisation spontanée. Dans ces conditions les phénomènes de précipitation et polymérisation sont en compétition et lors de l'émulsification de poly(alkylcyanoacrylates), des nanocapsules et nanoparticules sont obtenues.[142,143]

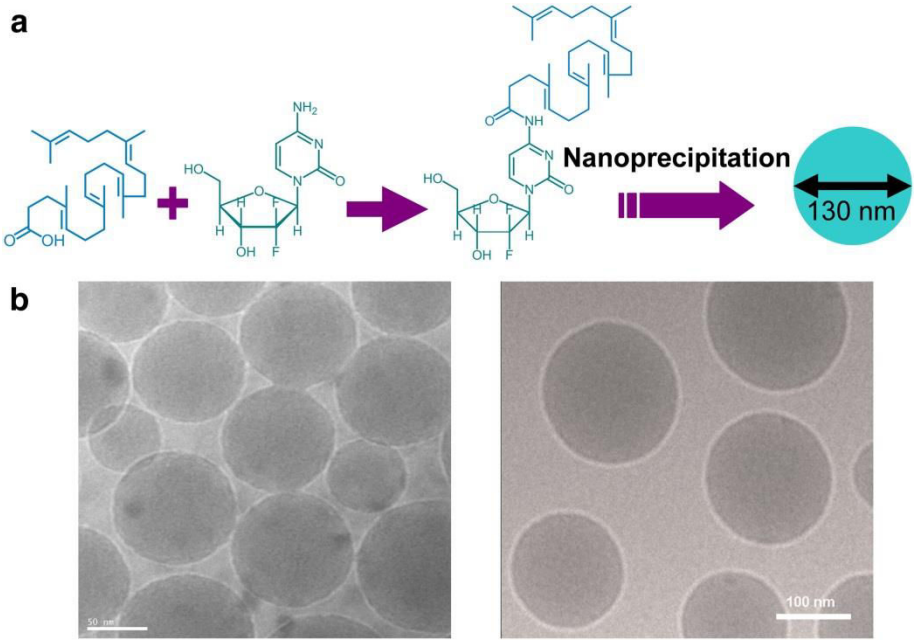

Figure 19: (a) couplage de l'acide squalenique avec la gemcitabine et auto-assemblage dans l'eau par nanoprécipitation, formant des particules d'environ 130nm. (b) Micrographies cryo-TEM des nanoparticules squalenoyl-paclitaxel (gauche) et squalenoyl-peniciline G (droit). Adaptée de [124].

Des observations similaires de nanocapsules avaient déjà été reportées en 1989 par Fessi *et al.* ont. Ces premiers travaux ont été réalisés dans un mélange d'acétone contenant de l'indométacine comme soluté et d'une phase aqueuse. En mettant également en présence un d'acide polylactique (PLA), Fessi *et al.* ont ainsi observé des capsules de l'ordre de 230 nm.[144] D'autres travaux mentionnés dans la partie 1.1 en présence de poly(g-benzyl-L-glutamate)-block-hyaluronan (PBLG-b-HA) conduisent à des capsules de polymères (polymersomes) et ont parfois été corrélés à l'effet Ouzo.[4,124]

Enfin, dans de rares cas l'utilisation de nanoparticules inorganiques a été mise en œuvre dans ces conditions. Sacana *et al.* ont mis en jeu du methacryloxypropyltrimethoxysilane (TPM) et des oxydes de fer en présence d'eau. L'émulsification spontanée conduit à des assemblages de 50 nm stables dans le temps. Cependant pour faciliter l'observation les auteurs initient polymérisation des gouttes de TPM qui solidifie le cœur et permet d'observer au microscope électronique les sphères sur lesquelles sont piégés les particules d'oxydes de fer.[145]

Dans la plupart de ces travaux, la nanoprécipitation de solutés polymériques conduit à l'assemblage de nanosphèsres. Cependant, dans certains cas l'observation de nanocapsules soulèvent des questions à propos des phénomènes interfaciaux. Le comportement et la structuration des solutés dans la zone Ouzo est sont encore

Dans ce contexte, nous nous sommes intéressés au comportement de nanoparticules dans ces conditions. Dans la suite de cette thèse, nous présentons un procédé d'assemblage de nanocristaux inorganiques d'environ 5 nm en nanocapsules d'environ 100nm. Nous aborderons dans le chapitre 2 le développement de ce procédé ainsi que la caractérisation des nanocapsules formées, et ce à plusieurs échelles. Ensuite, dans le chapitre 3, nous présenterons l'utilisation des nanocapsules comme building block dans la construction de films nanostructurés, multifonctionnels et sensibles. Dans un quatrième chapitre nous aborderons les potentialités des nanocapsules en tant que plateforme en nanomédecine. Enfin dans un ultime chapitre, nous élargirons le procédé d'assemblages à des clusters métalliques. Nous présenterons leur auto-assemblage en de nouvelles nanosphères et nanocapsules.

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Chapitre 2

Auto-Assemblage d'un nouveau type de nanocapsules hybrides: les Hybridosomes®

1. Contexte initial : des agrégats aux nanocapsules.

Dans ce chapitre nous allons nous intéresser à la première famille d'assemblages développée lors de cette thèse. Ce travail fait suite à la thèse du Dr. G. Casterou en co-tutelle entre l'ISCR (Dr. F. Gauffre) et le LCC à Toulouse (Dr. M. Kahn).

G. Casterou a consacré la première partie de son travail à la synthèse de nanoparticules d'oxydes de fer (IONP) par une approche organométallique. Ses travaux ont consisté à l'optimisation de la synthèse d'une phase cristalline pure de type maghémite Fe_2O_3, un contrôle de la taille des nanoparticules afin d'optimiser leurs propriétés superparamagnétiques (magnétisation à saturation M_v et relaxivités transversales r_2)[1]. Dans une seconde partie, il s'est ensuite intéressé à l'assemblage de ces nanoparticules afin d'exacerber à nouveau les propriétés magnétiques. En effet, il est reporté dans plusieurs travaux que les propriétés magnétiques des particules dépendent de leur taille et qu'une particule de taille inférieure à 30 nm présente de meilleures propriétés.[2] De plus, l'assemblage de telles particules sous formes d'agrégats permet des interactions interparticules différentes selon l'état de surface des particules, la taille et la forme des agrégats. Ainsi il est possible d'atteindre des valeurs de r_2 élevées avec de forts potentiels comme agent de contraste en imagerie de résonnance magnétique (IRM)[1,3]. La stratégie d'assemblage était basée sur l'effet solvophobe. Il s'agit de mettre les nanoparticules en présence d'un mauvais solvant et ainsi les déstabiliser, les contraindre à s'agréger par interactions hydrophobes. Les nanoparticules fonctionnalisées par un ligand de type octylamine, ont d'abord été dispersées dans le tétrahydrofurane (THF). Ensuite, plusieurs quantités d'eau ont été ajoutées et différentes compositions eau/THF ont été étudiées par spectroscopie UV-Visible afin de déterminer la stabilité colloïdale des suspensions. Dans les cas où les suspensions ne sont pas stables, l'étude du surnageant permet l'estimation de la fraction sédimentée. Les suspensions ont également été caractérisées par diffusion de la lumière (DLS) ainsi que par microscopie électronique à transmission (TEM) dans le but de caractériser la taille et la structure de ces agrégats. Il a été remarqué plusieurs types de comportements dans ces suspensions de nanoparticules dans des mélanges eau/THF. Pour des fractions volumiques en eau φ_w comprises entre 2 et 30 % les suspensions n'étaient pas stables et les particules sédimentaient 16 h après ajout du mauvais solvant. En revanche, pour des suspensions plus riches en eau, $35 < \varphi_w < 60$ %, les suspensions apparaissent stables 16 h après ajout du mauvais solvant[4] (Figure 20b).

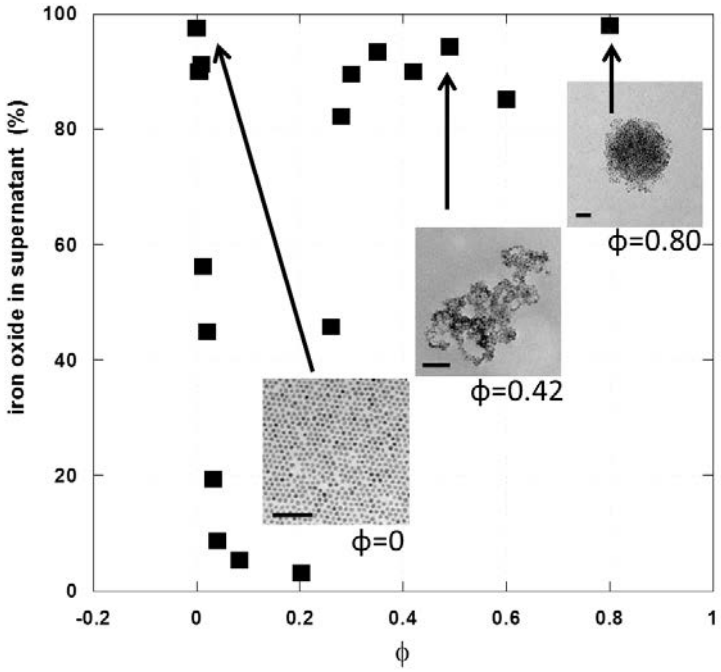

Figure 20: Stabilité des IONP dans le mélange THF/Eau en fonction de φ_w et micrographies TEM obtenus à partir des surnageants, barres d'échelle 50 nm.

Afin de figer ces agrégats et ainsi pouvoir les observer en TEM et les transférer en phase exclusivement aqueuse, la stratégie a été d'ajouter un polymère de type poly(vinylpyrolidone) (PVP), acide polyacrylique (PAA) ou encore un copolymère de type PEG-PAA (PEG= polyéthylène glycol). En effet, le PAA et la PVP sont connus pour avoir de fortes interactions avec les oxydes de Fer [5,6]. Le PEG quant à lui est reconnu depuis de nombreuses années pour apporter une stabilité colloïdale en milieu aqueux, une meilleure pharmacocinétique à de nombreux principes actifs [7] et est approuvé pour son usage en milieu clinique[8]. Plusieurs tailles de polymères et copolymères ont été étudiées mais le diamètre hydrodynamique des agrégats formés (entre 200 nm et 730 nm) ne semblait pas suivre de tendance particulière ni en fonction de la nature, ni de la taille des polymères. Enfin, les agrégats formés ont été évalués *in vitro* comme agents de contraste en IRM et les meilleurs résultats ont montré des relaxivités transversale (r_2) de l'ordre de 466 $mM^{-1}.s^{-1}$ et 275 $mM^{-1}.s^{-1}$ pour des agrégats stabilisés respectivement par du PAA450k (PAA M = 450 000g.mol^{-1}) et de la PVP360k (PVP M = 360 000g.mol^{-1})[1,4].

A la suite de ces travaux de thèse, nous nous sommes plus particulièrement intéressés aux fractions volumiques encore inexplorées dans les mélanges THF/eau en présence et absence de nanoparticules, c'est-à-dire les mélanges riches en eau.

En effet plusieurs travaux proposent l'existence de mésostructures,[9] inhomogénéités[10,11] ou encore des nanobulles de CO_2[12] au sein des mélanges miscibles utilisés qui pourraient avoir une influence dans le mécanisme de formation des agrégats formés jusqu'à présent.

Nous avons également étendu cette étude à d'autres types de nanoparticules dans le but d'investiguer des assemblages composés de particules plasmoniques de métal noble (Au), luminescentes comme des semi-conducteurs (quantums dots = QD de type CdSe@ZnS), des particules up-converting (UCNP) à base de lanthanides, ainsi que leur combinaisons. Ces premiers développements ont conduits à l'élaboration de nanocapsules, appelées Hybridosomes®. Le procédé d'auto assemblage de ces capsules hybrides a également conduit au dépôt d'un brevet[13] ainsi qu'à la publication d'un article présenté ci-après dans la partie 2.[14]

2. Article 1: Simple Engineering of Polymer - Nanoparticle Hybrid Nanocapsules

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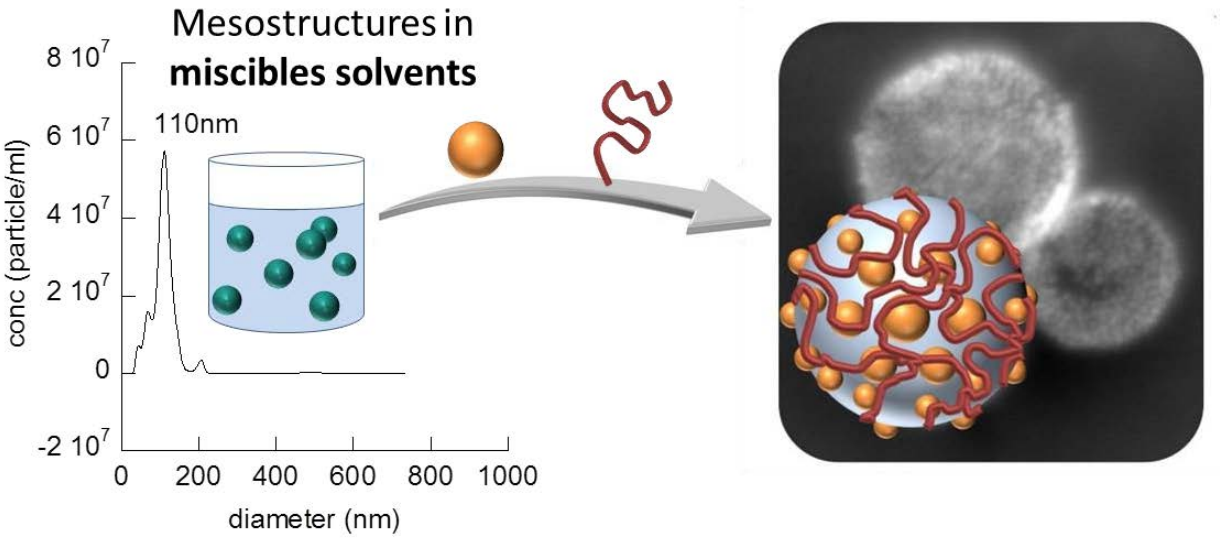

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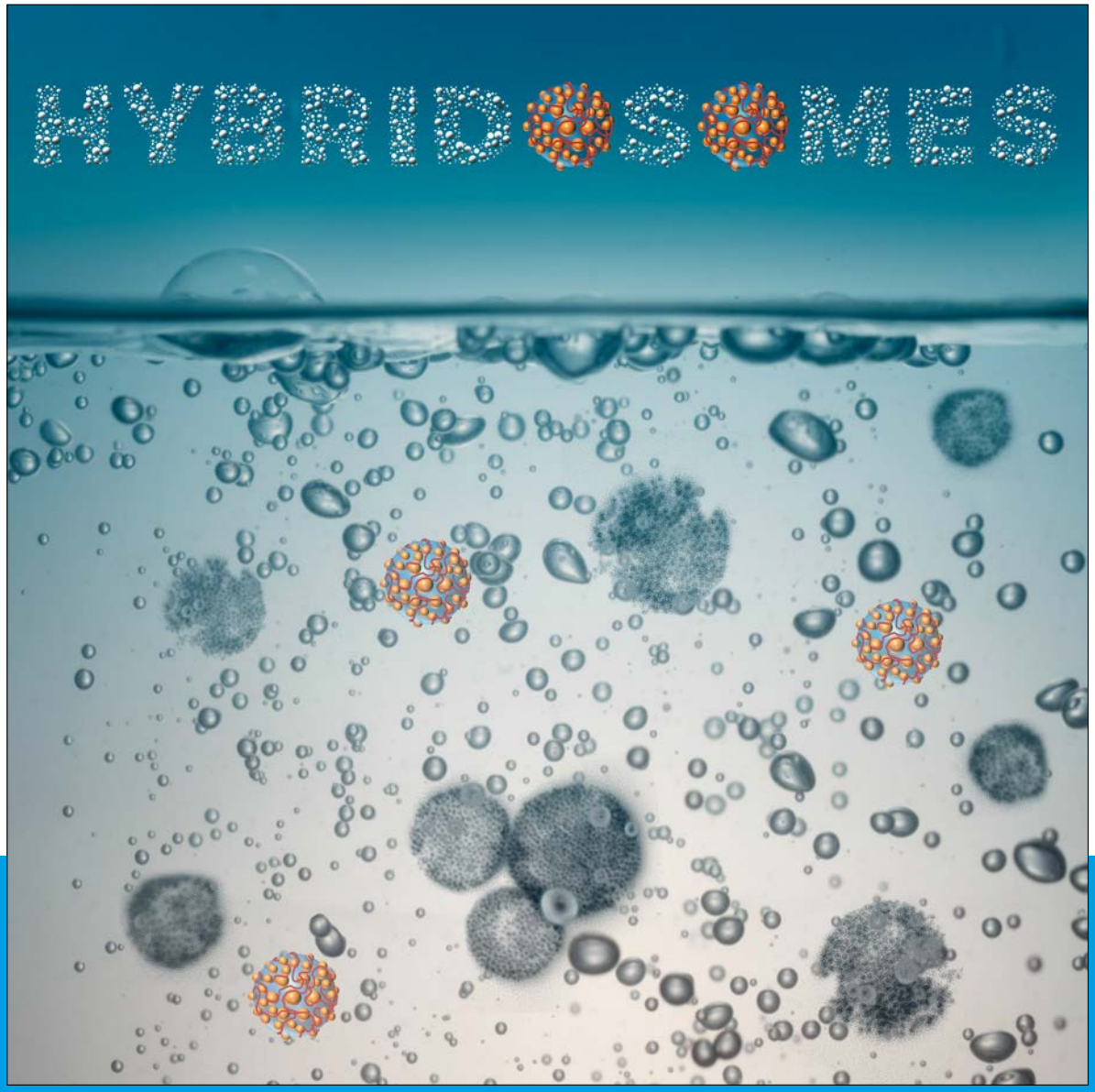

8/02
2016

A Journal of

ACES
Asian Chemical Editorial Society

A sister journal of **Chemistry – An Asian Journal** and the **Asian Journal of Organic Chemistry**

Cover Picture:
M. L. Kahn, F. Gauffre et al.
Simple Engineering of Polymer–Nanoparticle Hybrid Nanocapsules

WILEY-VCH

DOI: 10.1002/cnma.201600155

Self-Assembly

Simple Engineering of Polymer–Nanoparticle Hybrid Nanocapsules

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Abstract: A general method to generate hybrid hollow capsules is reported. The process is based on the stabilization of solvent droplets by nanoparticles, in macroscopically miscible mixtures of tetrahydrofuran (THF) and water. After addition of a crosslinking polymer and removal of the solvent core, capsules of diameter ca 100 nm are obtained. This novel strategy does not require the use of block copolymers. In contrast, most methods reporting the formation of hybrid nanocapsules incorporate nanoparticles into block-copolymer polymersomes or use nanoparticles tethered with block-copolymers. The nanocapsules were characterized using a full set of techniques including nanoparticle tracking analysis, electron microscopy and liquid phase atomic force microscopy. Our results show that the vesicular shape of the nanocapsules is templated by the liquid droplets. Nanocapsules were prepared from quantum dots, gold nanoparticles, superparamagnetic iron oxide nanoparticles and mixtures of particles. The entrapment of a fluorescent dye was also demonstrated. Thus, nanocapsules with dual properties (e.g., magnetic and fluorescent) are easily obtained. Interestingly, the magnetic nanocapsules enable magnetic resonance imaging contrast enhancement of tumors in vivo.

The interest in forming assemblies of inorganic nanoparticles and polymers is diverse. On the one hand, the nanoparticles bring optical or magnetic properties that can be used for sensing, imaging, or heating, whereas on the other hand the polymers provide functions such as response to external stimuli (pH, temperature), stealthiness and molecular recognition.[1] Although such assemblies hold much promise as multifunctional platforms for nanomedicine, the control of their size and morphology remains a daunting challenge. A variety of strategies have been proposed to design nanoparticle-polymer assemblies.[2] For instance, the in situ synthesis of nanoparticles within linear or hyperbranched polymers is generally a convenient one-pot procedure in which the polymer promotes the nucleation of the particle while preventing uncontrolled clustering.[3,4] Alternatively, nanoparticles can be encapsulated within block-copolymer micelles[5,6] or co-assembled with polymers via nanoprecipitation or electrostatic interactions.[7,8] Recently, many efforts have been devoted to the design of hybrid nanoparticle-polymer nanocapsules, which offer the possibility to encapsulate and potentially release drugs. Thus, polymersomes incorporating inorganic nanoparticles into their membranes were fabricated via the cooperative self-assembly of amphiphilic block-copolymers and nanoparticles.[9] Alternatively, preformed polymersomes were armored with a layer of nanoparticles via electrostatic interactions.[10] It was also demonstrated that inorganic nanoparticles tethered with block-copolymers self-assemble into superstructures such as clusters and vesicles.[11–15] All these strategies have in common the use of block copolymers which direct the self-assembly.

We report here for the first time a general process to generate hybrid hollow capsules without the need for block-copolymers. The formation of the vesicles is templated by solvent droplets in mixtures of water and THF. Indeed, although water and THF are macroscopically miscible, nanodroplets are formed in the water rich region, which we used as templates for the self-assembly of the nanoparticles. After crosslinking

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the nanoparticles with a suitable polymer the solvent core can be removed. The resulting vesicles possess a "composite" or "hybrid" nanoparticle-polymer shell. In the following we will refer to them as hybridosomes. Based on these findings, we elaborated hybridosomes from a variety of nanoparticles and evaluated the in vivo MRI efficiency of hybridosomes made from superparamagnetic iron oxide nanoparticles (SPION).

We have all learned from our textbooks that water is miscible in all proportions with THF. However, dynamic light scattering (DLS) and small angle neutron scattering measurements clearly evidence fluctuations of concentrations for specific compositions of water/THF mixtures. Such fluctuations have also been reported for mixtures of water with other "miscible" solvents such as acetone or alcohols,[16–18] the nature of which is still under debate.[19] It has been suggested that they could be either solute droplets[16] or air bubbles.[20] Using nanoparticle tracking analysis (NTA) and analytical centrifugation, Sedlak et al. have provided convincing evidence of the existence of droplets of about 100 nm in diameter in various mixtures of miscible liquids.[16]

Inspired by these results, we hypothesized that such droplets could template the formation of nanoparticle shells, similarly to Pickering emulsions. Nanoparticles are not amphiphilic but strongly adsorb at interfaces between polar and non-polar liquids.[21] We first looked for solvent droplets in water/THF mixtures in a wide range of compositions ($\varphi_w = 0.05$–0.9, water vol. fraction). Time fluctuations were clearly measured using DLS, with faster dynamics as more water was added (Figure S1 in the Supporting Information). Particularly, a mode corresponding to the Brownian motion of small objects (length scale ~ 100 nm) appeared for compositions close to $\varphi_w = 0.8$. Note that DLS is sensitive to any fluctuation and that the dynamic modes that were measured do not necessarily correspond to droplets. To verify the presence of droplets, NTA was undertaken for compositions with the fastest correlation modes ($\varphi_w = 0.8$–0.9). The results clearly evidenced finite structures with a hydrodynamic diameter $D_h = 110 \pm 35$ nm (Figure S2).

We then investigated the water/THF/NP (NP = nanoparticles) mixtures using dodecanthiol-coated gold nanoparticles (AuNP) (Figure S3a). In the presence of small or moderate amounts of water ($\varphi_w = 0.05$ to 0.4), a bad solvent for the NPs, they rapidly aggregate and sediment as expected. However, stable suspensions were found at higher water contents ($0.5 < \varphi_w < 0.9$), the most stable samples ($\varphi_w = 0.8$) showing no sign of sedimentation over more than one year. A similar behavior was found for γ-Fe_2O_3 (maghemite) SPION (Figure S3 b). Transmission electron microscopy (TEM) revealed relatively ill-defined structures for $\varphi_w = 0.42$, whereas regular spherical aggregates with a diameter of ca 100 nm were obtained at $\varphi_w = 0.8$. DLS measurements clearly evidenced the stabilization of the smaller droplets at the expenses of larger ones in the presence of the nanoparticles (Figure S4).

Based on these findings, we proposed a general strategy to elaborate stable nanoparticle–polymer capsules (Figure 1). Starting from THF solutions of hydrophobic nanoparticles the self-assembly is triggered by adding water to a final volume

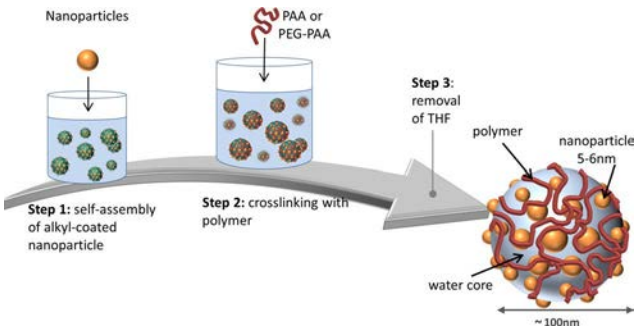

Figure 1. Creation process of the hybrid nanocapsules.

fraction $\varphi_w = 0.8$. This causes the nanoparticles to gather at the surface of solvent droplets (step 1). Upon addition of a suitable polymer, a crosslinked network of nanoparticles and polymers forms around the droplets (step 2). Finally, the removal of the organic solvent yields the hybridosomes (step 3).

As a proof of principle, we first created magnetic hybridosomes from SPIONs. Assemblies of SPIONs are of particular interest in a wide range of applications including magnetic resonance imaging (MRI)[22] and protein separation,[23] among others. However, due to their small size, single SPIONs are difficult to collect under a magnetic field and make poor MRI contrast agents. A large number of theoretical and experimental studies have demonstrated that clustering of SPIONs increases the transversal relaxivity r_2, thereby enhancing the efficiency as T_2 contrast agent.[24–27]

Magnetic hybridosomes were prepared following the scheme depicted in Figure 1. Poly(acrylic acid) (PAA) was selected as the polymer for its ability to link to iron oxide, via the multiple carboxylic acid functions.[28] Alternatively, we used a poly(ethylene glycol)-*co*-PAA diblock copolymer to prepare PEGylated hybridosomes (Figure 5 a, see below).[29] The scanning electron microscopy (SEM) analysis of the dried samples at step 1 and step 2 clearly evidenced deflated capsules (indicated by arrows, Figure 2) together with spherical ones. In their hydrated state, the hybridosomes adopt a swollen shape as shown by AFM analysis in water (Figure 3). To confirm their hollow structure, we imaged the hybridosomes using TEM tomography (Figure 4, Movie S1). Due to the small size of the pristine nanoparticles, it is difficult to identify them individually on the 3-dimensional reconstruction. However, the hollow structure is clearly confirmed both by the animated tilted projection (Movie S1) and the 3D-reconstruction. The templating effect of droplets clearly appears when a lower amount of nanoparticles is used as in Figure S5. In this case, the partial coverage of the surface by nanoparticles underlines the shape of the initial droplet. All together, these analyses strongly support the hypothesis that the hybridosomes are soft hollow capsules. The hybridosomes were easily separated from the supernatant by applying a magnet and then resuspended without any noticeable aggregation.

Mixed hybridosomes of AuNP and SPIONs as well as hybridosomes from quantum dots (QDs) were also prepared (Figure 5 b–c). TEM (Figure 5 b) and energy-dispersive X-ray spec-

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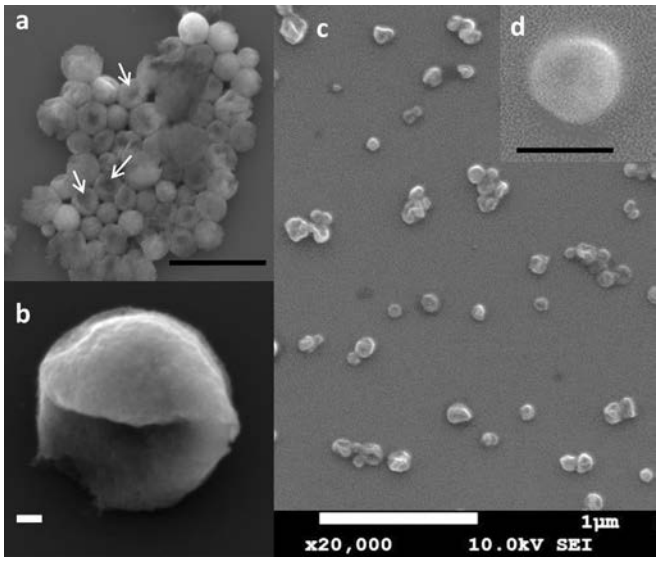

Figure 2. SEM images of (a, b) assemblies of SPIONs in water/THF mixtures; and (c, d) magnetic hybridosomes. (a) [Fe] = 65 µg mL^{-1}; (b) [Fe] = 80 µg mL^{-1} (c, d) [Fe] = 25 µg mL^{-1}; polymer: PAA450k. Bars: (a, c) = 1 µm; (b, d) = 100 nm. White arrows indicate deflated capsules.

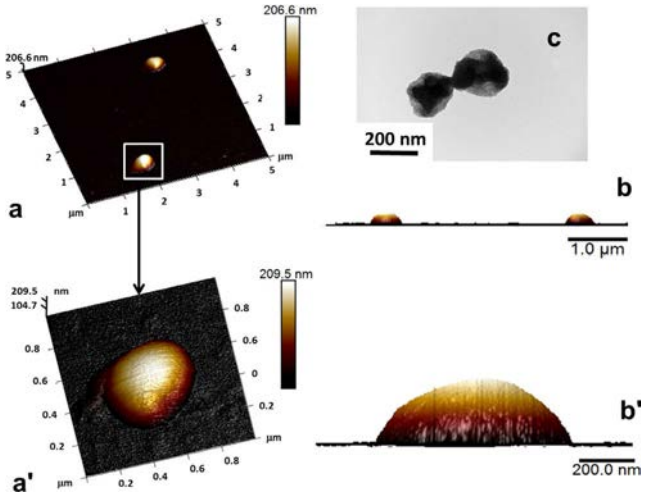

Figure 3. AFM of SPIONs hybridosomes in aqueous solution. (a, a′) 3D topography; (b, b′) lateral views; (c) TEM image of the corresponding sample. [Fe] = 65 µg mL^{-1}; PAA450k.

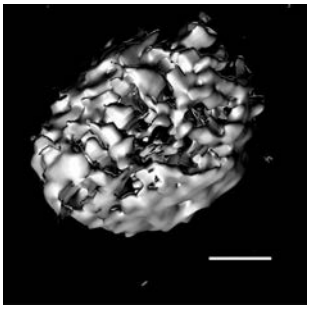

Figure 4. Top view of the 3D density map reconstructed from TEM-tomography of magnetic hybridosomes (SPION [Fe] = 50 µg mL^{-1}; polymer: PAA450k). Scale bar = 20 nm.

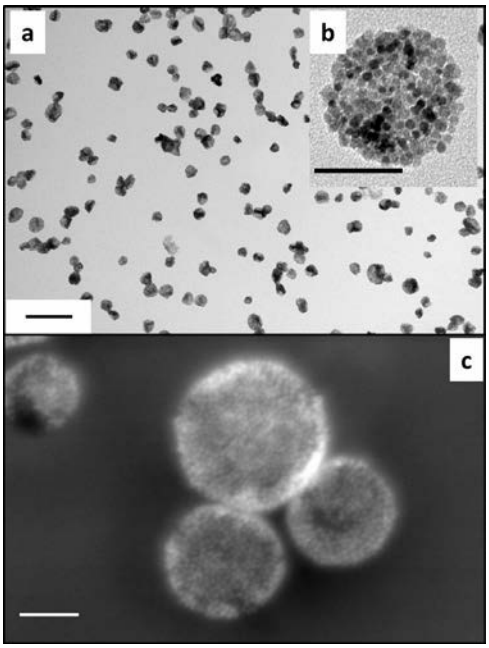

Figure 5. TEM of (a) SPION hybridosomes ([Fe] = 50 µg mL^{-1}; polymer: PEG-PAA); (b) mixed hybridosomes from AuNP and SPIONs, the AuNP appear with darker contrast ([Fe] = [Au] = 26 µg mL^{-1}; polymer: PAA450k); (c) SEM of (CdSe@ZnS)QD-hybridosomes ([QD] = 26 µg mL^{-1}, PAA450k). Bars: (a) 500 nm; (b) 50 nm; (c) 100 nm.

troscopy (EDX) (Figure S6) analysis of the mixed AuNP/SPION hybridosomes both confirm the co-localization of gold and iron. Since the PAA does not link to the AuNP, a collapse of the AuNP within the core of the hybridosomes on removal of THF cannot be excluded. However, the blue resonance characteristic of coupled AuNp was not observed.

SPIONs hybridosomes could also be loaded with a fluorescent BODIPY dye, simply by dissolving the dye in the THF solution at the initial stage of the process, yielding dual magnetic and fluorescent features (Figure S7).

The transversal relaxivities r_2 of the magnetic hybridosomes were found comparable to those of commercial contrast agents such as Feraspin™ in vitro (Figure S8). Therefore, we evaluated their imaging ability for in vivo magnetic resonance (MR) using a mouse model. The T_2*-weighted images show a significant extinction of the liver signal after retro-orbital intravenous injection (Figure S9). Interestingly, AuNP/SPION hybridosomes also yielded significant contrast, which augurs interesting prospects for theranostics with MR-imaging and gold-based thermotherapy (Figure 6a–b). A further beneficial effect is the increased tumor detection (Figure 6c–d). We evaluated the in vitro cytotoxicity (Figure S10) to HEK cells for hybridosomes made from two types of particles (SPION and SPION/AuNp) and stabilized with the two polymers used in this study (PAA450k and PEG-PAA). After 48 h of exposure at an iron concentration of 100 µg per 10^6 cells, both SPION/PAA450k and SPION/PEG-PAA hybridosomes show no detectable toxicity. Mixed SPION/AuNP hybridosomes stabilized with PAA450k show only a slight toxicity, with 85% of viability relative to the control, despite the high metal concentration ([Fe] = [Au] = 100 µg per 10^6 cells).

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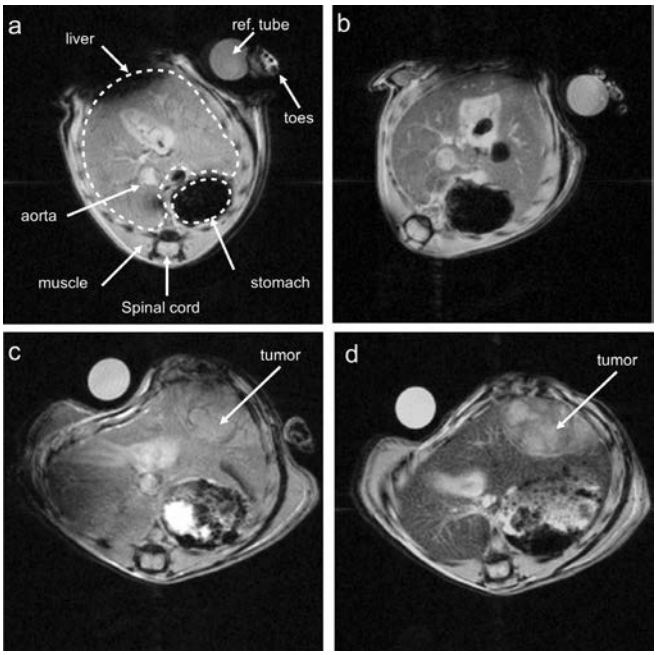

Figure 6. T_2*-weighted MR images of healthy (a, b) and tumor-bearing (c, d) mice acquired before (a, c) and at 30 min (b, d) after injection of the AuNP/SPION hybridosomes (0.5 to 0.9 mg kg^{-1}).

In summary, based on the observation that THF and water mixtures contain droplets of well-defined size, we propose a new strategy to form hybrid nanoparticle–polymer nanocapsules, using these droplets as templates. In contrast with previous methods, this novel strategy does not require the use of amphiphilic block-copolymers and produces vesicles of smaller size. We demonstrated that such vesicles can be readily obtained from nanoparticles of various chemical composition provided that the polymer crosslinks the nanoparticles as it is the case for PAA with iron oxide or with CdSe@ZnS. Particularly, SPION-containing hybridosomes provide efficient MRI contrast enhancement, enabling the imaging of tumor areas at low dose and show low if any toxicity. Not only does this study demonstrate a versatile method for the facile engineering of well-defined assemblies with combined plasmonic, magnetic and/or fluorescent properties, but it also brings into light the role of preformed solvent droplets in the formation of vesicular aggregates.

Acknowledgements

The authors thank O. Mongin (ISCR), M. Destarac (Univ. Toulouse) and O. Musso (INSERM) for providing BODIPY, PEG-PAA and the tumor bearing mice, respectively. We are grateful to BIOSIT-University of Rennes 1, CNRS UMS 3480, Inserm US 018 for financial support, to Biosit MRic core facility (A. Burel), CMEBA (V. Dorcet) and THEMIS for electronic microscopy and to Biosit ARCHE core facility for mouse housing.

Keywords: magnetic resonance imaging · nanocapsules · nanoparticles · polymer · self-assembly

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Manuscript received: May 24, 2016
Revised: June 14, 2016
Accepted Article published: June 20, 2016
Final Article published: June 30, 2016

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CHEMNANOMAT
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Supporting Information

Simple Engineering of Polymer–Nanoparticle Hybrid Nanocapsules

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1. Experimental section

Materials. Octylamine was purchased from Sigma–Aldrich and stored as received in an argon-filled glove box. $\{Fe[N(SiMe_3)_2]_2\}$ (purchased from Nanomeps) was stored as received in the fridge of the glove box. 5.4 nm dodecanthiol coated gold NP (AuNP) were purchased from Nanocomposix. Tetrahydrofuran (VWR) was used as received. Poly(acid acrylic) PAA (1.88k or 450k) was purchased from Aldrich. The BODIPY 1 was kindly provided by O. Mongin (ISCR) and synthesized following a reported procedure.[1] Octadecylamine-coated CdSe@ZnS core-shell quantum dots in powder form were purchased from Sigma-Aldrich. The MRI constrast agent Feraspin XXL™ was purchased from Miltenyi Biotec.

SPION synthesis. The synthesis of the maghemite (γ-Fe_2O_3) SPIONs was achieved via hydrolysis of an organometallic Fe(II) precursor following a recently reported procedure.[1] The reaction was achieved in pure octylamine and in mild conditions of pressure and temperature. H_2O was deoxygenated by bubbling argon 30 mins before the reaction and stored under argon. The precursor $\{Fe[N(SiMe_3)_2]_2\}$ and octylamine (2 eq. to the precursor) were introduced in vials in a glovebox under argon. After a dark homogeneous liquid was obtained the vials were placed in a closed reactor, removed from the glovebox, and set to 40°C. After 15 min, H2O (2.1 eq.) was introduced in the reactor. The reaction was stopped after 3 days by putting the reactor under vacuum. The mixture was then exposed to air and dispersed in THF. The solution was then centrifuged at 19 000 rcf for 10 min and the pellet discarded. The mean diameter measured from TEM analysis was 5.56 nm (0.60 nm

standard deviation). After air oxidation, superparamagnetic magnetite nanocrystals with a mean diameter d= 5.5 nm and a narrow size distribution (standard deviation 1.0 nm) are obtained.[1]

Elaboration of the hybridosomes. The hybridosomes were prepared by adding water to a THF solution containing the hydrophobic NP, in order to reach the composition φw=0.8, promptly followed by vortexing (total metal concentration C=26-66 µg/mL). After ca 15 h, PAA (2.1 mMol.L-1 in AA unit) or PEG-PAA (2.7 mMol.L-1, in AA unit) were added to the mixture. The THF was then totally removed by slow evaporation (15h at 40°C), after which the remaining volume was ca 500 µL. It was verified that after this treatment, no traces of THF could be detected by 1H NMR.

Nanoparticle Tracking Analysis (NTA) and Dynamic Light scattering (DLS). NTA was carried out with a Nanosight LM10 device system equipped with a 40 mW laser working at λ = 638 nm. Video sequences were recorded via a CCD camera operating at 30 frames per second and evaluated via the NANOSIGHT NTA 2.0 Analytical Software Suite. A blank NTA measurement with pure water was performed to exclude a possible contamination with scatterers. Calculations of the hydrodynamic diameters were achieved taking 1.22 cP as the viscosity of THF/water mixture at φ_w= 0.8, from Nayak et al.[2] DLS measurements were performed by using a NanoZS Instrument (Malvern) with Contin analysis to yield the size distribution by intensity.

Scanning Electronic Microscopy (SEM). A few microliters of a solution of hybridosomes were dropped onto a polished silicium wafer. Observation of sample was done with a JEOL 7100f ttls SEM at 10kV equipped with a tungsten tip and a Everhart-Thornley detector.

Transmission Electronic Microscopy (TEM). A 200mesh copper grid, covered with a carbon/formvar film was dipped into the solution of hybridosomes (at [Fe]=1mM) and allowed to dry in ambient conditions. The samples were examined using a JEOL 1400 TEM operating at 120kV and equipped with a GATAN Orius 1000 camera or alternatively a JEOL 2100 LaB6 equipped with a GATAN Orius 200D and a SDD detector for Energy-Dispersive X-ray Spectroscopy (EDS) analysis. For TEM tomography a GATAN dual orientation 927 tomography sample holder was used. Tilted projection images were recorded at 1° tilt intervals from -50° to +30° and we used Digital micrograph software to convert the TEM projections into the 3-dimensional reconstructed image.

Atomic Force Microscopy (AFM). Samples were imaged in fluid over an area of 500 nm^2 to 20 mm^2 at 0.2 to 0.5 Hz operated in PeakForce Tapping mode on a BioScope Catalyst AFM (Bruker AXS, Santa Barbara, CA) using a small volume liquid cell and a set-point of around 1-2 nN. SCANASIST-Fluid AFM tips (Bruker AFM Probes, Camarillo, CA) with a nominal spring constant, resonant frequency and radius of 0.7 N/m, 150 kHz and 20 nm respectively, were used for each experiment. AFM data were analyzed using the Nanoscope Analysis 1.5 software (Bruker AXS, Santa Barbara, CA).

Relaxivity measurements. Relaxivity measurements of hybridosomes were performed by measuring T_2 values at various iron concentrations in water (determined by ICP-OES). Acquisitions were performed at 4.7 T on a Bruker Biospec 47/40 horizontal MRI system equipped with a 72 mm inner diameter volume transmit/receive quadrature coil. The T_2 values were measured by using a multi-echo spin echo sequence with 16 echoes, an echo time spacing of 8 ms, and a repetition time (TR) of 2000

ms. The T_2 maps were generated with ImageJ using the MRI analysis Calculator V1 by fitting the signal decay to a monoexponential function. Other imaging parameters were an image matrix size of 128 x 128, a field of view (FOV) of 51.2 mm, a slice thickness of 3 mm, and one signal average per phase-encoding step. The measurements were performed at room temperature (20.0±0.5°C). For each sample, a circular region of interest (ROI) was drawn from which T_2 maps and then the mean T_2 and standard deviations were calculated.

Cytotoxicity. HEK cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 1% antibiotics. The hybridosomes were dialyzed against cell culture medium for 4 h prior to cell exposure. The cells were then exposed to hybridosomes (100 µg of iron per 106 cells) for 48 h at 37°C in cell culture medium. Viability assays were carried out by measuring MTS (3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) activity.

***In vivo* MRI.** Animal experiments were conducted in line with French Agriculture Ministry guidelines. The C57BL/6 healthy mice and tumor bearing mice received SPION hybridosomes (1mg iron/kg body weight, stabilized with PAA 450k) or SPION-AuNP hybridosomes (0.5 to 0.9mg iron/kg body weight, stabilized with PAA 450k or PAA 1.8k) by retro-orbital intravenous injection, after anesthesia with isoflurane. MR imaging sequences were recorded prior to the injection of the contrast agent, and 30 mins and 24 h after injection, using the 4.7 T (Brucker) small animal imager. Magnetic Resonance (MR) images were acquired using a Bruker Biospec 47/40 (Bruker Biospin, Wissembourg, France) with a 4.7 T magnet with a BG-06 gradient insert. Anesthetized animals were placed in a supine position in a 3.5-cm bird-cage resonator. Images were acquired using a gradient echo (FLASH) sequence with TR = 300 ms, TE = 5 ms, Flip Angle = 30°, 4 averages per phase-encoding step, a 212 x 212 matrix and FOV = 3.2 x 3.2. Fifteen 1 mm thick axial slices covering the mouse abdomen were acquired. Respiratory gating was used during the acquisition. A tube filled with water was used as reference. Respiratory triggering was performed using a highly sensitive pneumatic system placed under the mouse. Body temperature was maintained with a warming blanket.

2. Investigation of THF/water and THF/water/NP mixtures

a) THF/water mixtures

For dynamic light scattering measurements of THF/water mixture, great care was taken to avoid any impurities: water was filtered through a 0.2 µm cellulose filter before use and THF was never in contact with any plastic material. Only glassware was used for all syringes, flasks and measurements cell. Dynamic light scattering (DLS) measurements of mixtures of THF and water were achieved for various volume fractions of water (φ_w) in the range φ_w = 0.05-0.9 (Figure S1). The relaxation times extracted from the correlograms are interpreted in terms of Brownian motion of spherical particles. Figure S1 shows that fluctuations of concentrations are measured over the whole range of compositions. As the fraction of water increases, faster dynamics is observed, corresponding to smaller length scale.

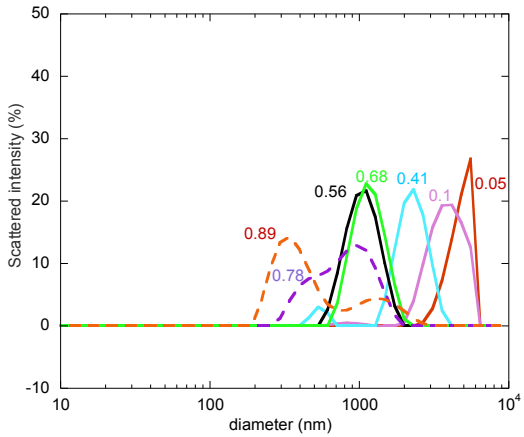

Figure S1: DLS measurements of THF/water mixtures. Volume fraction of water φ_w as indicated on graph.

A mode corresponding to the Brownian motion of spherical objects of ca 250 nm can be observed at φ_w= 0.78 and φ_w= 0.89. Since DLS is sensitive to any fluctuation, the dynamic modes that were measured do not necessarily correspond to defined long-lived structures. Therefore, Nanoparticle Tracking Analysis (NTA) analysis was undertaken for compositions where the fastest correlation modes were observed (φ_w= 0.8-0.9). NTA is a microscopic analysis of individual trajectories of spotlights scattered by scattering objects in solution. It was shown to accurately analyze the size distribution of polydisperse samples, which is not the case for DLS. Individual objects were clearly observed (Figure S2) using NTA. The size distribution profile shows a narrow distribution with a main mode at D_h = 110±35 nm.

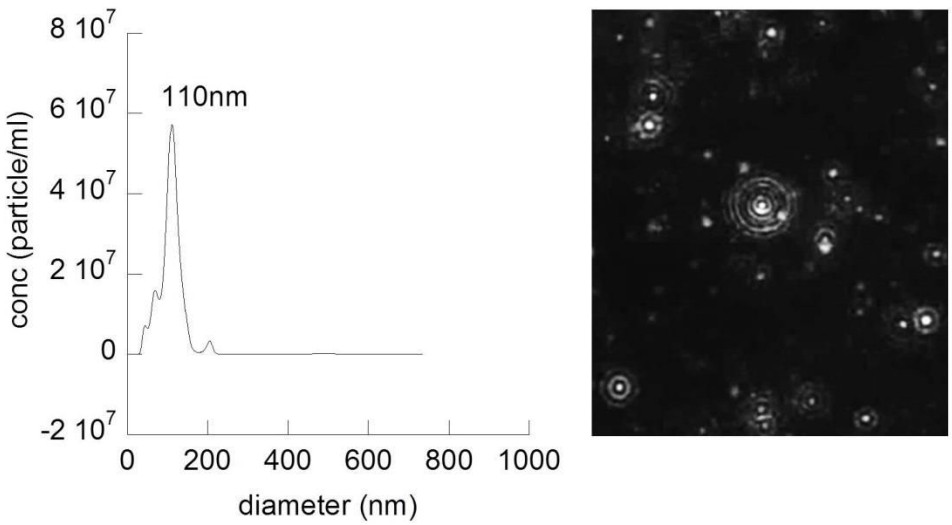

Figure S2: NTA analysis of a THF/water mixture for φ_w =0.8. Left: size distribution profile. Right: optical microscopy image of the scattered spotlights.

b) Investigation of water/THF/NP mixtures

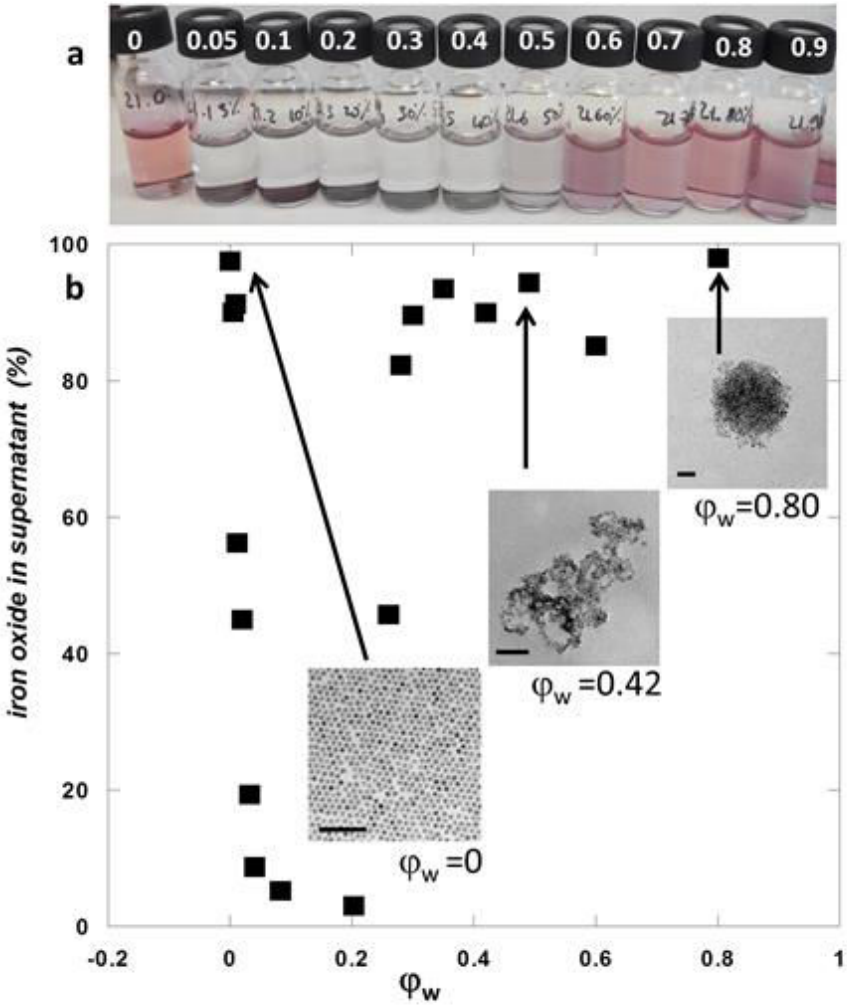

Figure S3: (a) Picture of solutions of AuNP in water/THF (the white figures on caps indicate the water volume fraction φ_w). (b) Stability of SPION in water/THF and TEM pictures obtained from the supernatants. Scale bars: 50 nm.

c) Effects of nanoparticles over THF/water mixtures (DLS)

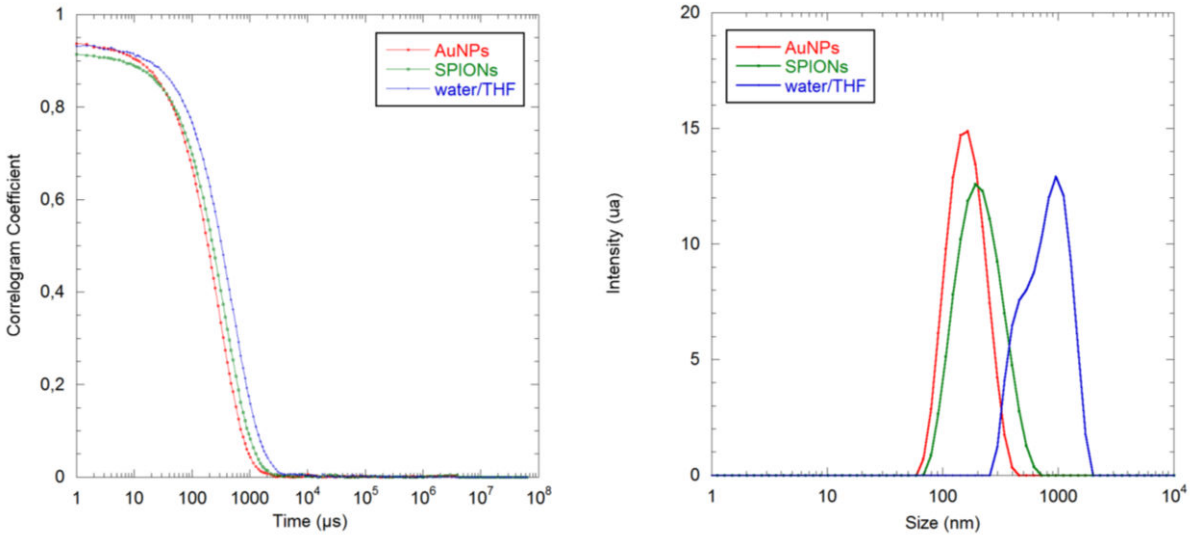

Figure S4: Correlograms (a) and size distribution (b) (by scattered intensity) from DLS measurements of water/THF mixtures (φ_w=0.8) in absence of NP (blue) and in presence of SPION (green) and AuNP (red).

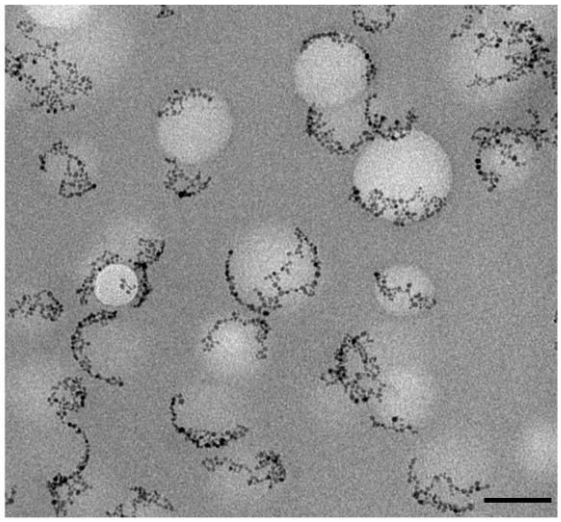

Figure S5: TEM picture of assemblies of SPION at low nanoparticle concentration, in order to evidence the templating effect of liquid droplets. [Fe]=5 µg/ml; polymer: PAA_{450k}. Scale bar: 100 nm

3. Energy dispersive X-ray spectroscopy (EDX)

EDX analysis was coupled to TEM observation to demonstrate the colocalization of iron and gold in a single AuNP/SPION hybridosome.

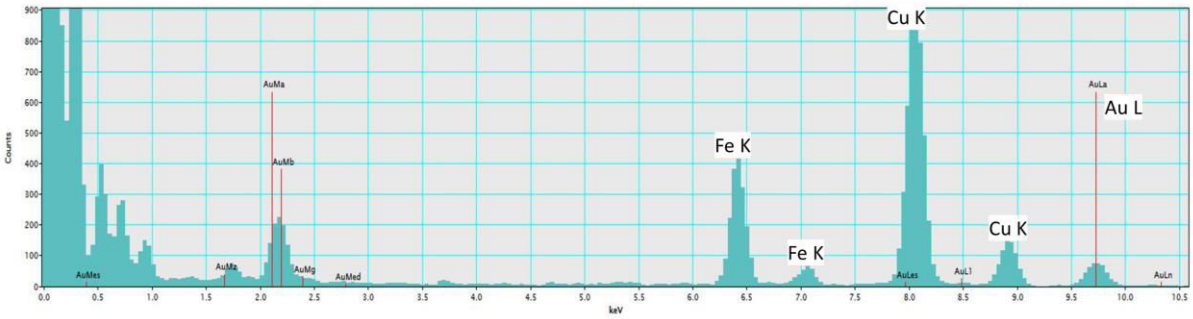

Figure S6: Energy dispersive X-ray spectroscopy of a single AuNP/SPION hybridosome showing the co-localization of iron and gold.

4. Fluo-magnetic hybridosomes

The magnetic SPION hybridosomes could also be loaded with a fluorescent BODIPY dye, simply by dissolving the dye into the THF solution at the initial stage of the fabrication process. The selected dye (BODIPY 1, Figure S7) is not soluble in water and fluoresces both in its dissolved and aggregated states. Two cycles of magnetic separation and washing with water were achieved to obtain bright fluorescent hybridosomes and a dye free supernatant as demonstrated by absorbance and emission measurements. Figure S7a shows the absorbance and emission spectra of the supernatant after the two cycles of washing, and Figure S7b shows the fluorescent hybridosomes after redispersion in water. No significant leakage of the dye was observed over months.

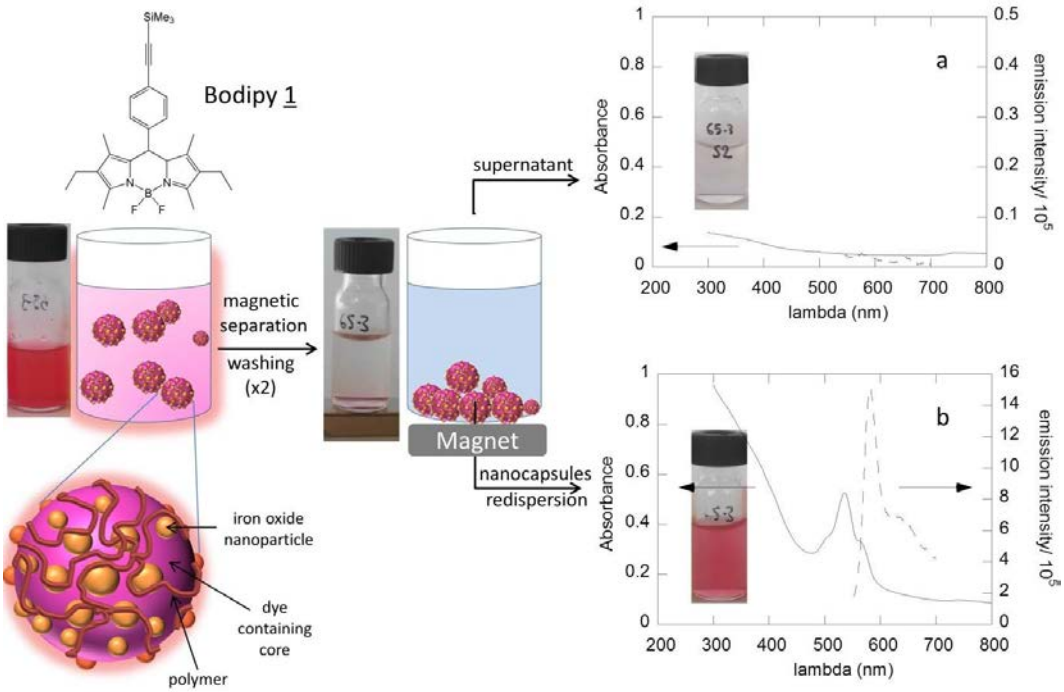

Figure S7: Elaboration of magnetic and fluorescent hybridosomes: chemical structure of the BODIPY 1. (a) Absorbance and emission spectra of the supernatant after removal of the fluo-magnetic hybridosomes using magnetic separation. (b) Absorbance and emission spectra of the suspension after redispersion of the hybridosomes in water.

5. Transversal relaxivities

The transversal relaxivities of SPION and SPION/AuNP hybridosomes stabilized with PAA or PEG-PAA were measured in water using a 4.7 T magnetic resonance (MR) scanner (Figure S8). The measured values were r_2 = 274±35 $mM^{-1}.s^{-1}$, 285±37 $mM^{-1}.s^{-1}$ and 257±15 $mM^{-1}.s^{-1}$ for the SPION/PAA, SPION/PEG-PAA, and SPION/AuNP PAA hybridosomes, respectively. All measured values compare Feraspin™, a commercial contrast agent for pre-clinical imaging, measured in the same conditions (r_2 = 307±80 $mM^{-1}.s^{-1}$). The choice of the polymer, PAA or PEG-PAA, has no significant effect on the relaxivity of SPION hybridosomes in water. Mixed SPION/AuNP hybridosomes show a sligthly lower relaxivity, most probably due to a lesser extent of magnetic coupling between SPION.

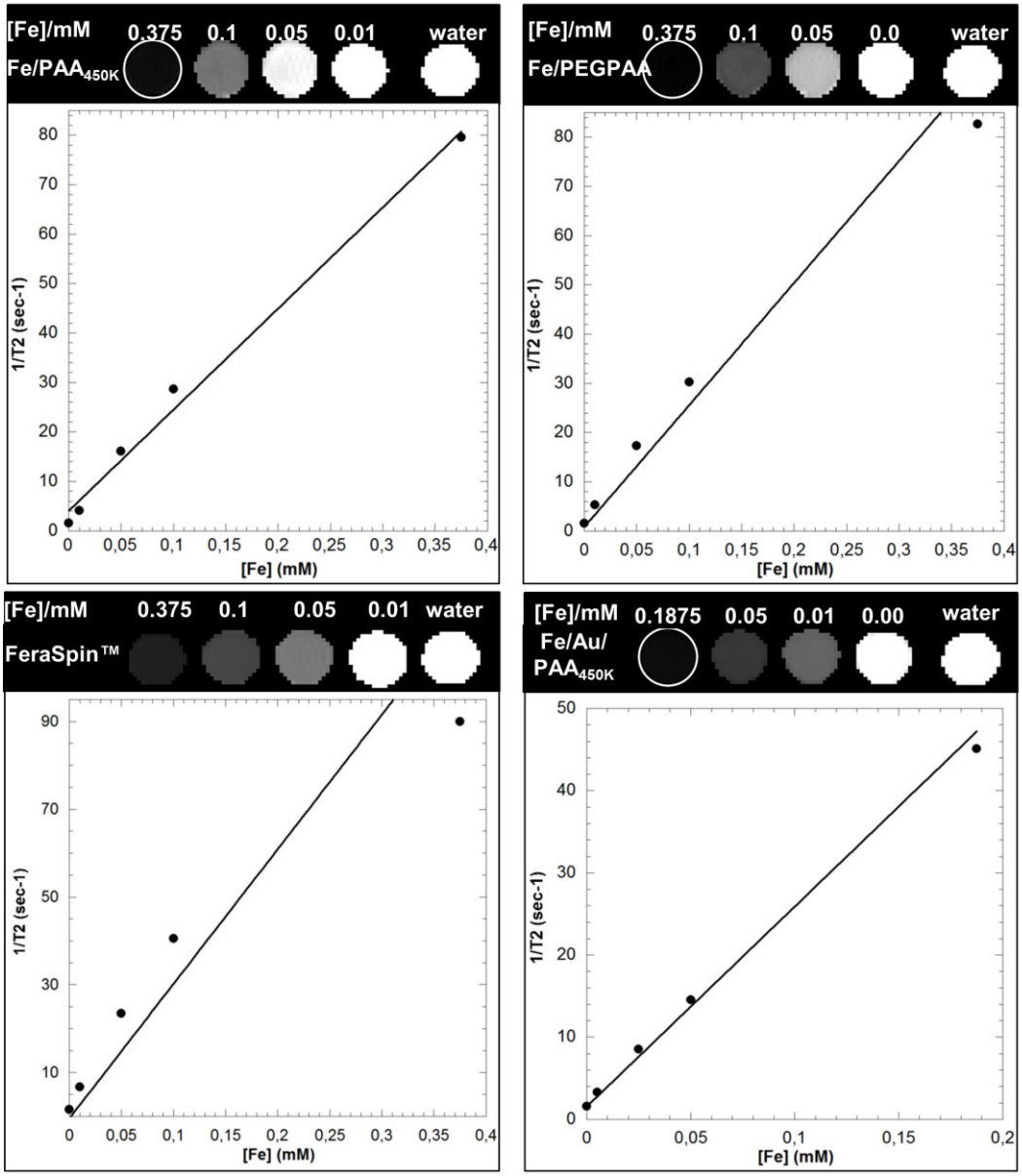

Figure S8: MR images obtained at 4.7 T of phantoms containing hybridosomes solutions of decreasing concentrations of (iron concentration as indicated) and graphs of reciprocal measured relaxation times *vs* iron concentrations for hybridosomes (a) SPION/PAA_{450k}, (b)SPION/PEG-PAA, (c)SPION/AuNP/PAA_{450k} and (d) commercial contrast agent Feraspin™ XXL.

6. *In vivo* MRI

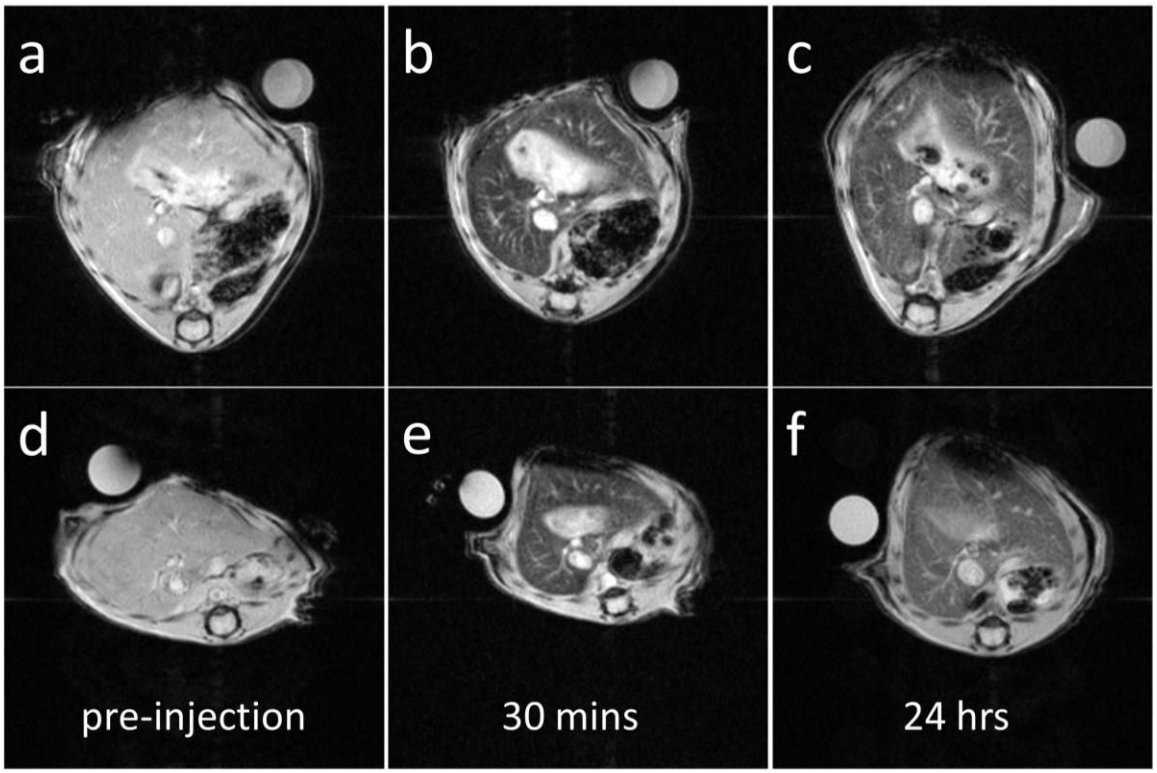

Figure S9: T_2*-weighted MR images of healthy bearing mice injected with SPION hybridosomes stabilized with PAA450k (a-c) or PEG-PAA (d-f) and acquired at t=0; 30 min and 24 h after intravenous injection, showing a clear extinction of the liver.

7. *In vitro* toxicity

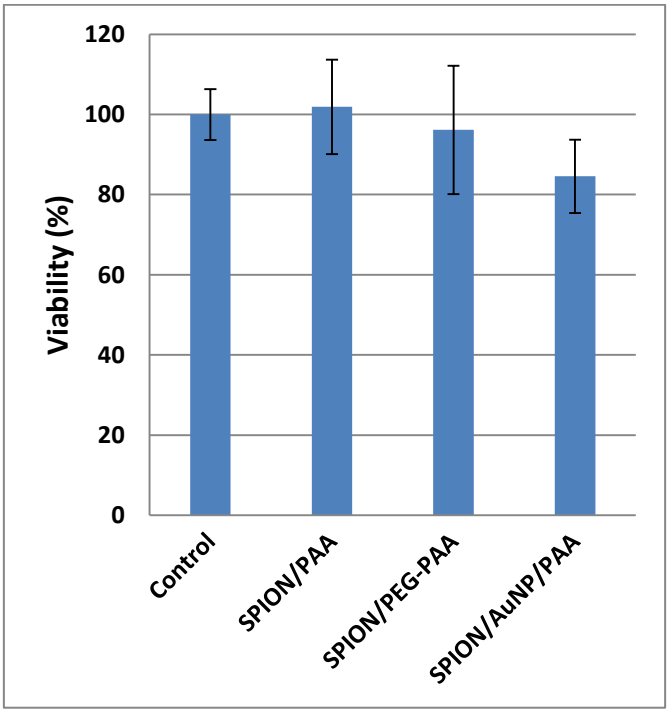

Figure S10: Viability of HEK cells after incubation 48h with the magnetic SPION ([Fe]=100µg/10^6cells) or SPION/AuNP ([Fe]=[Au]=100µg/10^6cells) hybridosomes used as MRI contrast agents.

8. TEM tomography of hybridosomes

Movie S1 shows animated slab of images collected at tilted intervals.

[1] G. Casterou, V. Collière, P. Lecante, Y. Coppel, P.-A. Eliat, F. Gauffre, M. L. Kahn, *Chemistry* **2015**, *21*, 18855–61.

[2] J. Nayak, M. Aralaguppi, B. V. Naidu, T. Aminabhavi, *J. Chem. Eng. Data* **2004**, *49*, 468–474.

Lors de ce premier article, nous ne faisons pas mention de l'effet Ouzo pour éventuellement expliquer le procédé de formation de « gouttes ». Plusieurs expériences de diffusion de la lumière ont été menées sur les mélanges THF/eau et d'autres mélanges miscibles pour comprendre le phénomène. Plusieurs mesures préliminaires de DLS présentent des « structures » dont la taille est comprise entre 100 et 300 nm. Plusieurs études ont été également initiées plus particulièrement sur le système THF/eau en variant le grade et la nature du THF. En réalité, le THF utilisé jusqu'à présent pour observer ces structures de « gouttes » contient un stabilisant, utilisé comme antioxydant : le BHT (butylhydroxytoluène). Il s'agit d'un alcool plutôt hydrophobe qui prévient la formation de peroxydes, composé parfois explosifs. En milieu fortement acide le THF peut également subir une polymérisation et les produits de cette polymérisation ou bien de dégradation du THF pourraient aussi être à l'origine des gouttes observées. Des premières expériences de GC-MS (chromatographie en phase gazeuse couplée à une détection de masse) sur différents types de THF stabilisés, ont permis de mettre en évidence plusieurs autres espèces. De ce fait, ces observations peuvent suggérer la présence de nombreuses autres espèces au sein d'un THF stabilisé (résidus de synthèse, produits de dégradation etc), dont le grade de pureté mentionné par les fournisseurs se situe entre 99 et 100%.

Ces espèces pourraient être des produits de dégradation du BHT, sous la forme d'espèces oxydées de type alcool aromatiques. Les espèces formées lors de l'oxydation du BHT ont un caractère amphiphile encore plus prononcé que le BHT lui-même, ce qui peut laisser penser que toutes ces espèces en petite quantité (BHT de l'ordre du ppm selon les spécifications fournisseur) aient un rôle dans l'existence de ces gouttes. Ces espèces présentent des solubilités très faibles dans l'eau ($0.6 mg/L^{-1}$) et des solubilités bien plus importantes dans les solvants de types alcanes, alcools ou éthers cycliques et semblent des candidats idéaux dans l'effet Ouzo.

Dans la partie 3 de ce chapitre, nous allons nous intéresser un peu plus à la structure et la composition des Hybridosomes en fonction des quantités d'IONP et de PAA, ainsi qu'à leurs propriétés mécaniques.

Les observations faites jusqu'à présent nous ont permis de d'identifier la structure de capsule de l'assemblage. Une étude de tomographie TEM a pu être réalisée au sein de la plateforme THEMIS (UMS 2001 ScanMAT) avec l'aide de V. Dorcet. La reconstruction de cette étude est présenté sous la forme d'une vidéo accessible au lien ci-dessous et une description de cette analyse plus détaillée est présente dans le chapitre suivant (Supporting Info article 3). On y aperçoit un Hybridosome d'environ 80 nm dans un état non dégonflé. L'acquisition de 76 images de -60° à +15° permet la reconstruction de la vidéo. On y voit distinctement la paroi mono-nanoparticule.

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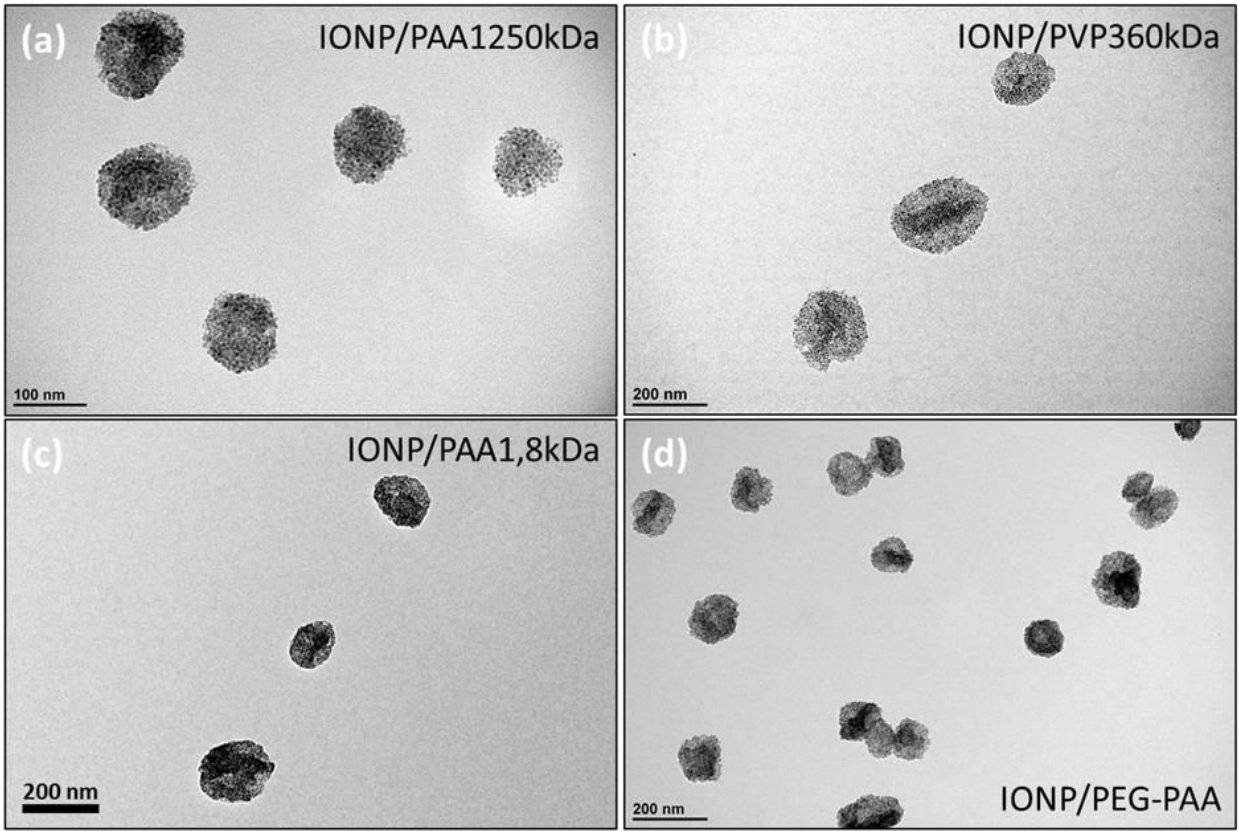

Figure 21: Hybridosomes assemblés avec différents types de polymères (a) PAA1250k, (b) PVP360k, (c) PAA1.8k et (d) PEG-PAA

Tout comme la vidéo, les micrographies TEM présentés précédemment laissent apparaître des interstices entre nanoparticules suggérant une certaine porosité de la paroi. La question relative à la nature et la taille du polymère semble être pertinente. En effet, plusieurs Hybridosomes ont été préparés avec succès à partir de PAA de différents poids moléculaires, ainsi que de PVP et de PEG-PAA comme le montre la Figure 21.

De plus, les premières observations TEM à différentes concentrations d'IONP semblaient suggérer que le ratio IONP/Polymère avait un impact sur la formation des Hybridosomes dû à la présence de particules non assemblées Figure 22.

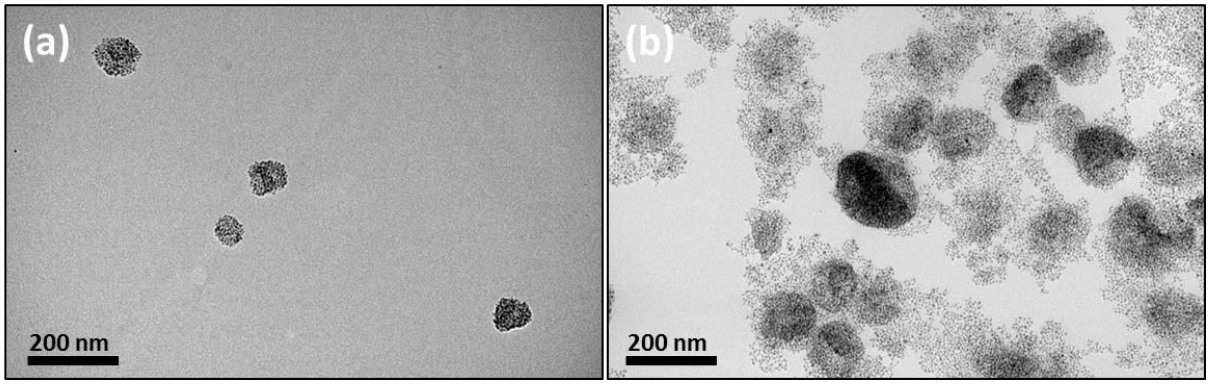

Figure 22: Assemblage d'Hybridosomes IONP/PAA1250k avec [AA] = 2.1mM et (a) [Fe] = 25 µg/mL, (b) [Fe] = 50 µg/mL.

Nous avons par la suite décidé d'étudier ce phénomène sur le système IONP/PAA450K uniquement bien que de très beaux Hybridosomes aient été obtenus avec d'autres polymères. Les résultats présentés ci-après ont été obtenus en grande majorité par M.Thivolle, étudiant de l'ENSC Rennes que nous avons accueilli lors de son stage de dernière année. Ils ont conduit à la publication d'un article que nous présentons ci-après.

3. Article 2: Structure and Elasticity of Composite Nanoparticle/Polymer Nanoshells (Hybridosomes®)

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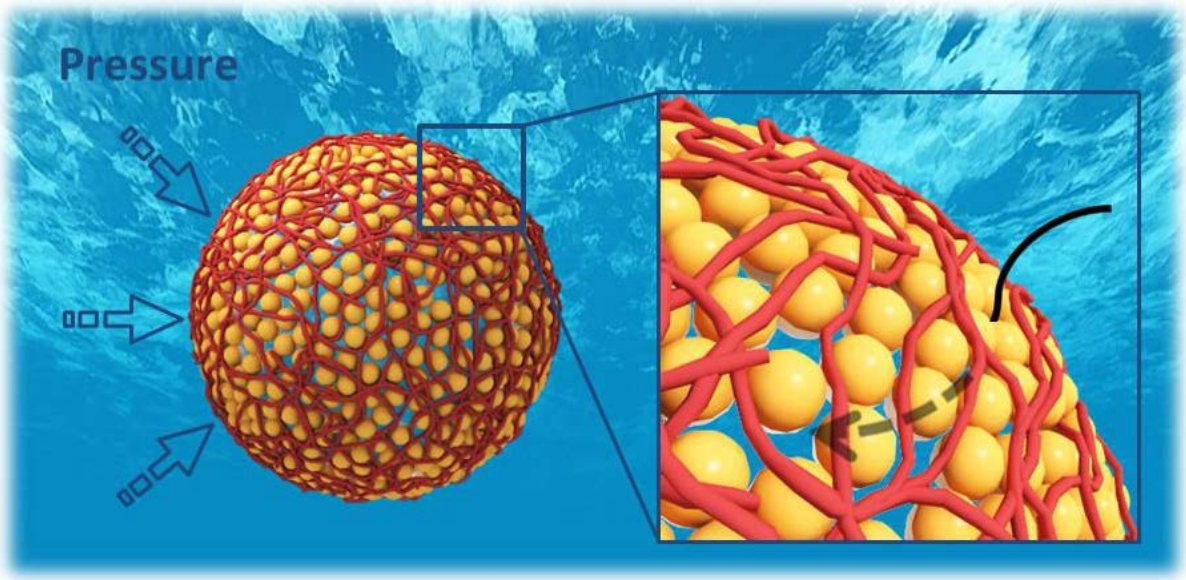

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Structure and elasticity of composite nanoparticle/polymer nanoshells (hybridosomes®)†

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Received 7th April 2017,
Accepted 13th May 2017

DOI: 10.1039/c7sm00705a

rsc.li/soft-matter-journal

Our group recently introduced a new process to synthesize nanoparticle shells of about 100 nm, named "hybridosomes®". Here, the structure and mechanical properties of hybridosomes® made from iron oxide nanoparticles and poly(acrylic acid) are characterized using TEM, AFM and an osmotic compression technique. For the latter, the size distribution of the hybridosomes is monitored by nanoparticle tracking analysis (NTA) in the presence of poly(ethylene glycol)s of different molecular weights. It is found that the size of the hybridosomes® can be tuned from *ca.* 80 nm to over 110 nm by adjusting the amount of nanoparticles and that their shell consists of a single layer of nanoparticles, with a porous structure. The size of the pores is estimated from osmotic compression experiments at *ca.* 4000 g mol^{-1}. The mechanical properties are measured both at the ensemble level using size measurements under osmotic pressure and at the single nanoparticle level by atomic force microscopy nanoindentation. Both osmotic and AFM experiments are analyzed in the framework of the continuum elastic theory of thin shells and yield a value of Young's modulus of the order of MPa.

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A. Introduction

Capsules are abundant in nature where membranes (of cells, nucleus, exosomes...) and shells (of eggs) provide a protective barrier, while enabling selective exchange. At the age of modern technology, micro- and nanocapsules (or vesicles) are used as protection shells for enzymes, vessels for drug or gene vectorization, nanoreactors for confined chemical reactions and heterogeneous catalysis, or to template the formation of new materials.[1–3] A lot of effort has been devoted to the elaboration of submicronic synthetic capsules, each of them having their own advantages and disadvantages. Among them, liposomes, polymersomes and multilayered polyelectrolyte nanocapsules are the most documented.[4–9] More recently, nanoparticle shells have appeared as highly desirable for the elaboration of metamaterials[10–12] as well as for biomedical applications such as imaging and theranostics.[13,14] The methods that have been developed so far to elaborate nanoparticle shells fall into two categories. The first one makes use of amphiphilic block copolymers to direct the self-assembly.[15,16] The second family is based on the use of sacrificial templates, either silica or latex particles over which a layer of nanoparticles is deposited.[6] A calcination step is generally required to remove the templating core.

Recently, our group introduced a simple and robust procedure to elaborate hybrid polymer/nanoparticle capsules (diameter ~ 100 nm), which were coined hybridosomes®.[17] The shell of the capsules is made from inorganic nanoparticles crosslinked by polymers. Interestingly, hybridosomes made from superparamagnetic iron oxide nanoparticles (IONPs) (maghemite) and crosslinked with poly(acrylic acid) (PAA) showed excellent properties as MRI contrast agents and can easily encapsulate hydrophobic compounds. Their bench stability as dispersions was demonstrated to be at least 1 year. In addition, hybridosomes® were prepared from various inorganic nanoparticles including quantum dots, plasmonic and upconverting nanoparticles, with the only limitation being that they should be coated by a hydrophobic layer. Thus, these capsules of a new type hold much promise particularly for imaging and theranostic applications.

Obviously, the shell permeability and mechanical properties of the capsules are of great importance in many aspects such as encapsulation, triggered release, deformation into flow, rheology, and even adhesion. For instance, the protein shells of viruses are extremely rigid and tailored to resist internal pressure generated by the encapsulated genetic material, as high as 10 MPa.[18,19] In contrast, red blood cells are among the

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7sm00705a

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less rigid materials, but nevertheless can withstand extreme stretching forces and squeeze into very small capillaries.[20] Both liposome and polymersome membranes are bilayers composed of phospholipids or amphiphilic block copolymers, respectively. Liposomes are generally characterized by thermal membrane fluctuations and a high lateral mobility due to the low molecular weight (<1 kDa) of their constituent phospholipids. Polymer membranes are more resistant to rupture, and interestingly the membrane properties of polymersomes can be tailored by a precise molecular design of the constituting blocks.[21,22] There has also been much interest in the investigation of the mechanical properties of multilayered polyelectrolyte nanocapsules because the thickness of the shell is directly related to the number of layers and can be tuned to a large extent.[23–27]

In this paper, we investigated the shell structure and mechanical properties of hybridosomes® made from IONPs and PAA. An osmotic technique, associated with nanoparticle tracking analysis (NTA) was introduced to investigate the effect of isotropic compression. To this aim, hybridosomes® were suspended into solutions of poly(ethylene glycol) (PEG) of various sizes, and the size distribution of the capsules was measured. This technique also enables us to determine the porosity of the shell, since low molecular weight PEGs obviously equilibrate between the interior and the exterior of the capsules. In complement to osmotic pressure experiments, the effect of local stress was investigated using the Atomic Force Microscopy (AFM) nanoindentation technique. The results were interpreted using the theory of thin shell elasticity to determine the Young's modulus of the composite shell. The compositions of the polymer and nanoparticles were varied, to determine their respective role in the size and shell properties of hybridosomes®.

B. Experimental

Materials

Octylamine was purchased from Sigma-Aldrich and stored as received in an argon-filled glove box. The iron precursor, $\{Fe[N(SiMe_3)_2]_2\}$, was purchased from Nanomeps and stored as received in the fridge of the glove box. (VWR) was used as received. Poly(acrylic acid) PAA (450 000 g mol^{-1}) was purchased from Aldrich.

Nanoparticle synthesis

The synthesis of the maghemite (γ-Fe_2O_3) IONPs was achieved *via* hydrolysis of an organometallic Fe(II) precursor following a recently reported procedure.[40] The reaction was achieved in pure octylamine and under mild conditions of pressure and temperature. Water was deoxygenated by bubbling argon 30 min before the reaction and stored under argon. The precursor $\{Fe[N(SiMe_3)_2]_2\}$ and octylamine (2 eq. to the precursor) were introduced in vials in a glovebox under argon. After a dark homogeneous liquid was obtained, the vials were placed in a closed reactor, removed from the glovebox, and the temperature was set to 40 °C. After 15 min, H_2O (2.1 eq.) was introduced into the reactor. The reaction was stopped after 3 days by placing the reactor under vacuum. The mixture was then exposed to air and dispersed in THF. The solution was then centrifuged at 19 000 rcf for 10 min and the pellet was discarded. After air oxidation, superparamagnetic magnetite nanocrystals with a mean diameter d = 5.5 nm and a narrow size distribution (standard deviation 1.0 nm) were obtained.

Preparation of stock dispersions of hybridosomes

The hybridosomes were prepared by adding water into a THF solution containing the IONPs, in order to reach a water volume fraction of 0.8, promptly followed by vortexing. After *ca.* 15 h, PAA was added into the mixture as a concentrated aqueous solution. Then, THF was totally removed by slow evaporation (15 h at 40 °C), after which the remaining volume was *ca.* 500 μL. It was verified that after this treatment, no traces of THF could be detected by ^{1}H NMR.

Nanoparticle tracking analysis

Stock suspensions of hybridosomes were diluted 100 times prior to NTA measurements. For each sample, 3 acquisitions with an acquisition time of 60 s were performed. The relative standard deviation was calculated using SD × 100/mean.

Osmotic compression

5 μL of the stock dispersion were first diluted into water and then mixed with appropriate volumes of PEG aqueous solution and 200 nm calibrated latex solution to reach a final volume 500 μL and the desired PEG concentration. The osmotic pressure was determined from the PEG concentration. At very low PEG concentrations, the osmotic pressure (P) can be derived from the molar concentration (C) using the law of ideal gas $P = RTC$. However, one should be careful to take into account the divergence from ideality at higher concentrations.[41] The conversion from molarity to osmotic pressure was extrapolated from experimental data by Money (PEG 1k, 4k, 10k)[41] and Shaykewich (PEG 20k).[42]

Transmission electron microscopy

A 40 μL drop of the solution was first placed on a carbon-coated TEM copper grid (Quantifoil, Germany). The samples were then negatively stained with 2% w/w uranyl acetate (Merck, Germany) previously filtered using a 0.2 μm filter unit. The grid was then air-dried before introducing it into the electron microscope. The samples were viewed using a JEM 1230 'Cryo' microscope (JEOL, Japan) operated at 80 kV and equipped with a LaB_6 filament. All the micrographs were recorded on a Gatan 1.35k × 1.04k × 12 bit ES500W CCD camera.

Atomic force microscopy

Samples were imaged in fluid over an area of 500 nm^2 to 20 μm^2 at 0.2 to 0.5 Hz operated in the peak force tapping mode on a BioScope Catalyst AFM (Bruker AXS, Santa Barbara, CA) using a small-volume liquid cell and a set-point varying from 4 nN to 115 nN according to the strength applied in order to progressively crush the particles. SCANASYST-Fluid AFM tips (Bruker AFM Probes, Camarillo, CA) with a nominal spring constant,

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resonant frequency and radius of 1.5 N m^{-1}, 150 kHz and 20 nm, respectively, were used for each experiment. The thermal noise method was systematically used to refine the spring constant value of the cantilevers. Force spectroscopy curves were acquired in water by approaching a tip onto the sample at a pulling speed of 1 μm s^{-1}. Sensitivity calibration was determined in water solution over a piece of freshly silicon substrate at room temperature (25 °C). Force curves were recorded at different locations on the particles, in order to account for any heterogeneity in the coverage of the surface. Force measurements were acquired from around 500 different points at the surface. AFM data were analyzed using the Nanoscope Analysis 1.5 software (Bruker AXS, Santa Barbara, CA) to extract the effective Young's modulus as the slope of the linear elastic region of the approach–retract curves.

C. Results and discussion

All details about the synthesis of the hybridosomes® were described previously.[17] Briefly, the process consists in three steps (Fig. 1). First, liquid droplets are coated with the hydrophobic nanoparticles in a water/THF (80 : 20) mixture forming the primary nanoparticle shell (Step 1). Then, the nanoparticle shell is crosslinked by a coordinating polymer (Step 2). Finally, the solvent is removed by evaporation, yielding the hybridosomes® with an aqueous core, and the samples are purified by magnetic separation (Step 3). All the hybridosomes® used in this study were prepared from iron oxide nanoparticles (IONP; 5.5 nm ± 1 nm) crosslinked with poly(acrylic acid) (PAA 450 000 g mol^{-1}). TEM analysis of hybridosomes® after negative staining occasionally shows some open structures (urns, Fig. 1). From such pictures, it is clear that the shell is only a few nanometers thick, which is consistent with a single layer of particles. For the following discussion, we will consider that the shell thickness (h) is equivalent to the diameter of the IONPs ($h \approx 5$ nm).

We investigated the influence of the composition of the hybridosomes® (amount of IONPs and of PAA) on their structure. First, we varied the amount of IONPs introduced. Fig. 2a shows the size histograms at iron concentrations in the range 0–25 μg L^{-1} and at a given concentration of the polymer ([AA] = 2.25 mM) determined by NTA. NTA tracks individual trajectories, allowing the calculation of the diffusion coefficient and thus the hydrodynamic diameter (d_h) of each particle. Reporting the main mode (and when present a secondary mode) *vs.* the iron concentration evidences two regimes (Fig. 2b). The low concentration regime is characterized by a hydrodynamic diameter (d_h) of *ca.* 80 nm or lower, whereas in the higher concentration regime d_h is over 110 nm. Note that at the transition between the two regimes, the coexistence of the two populations is observed. It is common to report the width of the distribution obtained by NTA as the relative standard deviation (RSD, see the Experimental section for details). The histograms are of moderate width (RSD = 17–25%) except in the vicinity of the transition where the distributions can be considered as polydisperse (RSD ∼ 40%), as a result of the coexistence of the two populations. Interestingly, it was also possible to form capsules from PAA only, without the use of nanoparticles. In contrast, varying the amount of PAA to form the hybridosomes® does not significantly change the measured diameter (Fig. 3), but the distribution widens significantly at lower amounts of PAA and the total amount of capsules decreases. To summarize, the IONPs hybridosomes® can adopt two possible equilibrium conformations, a small size ($d_h \sim 80$ nm) and a larger size ($d_h \sim 110$ nm), at low and high particle concentrations, respectively. In the low concentration regime, the size is obviously determined from the polymer properties, whereas in the large concentration regime it is influenced by the presence of the particles. Note that the presence of a population of a relatively small size is important for future imaging and theranostic applications, since the body clearance of circulating particles increases with particle size.[28]

Two types of experiments were performed to investigate the shell properties of the hybridosomes®. The first type consists in applying an osmotic pressure and the second type a localized force, by nanoindentation. With regard to the osmotic compression, we reasoned that hybridosomes® dispersed in a polymer solution would experience an inward osmotic pressure if the polymer does not diffuse across the shell. In the first set

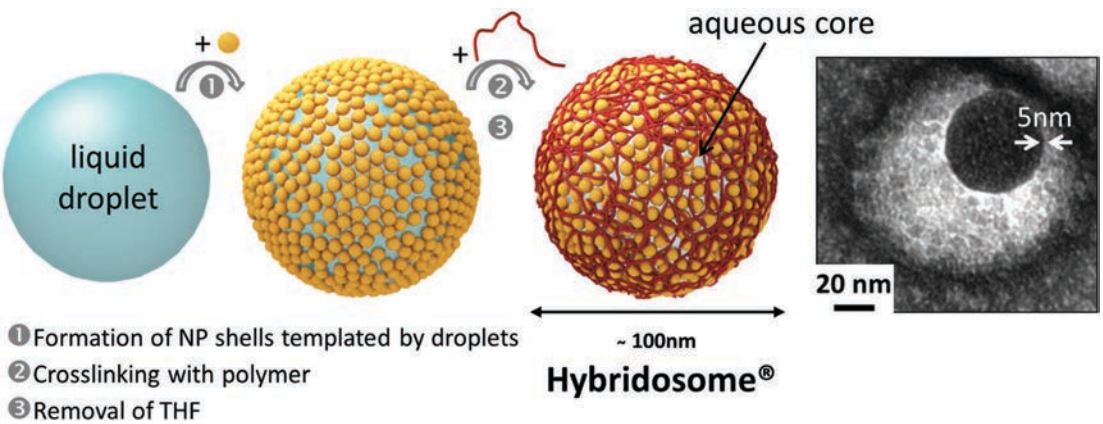

Fig. 1 Process of elaboration of the hybridosomes and TEM observation with negative staining, showing an "open" hybridosome ([AA] = 2.25 mM; [Fe] = 12.5 μg mL^{-1}).

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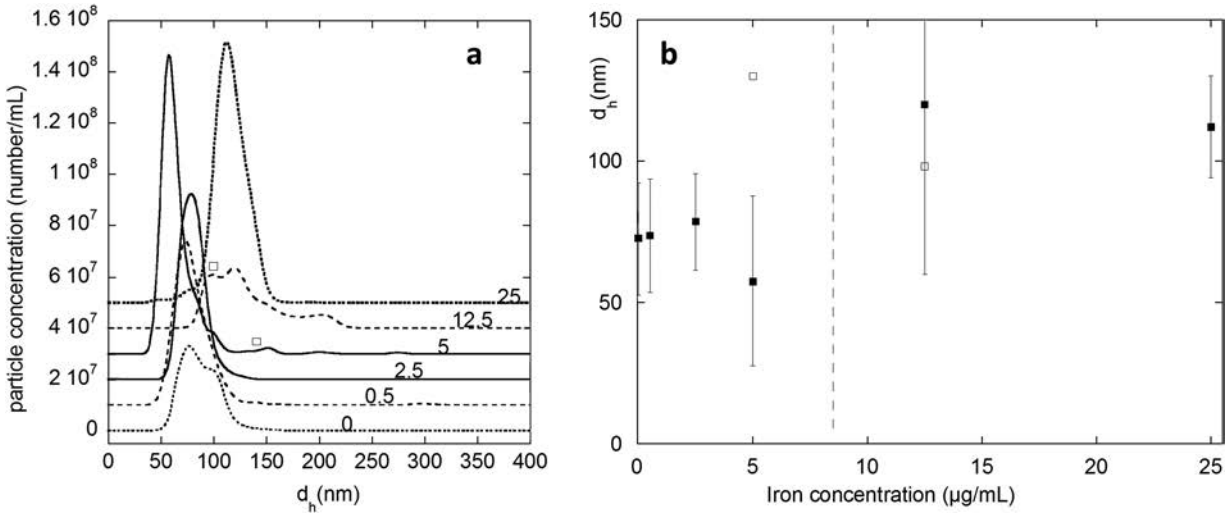

Fig. 2 Influence of the amount of IONPs on the size distribution (hydrodynamic diameter d_h) of hybridosomes. (a) Size histograms determined by NTA. The symbol □ points out a secondary mode. (b) Main mode (■) and secondary mode (□) of the distribution. Error bars represent the standard deviation (SD) of the distribution. Concentrations of stock solutions: [Fe] = 0, 0.5, 2.5, 5, 12.5; and 25 µg mL^{-1} as indicated in the graph; [AA] = 2.25 mM.

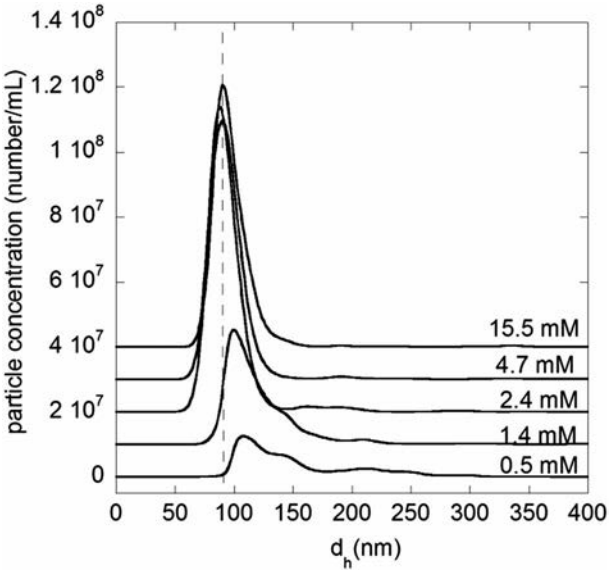

Fig. 3 Influence of the amount of PAA on the size distribution (hydrodynamic diameter d_h) of hybridosomes measured by NTA. Concentrations of stock solutions: [Fe] = 12.5 µg mL^{-1}; [AA] = 0.5, 1.4, 2.4, 4.7, and 15.5 mM as indicated in the graph.

of experiments, PEGs of four molecular masses: 1000 g mol^{-1}, 4000 g mol^{-1}, 10 000 g mol^{-1} and 20 000 g mol^{-1} (marked as 1k, 4k, 10k and 20k, respectively) were used, at increasing concentrations (15; 45; 75 and 100 g L^{-1}). The size distribution of hybridosomes® subjected to the osmotic pressure was analyzed using NTA and renormalized from viscosity effects using a 200 nm calibrated latex solution (Fig. 4a). Two facts are remarkable. The first one is that the influence of the shortest PEGs over the size distribution of the hybridosomes® is negligible (PEG 1k) or weak (PEG 4k), whereas a significant amount of shrinkage is observed for PEG 10k and 20k. These results evidence the porous nature of the shell, with an estimated molecular cut-off of approximately 4000 g mol^{-1}, or 2 nm in terms of the hydrodynamic radius.[29] Thus, PEGs smaller than 4k diffuse across the shell, equilibrating inner and outer osmotic pressures. The second observation is a sharp transition from a monomodal to a multimodal distribution with measured sizes spanning down to 25 nm (which is the detection limit of NTA) at the highest concentrations for PEG 10k and PEG 20k. We interpret this result as a transition between a regime of isotropic compression and a buckling instability, leading to the formation of wrinkles or possibly of inverted hemispheres, as previously observed for polyelectrolyte vesicles.[23,25,30] It is worthwhile to note that at 0.1 MPa and above, a population of hybridosomes® with a broken shell can be distinguished by the absence of shrinkage (indicated by the symbol (*) in Fig. 4d). Fig. 4e shows the measured hydrodynamic diameter as a function of the osmotic pressure, when a main mode can be clearly identified. We estimate the onset of the symmetry breaking instability at a critical pressure P_c = 0.075 MPa (denoted by the dotted line) and a critical radius R_c = 95/2 = 47.5 nm.

Can deformed hybridosomes recover their initial shape if the pressure is relaxed? To address this issue, we took advantage of the magnetic behavior of the IONPs hybridosomes®. Thus, the latter were separated from the PEG solution using a permanent magnet. After 2 steps of separation and washing with plain water, the hybridosomes® were incubated for 1 hour at 35 °C and the size distribution was measured again (Fig. S1, ESI†). A large fraction of the deflated shells swell back to their initial size. In addition, a larger population is observed, reflecting the presence of aggregates. The observation of the scattered light during NTA acquisition clearly shows some aggregates in the form of straight chains of a few hybridosomes. Such anisotropic aggregation most probably results from magnetic interactions between hybridosomes. Indeed, hybridosomes exhibit a non-zero magnetic moment in their swollen state (as

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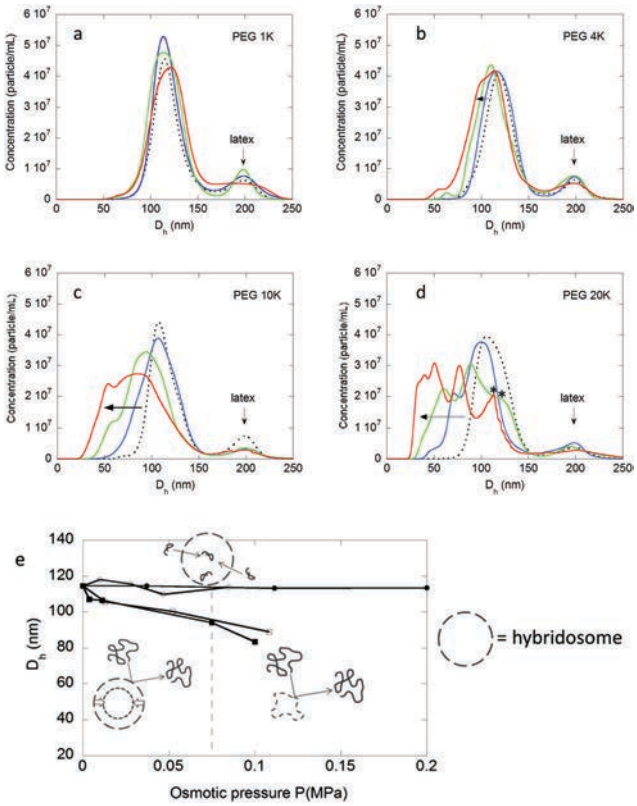

Fig. 4 (a–d) Osmotic compression of hybridosomes dispersed in solutions of PEG 1k, 4k, 10k, and 20k. A 200 nm calibrated latex solution was added into the dispersion for renormalization of the viscosity. Symbols (*) indicates broken hybridosomes. The PEG concentrations are (···) 15 g L^{-1}, (—) 45 g L^{-1}, (—) 75 g L^{-1}, and (—) 100 g L^{-1}. (e) Evolution of the size (main mode) of the hybridosomes as a function of osmotic pressure for the 4 sizes of PEG: 1k (full circles); 4k (open circles); 10k (full squares); 20k (open squares). The dotted line shows the onset of buckling. Hybridosomes: [Fe] = 25 μg mL^{-1}; [AA] = 2.25 mM.

shown by the fact that they are attracted by a permanent magnet) resulting from coupling between individual IONPs, which is increased under compression because the distance between the IONPs decreases. Interestingly, the compressed hybridosomes do not swell back at room temperature but require some thermal energy to overcome the magnetic interaction.

Our results can be interpreted in the framework of the continuum theory of thin spherical shells. The shell will be regarded as a homogeneous body (which is obviously not the case at the microscopic scale), characterized by a Young's modulus (E) and a Poisson ratio (σ). In contrast to lipid vesicles where shear is dominant, polymer vesicles are rather prone to bend (out-of-plane deformations) and stretch (in-plane deformations).[24,31] Let us first examine the case of an isotropic constraint, for instance the evaporation of a solvent core or osmotic compression.[28]

The isotropic compression (or alternatively stretching) of an isotropic hollow shell obeys:[32]

$$R_0 - R = \frac{1-\sigma}{2Eh} P R^2 \qquad (1)$$

Compression is costly and the shell is rapidly forced to give up its spherical symmetry to adapt to this new constraint at a lower energy cost.[33] At the onset of instability, the relation between the critical pressure (P_c) and the radius (R_c) is:[32]

$$P_c = \frac{2E}{\sqrt{3(1-\sigma^2)}}\left(\frac{h}{R_c}\right)^2 \qquad (2)$$

The main consequence of considering the shell as a continuum is that no length scale other than h and R enters the models. Therefore, eqn (1) and (2) apply at all scales from viruses to ping-pong balls and buildings. It was used previously to determine the mechanical properties of polyelectrolyte capsules.[23,25,34] Most previous reports on polyelectrolyte capsules monitored shape changes by optical microscopy and analyzed the fraction of deformed capsules as a function of the osmotic pressure, but were unable to characterize the evolution of the capsule radius with pressure in the subcritical regime, due to high dispersity in size.[24] Remarkably in our case, the narrow dispersity of hybridosomes® enables us to follow shrinkage during isotropic osmotic compression. Therefore, both the regime of isotropic compression and the onset of instability can be exploited using eqn (1) and (2), respectively. The values h = 5 nm and R_0 = 56.5 nm were taken as fixed geometrical parameters in both models. The Poisson ratio is an unknown parameter generally ranging from 0.25 to 0.45. We combined eqn (1) and (2) to evaluate E using arbitrary values of σ in the range 0.25–0.45. Our results were analyzed firstly in the subcritical regime using eqn (1). We fitted pressure *vs.* equilibrium radius during isotropic compression (Fig. S2, ESI†). Then, using the estimated values of the critical pressure and radius (P_c = 0.075 MPa and R_c = 47.5 nm, Fig. 4e), eqn (2) allows an independent determination of E. The results show that the value of the Poisson coefficient has in fact little influence (less than 1 MPa) on the determination of E. Therefore, we arbitrarily used the value σ = 0.3, which yielded E = 1.3 MPa and E = 5.6 MPa using eqn (1) and (2), respectively. Note that these values are one to two orders of magnitude lower than these commonly reported for polyelectrolytes capsules,[23,24] which is consistent with a weaker interaction between IONPs and PAA, than between layers of oppositely charged polyelectrolytes. Thus, hybridosomes® show a relative ability to shrink and swell, which might be exploited for encapsulation and release applications. Despite all our efforts, we were unfortunately unable to image the hybridosomes® under osmotic compression, neither by TEM nor by liquid atomic force microscopy, due to the high concentration of PEGs. However, the progressive flattening of the hybridosomes® was imaged by AFM in liquid, by applying a force of increasing intensity over their surface (Fig. S3, ESI†).

In contrast to osmotic compression for which experiments are at the ensemble level, nanoindentation enables us to probe the elasticity of the shell by applying a localized force on a single hybridosome®. Nanoindentation was investigated using AFM maintaining an aqueous environment (pure water). In the first set of experiments, force–displacement (FZ) curves were acquired in water by approaching a tip toward the sample at a speed of 1 μm s^{-1} (Fig. 5). The relative indentation extends

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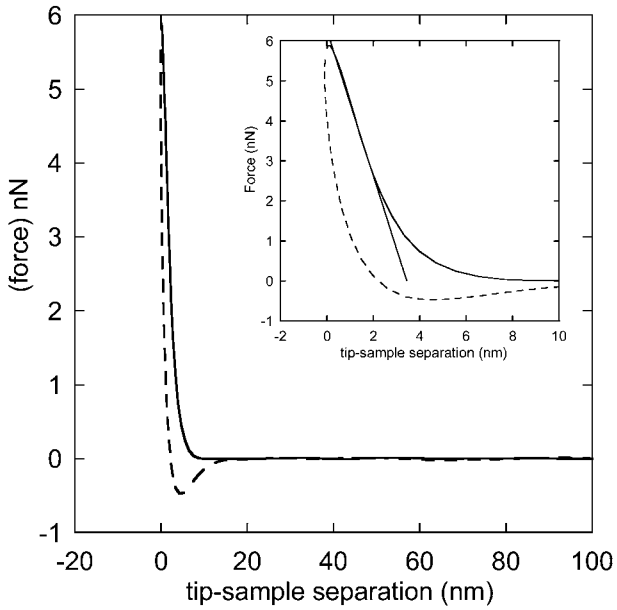

Fig. 5 Typical AFM force–distance approach (—) and retract (- - -) curve in aqueous solution. Hybridosome: [Fe] = 25 μg mL^{-1}; [AA] = 2.25 mM, initial radius 39 nm. The estimated slope from the linear part of the approach curve is 1.8 N m^{-1} (inset).

to 10% of the diameter and forces up to 7 nN. FZ curves were acquired from around 500 different locations at the surface in order to account for any heterogeneity, and yielded similar results with a standard deviation of ~25%. No alteration of the vesicle was observed after 500 measurements. The approach curves are mostly linear after build up (Fig. 5, inset) and retract curves clearly show hysteresis owing to the viscous and plastic behavior of the nanoparticle surface. We assume that the dynamics of water diffusion across the membrane is fast enough to ensure equilibrium of internal and external pressures.

To analyze the indentation data, we assume that the vesicle remains in a regime of small deformation without developing a buckling instability. This seems reasonable since the maximal displacement (~8 nm) is less than twice the shell thickness. In addition, buckling is characterized by a sudden drop in the measured force, which was not observed here.[18,24] The Young's modulus for the shell can be extracted from the linear slopes of the forward curve in the linear regime using:[35–37]

$$F = \frac{4}{\sqrt{3(1-\sigma^2)}}\frac{\delta E h^2}{R} \qquad (3)$$

where δ stands for the displacement of the surface. Using eqn (3) to fit the linear part of the forward FZ curve, we obtain E = 1.2 MPa, which again falls in the range of the values determined by osmotic pressure measurements. This value reflects a relatively low degree of interactions between the PAA polymer chains (*via* hydrogen bonding) and between the polymer and the particles (*via* interaction between the iron oxide surface and the particles). By comparison, the Young's modulus of multilayered polyelectrolyte capsules is over 100 MPa at room temperature. This relatively high value is due to their glassy state. Values measured in this work for hybridosomes are typically of the same order as those reported for multilayered polyelectrolyte capsules above their glass-melt temperature.[37]

It is interesting to note that, due to their relatively low Young's modulus, the hybridosomes® can withstand a certain amount of isotropic compression before buckling, making the elastic deformation domain experimentally observable, in contrast to most vesicular systems reported in the literature. It was reported that hollow thin shells buckle into a variety of complex conformations, including reversed spherical shells and crumpled spheres.[23,38,39] In the present case, it is difficult to characterize the shape of the hybridosomes® under pressure because of their small size.

In the following section, we investigate the effect of hybridosome composition (amount of IONPs and of PAA) on their elastic properties. In the first set of experiments, the effect of osmotic pressure was compared for two samples with different amounts of IONPs (12.5 μg mL^{-1} and 25 μg mL^{-1}). Fig. S4 (ESI†) shows that both samples have similar properties in terms of porosity and critical pressure. As mentioned previously, during their elaboration, the hybridosomes® prepared with relatively low amounts of particles contract on removal of the solvent until close contact of the IONPs. This explains both the lower mean size of the sample with [Fe] = 12.5 μg mL^{-1} compared to that with [Fe] = 25 μg mL^{-1}, and the absence of a significant effect of IONPs on the mechanical properties. In the second set of experiments, hybridosomes® with various contents of PAA were prepared in the range 0.5–15 mM (in terms of acrylic acid units). PEG 10k was selected for osmotic compression, assuming that it will not penetrate through the shells of hybridosomes®. Fig. 6 demonstrates that, at low PAA contents ([AA] = 0.4 and 1.4 mM), a mechanical instability develops and the hybridosomes® deform under

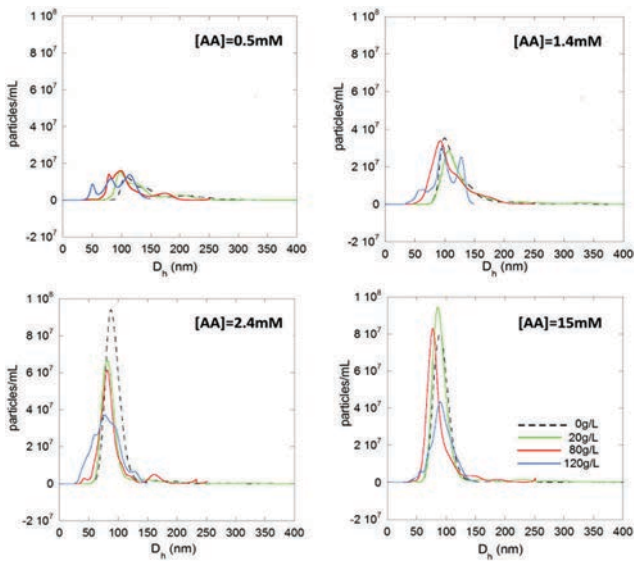

Fig. 6 Effect of osmotic compression for hybridosomes prepared with [Fe] = 12.5 μg mL^{-1} and different amounts of PAA (concentration in terms of AA units). Compression with PEG 10k at 0, 20, 80, and 120 g L^{-1}. The concentrations of PAA are indicated in the graphs.

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osmotic pressure. This is consistent with the hypothesis that PEG 10k is prevented from diffusing across the shell. The shell of hybridosomes® prepared with larger amounts of PAA should be even more impermeable to PEG 10k. Therefore, an osmotic pressure difference is exerted on the shell for all samples in the investigated range. Interestingly, we observe that increasing PAA contents provide improved mechanical stability under osmotic pressure. At the highest PAA concentration (15.5 mM), hybridosomes® are resistant to deformation exceeding 0.18 MPa (corresponding to 120 g L^{-1} of PEG 10k).

In summary, these results show that the mechanical properties of the hybridosomes® are primarily dependent on the content of the polymer in the composite shell. Increasing the amount of the polymer enables the preparation of hybridosomes® with improved mechanical stiffness.

D. Conclusions

Our result shows that hybridosomes® have a unique shell structure made of a single layer of nanoparticles, crosslinked with a polymer. In the present case, the thickness of the shell was estimated to be *ca.* 5 nm, which corresponds to the size of the individual iron oxide nanoparticles. In addition to conventional TEM and AFM analysis, we performed mechanical measurements using NTA under osmotic pressure. This is to our knowledge, the first report of mechanical measurements at the ensemble level of submicronic vesicular structures. Osmotic compression experiments demonstrated that this shell is porous, enabling the size selection of solute with a MWCO of *ca.* 4 kDa, which may be further used to protect encapsulated compounds from enzymatic degradation. The experimental results were interpreted in the framework of the continuum thin shell theory. It was found that the hybridosomes® have a soft deformable shell, with a Young's modulus of the order of MPa, which explains their outstanding mechanical properties, such as long term stability, easy deformation and shape recovery. In addition, the size and their shell properties of hybridosomes® can be tuned by varying their composition. These features are highly valuable for future biomedical applications, as contrast agents and drug carriers, to withstand high osmotic pressure in blood vessels and squeeze into small capillaries. The mechanical deformations of hybridosomes® made from IONPs should induce changes in their response to a magnetic field, which is important for imaging (MRI) and sensing applications. This will be investigated in further studies.

Acknowledgements

This work was supported by the French Ministry of Education and Research (F. Sciortino's grant) and the CNRS. F. G. warmly thanks I. Cantat and B. Dollet for fruitful discussions around the book of Landau and Lifshitz and hot chocolate.

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Structure and Elasticity of Composite Nanoparticle/Polymer Nanoshells (hybridosome®)

F. Sciortino, M. Thivolle, M. L. Kahn, C. Gaillard, S. Chevance, F. Gauffre

1. Re-swelling of the hybridosomes after osmotic compression

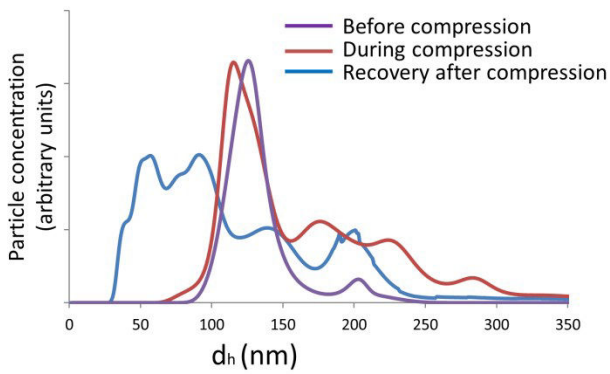

Figure S1: Size distribution of SPION hybridosomes before compression, during osmotic compression (PEG 10k, 150 g/L) and after magnetic separation and washing.

2. Fit of the linear compression domain.

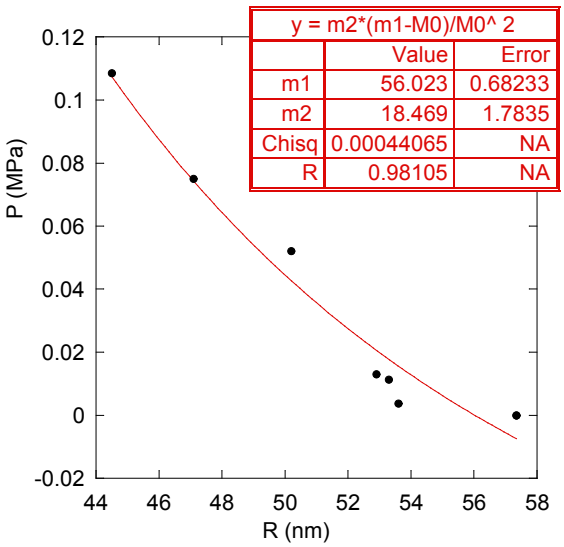

Figure S2: The data of figure 4a were merged for PEG 10K, and 20K and plotted as Pressure vs Radius and then fitted with Eq. 1.

3. Liquid AFM: Imaging applying increasing tip-sample interaction forces

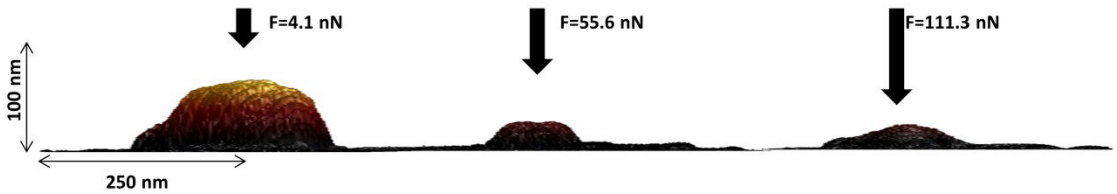

Figure S3: Height AFM images showing the evolution of the morphology of a single hybridosome at increasing values of loads from around 4 nN to 111 nN.

4. Influence of the IONPs composition of the membrane on susceptibility to osmotic pressure

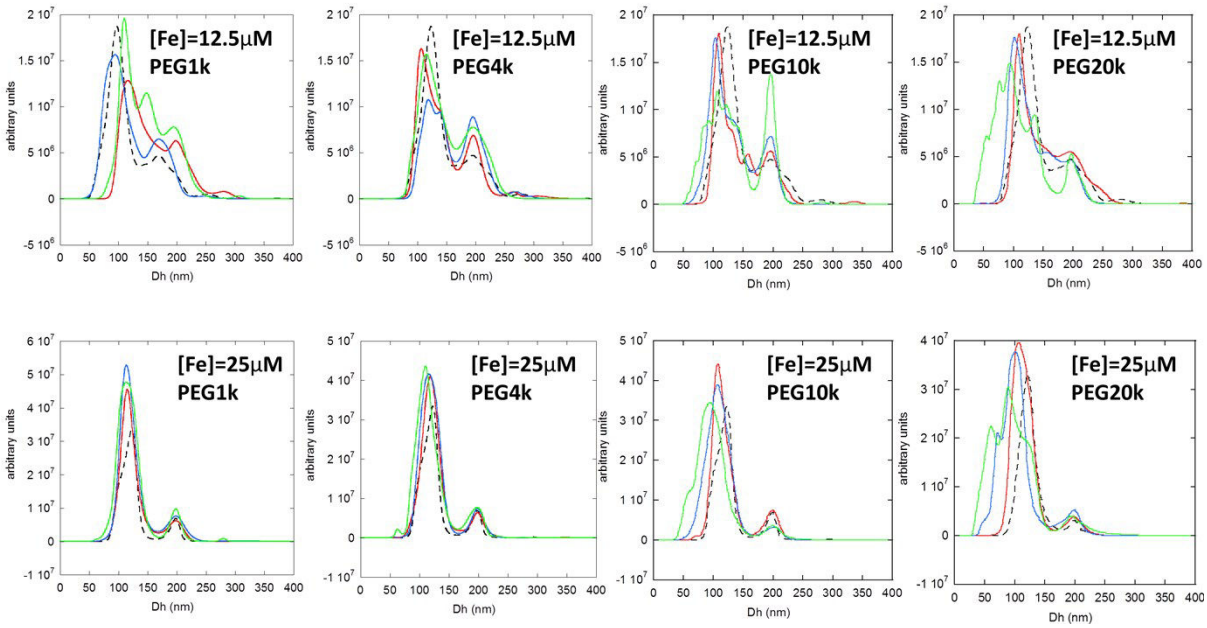

Figure S4: Osmotic compression of hybridosomes prepared from two different concentrations of IONPs ([AA]=2.25mM) : [Fe]=12.5 µg/ml (top row) and [Fe]=25 µg/ml (bottom row), using PEG of different molecular weights: 1k; 4k; 10k; 20k, as indicated on graph. PEG concentrations: 0 g/L (black, dashed), 15 g/L (red), 50 g/L (blue), 75 g/L (green).

4. Résultats complémentaires

4.1 Influence de la quantité de nanoparticule sur la structure et la paroi de l'Hybridosome

Au regard des résultats présentés dans les deux articles, on remarque que les Hybridosomes formés semblent parfois plus denses et que la structure de capsule est plus difficilement observable pour des quantités de particules plus importante ([Fe] > 50 µg.mL^{-1}). Ces observations ont été confirmées lors de l'analyse complémentaires par TEM d'Hybridosomes réalisés avec de plus grande quantité d'IONP et à quantité de PAA constante ([AA] = 2.25 mM). La Figure 23 présente les micrographies TEM et SEM des différentes conditions d'assemblage à quantité d'IONP croissante 0 µg/mL < [Fe] < 80 µg/mL.

Les micrographies Figure 23a-d montrent la présence de « gouttes » dans les milieux THF/eau. Dans les trois derniers cas, les suspensions n'ont pas pu être aimantées, ceci étant probablement dû à la faible quantité d'IONP Figure 23b-d. Par conséquent, les observations ont été réalisées au stade où les assemblages ne sont pas lavés magnétiquement. En effet la quantité d'IONP ne suffit pas pour recouvrir les gouttes et le taux de recouvrement n'est que partiel, ne créant ainsi pas suffisamment d'interactions magnétiques pour aimanter les assemblages.

C'est à partir de [Fe] = 26 µg/mL (Figure 23e) que l'on observe les Hybridosomes totalement structurés, avec un taux de couverture de goutte optimal. Les observations TEM laissent apparaitre des Hybridosomes peu contrastés, suggérant une épaisseur de la paroi de l'ordre d'une seule nanoparticule (~ 5nm). A plus forte concentration, [Fe] = 52 µg/mL (Figure 23f), les observations TEM montrent les Hybridosomes plus contrastés puisqu'il y a d'avantage de matière. L'état « dégonflés » des Hybridosomes est plus facilement discernable puisque la taille augmente aussi légèrement. L'observation des Hybridosomes au TEM se fait en condition sèche et sous vide. Les capsules apparaissent donc le plus souvent « dégonflées », « contractées » lors des observations en conditions sèches. A quantité de fer encore plus importante (Figure 23g), on observe des Hybridosomes beaucoup plus denses et de taille plus grande encore (200 - 300 nm). Enfin dans le cas [Fe] = 80 µg/mL (Figure 23g), la micrographie a été réalisée avant même d'avoir ajouté le polymère. A ce stade également on observe très bien les assemblages sphériques, qui apparaissent nettement plus denses et de taille d'environ 600 - 800 nm. Leur structure est bel et bien définie mais leur stabilité colloïdale est nettement affectée.

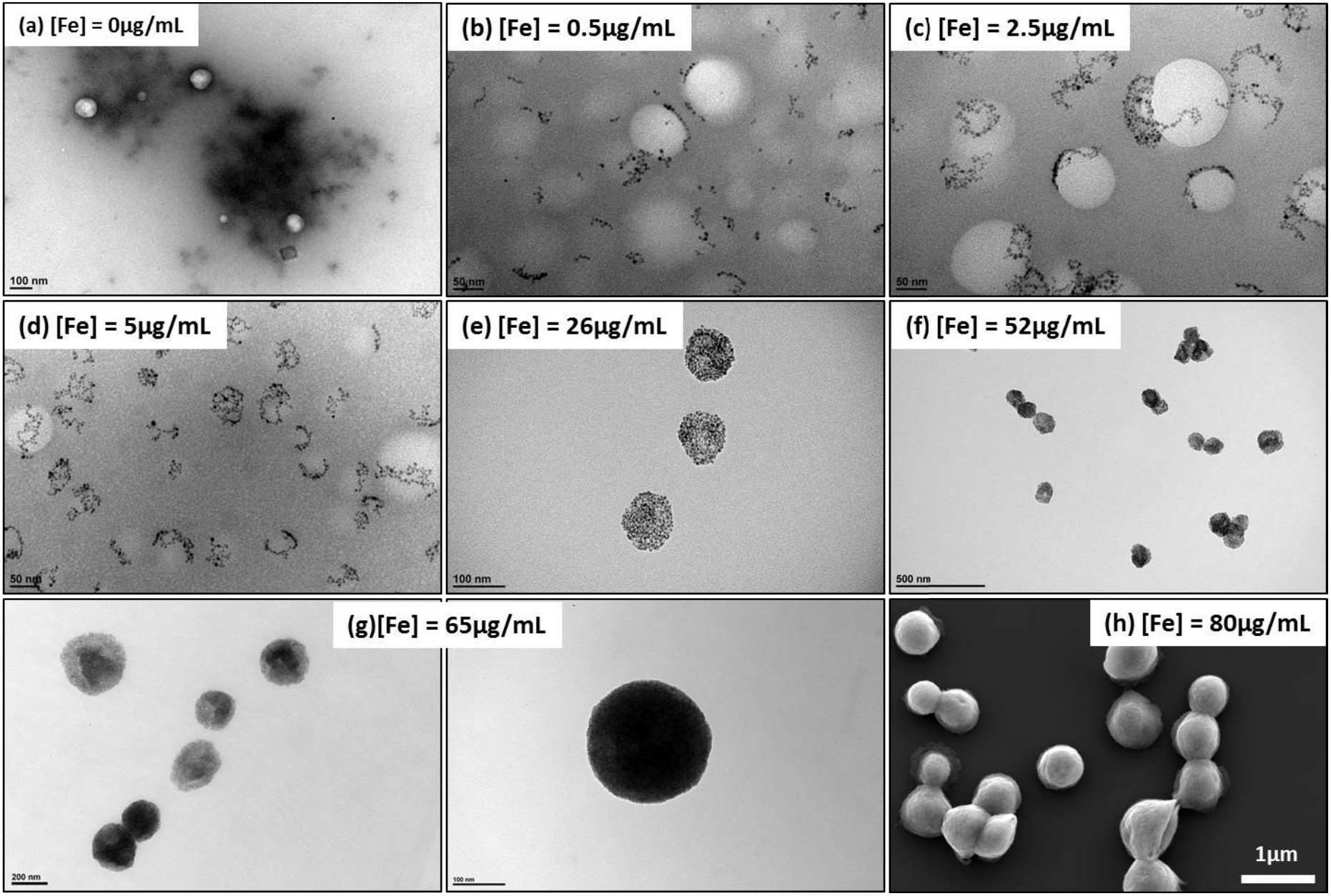

Figure 23: Micrographies TEM (a-g) et SEM (h) d'assemblages et d'Hybridosomes à concentrations croissantes en IONP (a-h) telles que [Fe] = 0, 0,5, 2,5, 5, 26, 52, 65 et 80 µg/mL.

4.2 Autres types d'Hybridosomes

D'autres types de particules ont également été utilisés pour élargir la famille des Hybridosomes comme des nanoparticules up-converting (UCNP). Les particules UCNP à base de lanthanides, émergent depuis plus d'une dizaine d'années dans les domaines de l'énergie, l'optique mais aussi en nanomédecine. En effet les propriétés électroniques remarquables des quinze éléments de la famille des lanthanides apportent d'intéressantes propriétés magnétiques et luminescentes notamment. Au cours des dernières décennies, le développement de la science des nanomatériaux a permis la synthèse de nombreux types de nanoparticules, de différentes formes et de différentes tailles. Les multiples compositions et les différents états de surface permettent aux UCNP de trouver déjà de nombreuses applications en biodétection[15], bioimagerie[16], thérapie guidée[17], chimiothérapie[18], imagerie multimodale[19].

4.2.1. Hybridosomes up-converting

Les particules UCNP utilisées sont issues d'une collaboration avec Dr. C. Coudret et B. Amouroux du laboratoire des Interactions Moléculaires Réactivité Chimique et Photochimique de Toulouse, au sein du projet ANR BLINK. Il s'agit des particules coeur@coquille de type $NaGdF_4$:Yb/Tm@$NaYF_4$ (Yb: 20 %, Tm: 1 % massique), dont la surface est fonctionnalisée par un ligand de type acide oléique et dont la taille est d'environ 8 nm (Figure 24a). Initialement dispersées dans du THF et en suivant le protocole dans des conditions similaires, des UCNP-Hybridosomes ont été formés (Figure 24b).

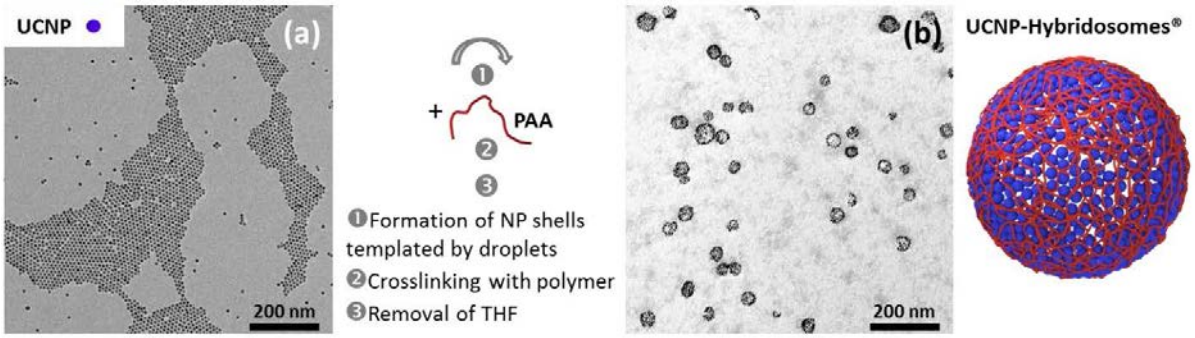

Figure 24: Assemblages d'UCNP en Hybridosomes selon le procédé classique. (a) Micrographie TEM d'UCNP avant assemblage (b) Micrographie TEM et Représentation schématique d'Hybridosomes UCNP/PAA.

On remarque que la taille des Hybridosomes est sensiblement plus petite, de l'ordre de 60-80nm et les particules sont donc facilement dénombrables grâce à leur taille légèrement supérieure.

4.2.2. Vers des Hybridosomes bi- et tri-fonctionnels

- IONP/QD-Hybridosomes

Comme vu précédemment dans l'article 1, des Hybridosomes mixtes IONP/AuNP ont déjà été préparés avec succès. De la même manière nous avons également combiné d'autres types de particules au sein de l'Hybridosome. Une première famille bimodale magnétique/luminescente a été obtenue grâce à l'utilisation des IONP ainsi que des QD (Figure 25). Les Hybridosomes formés présentent une taille autour de 100 nm et un pic d'émission de fluorescence à 625 nm (λ_{exc}= 350 nm). Nous n'avons pas cherché ici à observer un effet synergique au sein des Hybridosomes en comparant les spectres d'émission avec une suspension de QD seuls, ou en faisant varier la quantité de particules. La combinaison des particules magnétiques et semi conductrices pourraient conduire à des effets de transfert d'énergie lors d'une excitation par une source lumineuse. Cela conduit dans la plupart des cas à une diminution de l'émission de fluorescence. Avec un stimulus de nature magnétique ou électrique par exemple des hausses de température pourraient conduire à un effet mécanique et au désassemblage des capsules. Cette famille de capsules sera utilisée ensuite dans le chapitre 3.

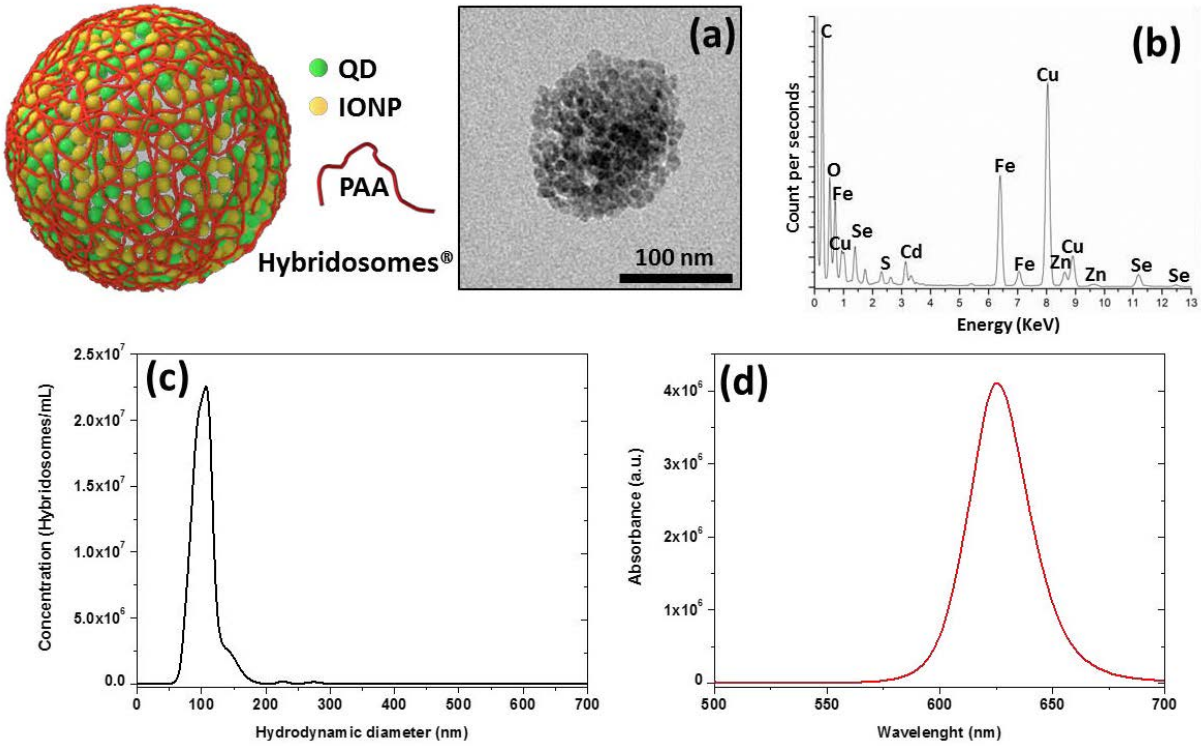

Figure 25: Représentation schématique desHybridosomes IONP/QD/PAA (a) Micrographie TEM d'un Hybridosome IONP/QD/PAA, (b) Analyse EDX obtenue à partir de (a), (c) Distribution obtenue par Nanoparticle Tracking Analysis (NTA), (d) Spectre d'émission des Hybridosomes IONP/QD/PAA (λ_{exc} = 350nm).

- IONP/AuNP/QD-Hybridosomes

Nous avons également montré qu'il était possible en faisant varier simplement la proportion de particules utilisées, de combiner trois types de particules avec dans ce cas des IONP, des AuNP et des QD. Ainsi les Hybridosomes formés sont à la fois magnétiques, plasmoniques et luminescents (Figure 26). L'analyse EDX (Figure 26b) montre la présence des six éléments attendus (Fe, Au, Cd, Se, Zn, S) à partir de la Figure 26a, confirmant la présence des trois types de particules au sein d'un Hybridosome. Le spectre d'absorbtion (Figure 26d) laisse apparaitre un signal de diffusion important et l'on discerne peu le signal plasmonique de l'or à 525 nm. Le spectre d'émission de fluorescence confirme la présence des QD avec un pic à 625 nm (λ_{exc}= 350 nm). Enfin l'analyse de taille NTA (Figure 26c) laisse apparaître une population majoritaire à 103 nm.

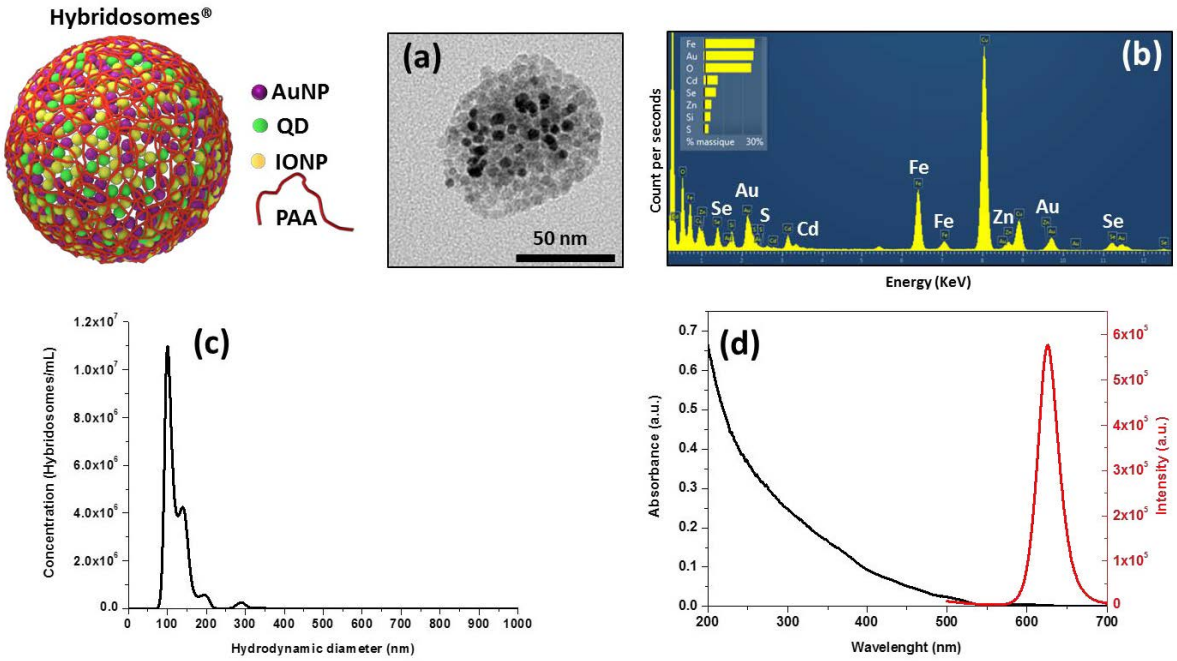

Figure 26 : (a) Micrographie TEM d'un Hybridosome IONP/AuNP/QD/PAA, (b) Analyse EDX obtenue à partir de (a), (c) Distribution obtenue par NTA, (d) Spectre d'absorption des Hybridosomes IONP/AuNP/QD/PAA (noir) et spectre d'émission (λ_{exc} = 350nm).

- UCNP/QD-Hybridosomes, UCNP/Au-Hybridosomes

Certains travaux ont montré des effets de transfert d'énergie de type FRET avec des assemblages d'UCNP et d'autres chromophores, d'autres particules de type QD ou encore plasmoniques[20]. Dans le but d'observer ces effets au sein d'assemblages de particules, des Hybridosomes UCNP/QD ainsi que UCNP/AuNP ont également été préparés. Cependant la manipulation des UCNP n'est pas aussi simple que les IONP car leur stabilité colloïdale est moindre. La sédimentation de la suspension mère d'UCNP ainsi que le faible rendement quantique de leur émission rend l'analyse et la comparaison des résultats d'absorbance et d'émission de fluorescence plus compliquée.

4.2.3. Vers les assemblages « coeur@coquille»

L'étape de stabilisation des capsules par les chaînes de polymères a également été envisagée avec un autre type de matériau comme la silice. Depuis les années 1970 avec les premiers travaux de Stöber[21] sur la synthèse de nanoparticule de silice colloïdale, le développement de matériaux à base de silice n'a cessé de croître et la littérature est aujourd'hui très riche. De très nombreux aspects du procédé de synthèse ont été étudiés et modifiés (nature des précurseurs, ratio entre précurseurs, cinétiques de réactions, température, etc) et permettent aujourd'hui la synthèse d'un grand nombre de particules de différentes tailles[22] et porosités[23,24]. La silice est souvent utilisée pour recouvrir des nanomatériaux dans le but de créer une couche qui apporte une stabilité chimique et colloïdale[25–27]. On décrit souvent ces matériaux comme cœur@coquille et un grand nombre de nanoparticules hydrophiles et hydrophobes ont été encapsulées. Au moment de l'étape de l'ajout du polymère, la suspension a été mise en présence du précurseur tétrahydroxysylane (TEOS) au lieu du PAA, et a été engagée dans un protocole modifié de Stöber. Les assemblages ont été uniquement caractérisés par microscopie électronique (Figure 27).

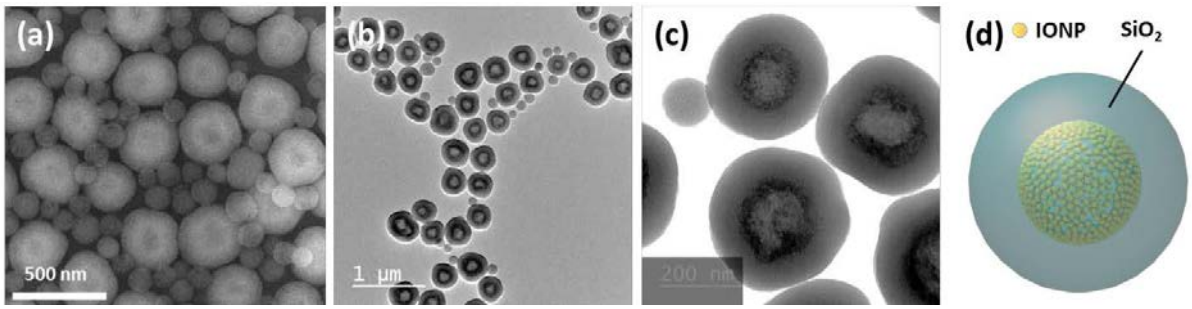

Figure 27: Enrobage des assemblages d'IONP par une coque de silice. (a) Micrographie SEM et (b,c) Micrographie TEM des assemblages recouverts par une coque de silice, (d) Représentation schématique des assemblages.

Les observations montrent que les assemblages d'environ 200nm ont bien été recouverts par une couche de silice d'environ 100 nm. On remarque cependant que des particules de silice d'environ 100 nm sont aussi présentes et ce malgré plusieurs lavages magnétiques. Cela est probablement dû à l'excès de précurseur. Ces résultats nous permettent de valider la preuve du concept de recouvrement des assemblages par une coque de silice. Cependant la question de l'évaporation des solvants reste ouverte dans ce cas. Effectivement si le THF est présent dans le cœur de la capsule, alors il se peut que son évaporation soit différente lorsqu'une couche de silice est présente. La porosité de la coquille reste à considérer.

4.3 Etudes de stabilité des Hybridosomes

4.3.1. Stabilité colloïdale des Hybridosomes à pH 4.5 et au cours du temps

Des premiers suivis de la taille au cours du temps ont été effectués à pH 4.5. Il s'agit du pH auquel s'initie la réaction de bioconjugaison envisagée dont nous parlerons dans le chapitre 4. Les diagrammes montrent une population majoritaire autour de 100 nm ainsi que des populations mineures entre 150 et 300 nm. La Figure 28b montre un cliché TEM des Hybridosomes dans leur état initial après lavage à pH 7. On remarque des objets de 100 nm et de 150 nm ce qui est en adéquation avec la mesure NTA. Ces résultats semblent montrer que à pH 4.5, les Hybridosomes ne subissent pas de changements dans les premières 24h, qui conduiraient à une variation de taille.

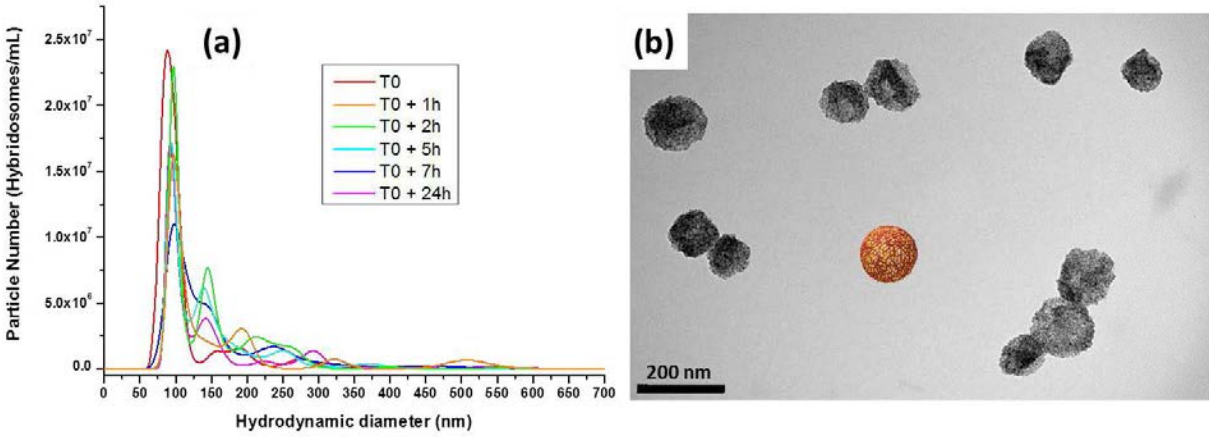

Figure 28: Caractérisation par NTA de la taille des Hybridosomes au cours du temps à pH 4.5. (a) Distributions obtenues par NTA à T_0 (rouge), T_{0+1h} (orange), T_{0+2h} (vert), T_{0+5h} (cyan), T_{0+7h} (bleu), T_{0+24h} (magenta). (b) Micrographie TEM des Hybridosomes à T_0, pH 7 et représentation schématique d'un Hybridosome.

4.3.2. Caractérisation de la charge de surface des Hybridosomes et étude de stabilité en fonction du temps à différents pH.

Des mesures de potentiel zeta (ζ) ont été menées afin de déterminer l'état de charge de surface des Hybridosomes en fonction du pH. L'étude porte sur des Hybridosomes de type IONP/PAA ainsi que IONP/BODIPY/PAA.

Cependant, les capsules ont été préparées à partir d'une suspension mère d'IONP de concentration inconnue mais visiblement supérieure. La quantité de fer introduite dans la préparation des Hybridosomes a donc été sous-estimée $[Fe]_{est}$ > 50 µg/mL. Comme nous l'avons vu précédemment dans les articles 1 et 2, la taille des Hybridosomes peut être variable selon la quantité de nanoparticules. La Figure 29 montre l'observation TEM et l'analyse NTA des deux types d'Hybridosomes.

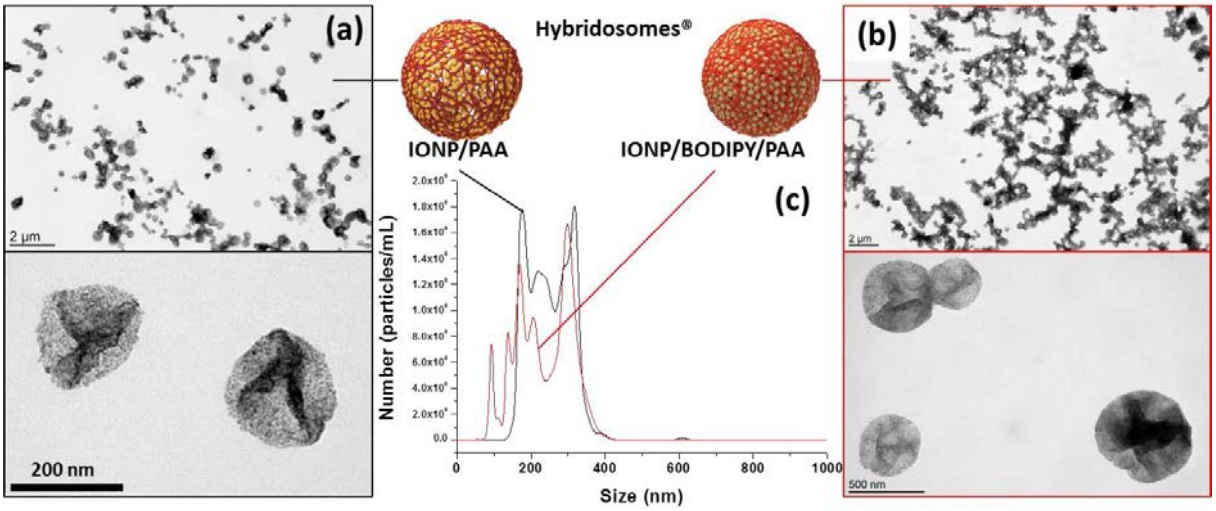

Figure 29: Caractérisation d'Hybridosomes à base d'IONP et IONP/BODIPY,à pH 7. (a,b) Micrographie TEM des Hybridosomes IONP/PAA et IONP/BODIPY/PAA respectivement. (c) NTA des Hybridosomes IONP/PAA (noir) et IONP/BODIPY (rouge)

Dans ce cas, à une concentration en fer supérieur à 50 µg/mL la distribution de taille est effectivement un peu plus large avec des objets entre 100 et 300nm ce qui rejoint les observations faites précédemment (partie 4.1)

Les mesures de potentiels zeta ont été effectuées à t_0, c'est-à-dire après dilution et ajustement du pH, et à t_{0+24h}. La première observation montre que le potentiel zeta des deux types d'objets (avec et sans Bodipy) suit la même tendance de pH 1.0 à pH 6.5 (Figure 30a). Autrement dit, il n'y a pas de différence d'état de charge entre les Hybridosomes avec et sans Bodipy sur la gamme de pH étudiée.

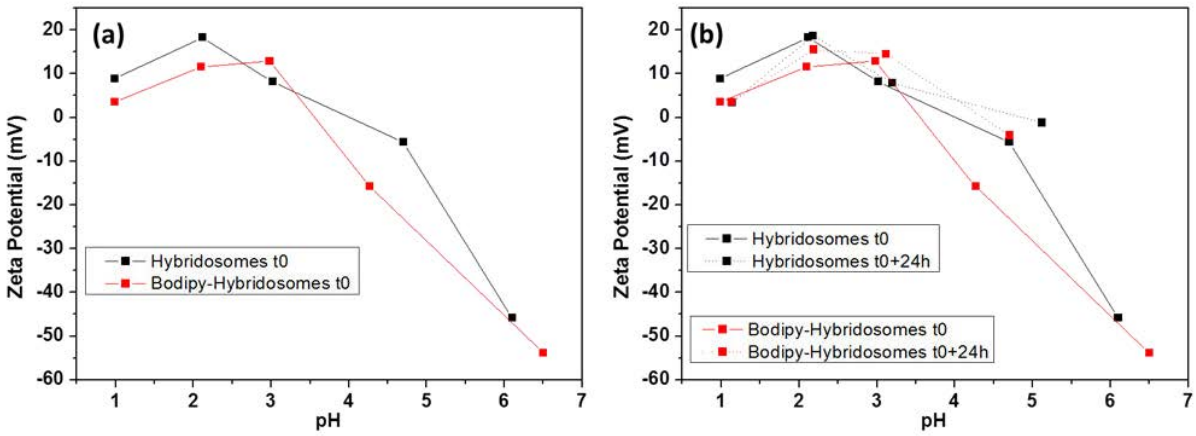

Figure 30: Potentiel Zeta (ζ en mV) des Hybridosomes IONP/PAA (noir) et IONP/BODIPY/PAA (rouge) en fonction du pH (a) à t_0 et (b) à t_0 (traits pleins) et à t_{0+24h} (traits pointillés)

La seconde observation montre un point isoélectrique (pHi) aux alentours de pH 4,0 – 4,5. Ce point correspond à la valeur de pH pour laquelle le potentiel zeta est nul. Dans notre cas, il renvoie à l'état de protonation du PAA : en effet, La valeur de 4-4.5 correspond avec le pKa de l'acide acrylique (pKa = 4,25) et confirme les observations suivantes. Aux grandes valeurs de pH > pHi + 1 le polymère non protoné donne des valeurs de potentiel zeta négatives (-53 mV < ζ <-15 mV). A l'inverse, en deçà de pHi -1, les chaînes de polymère sont protonées sous la forme COOH ce qui se traduit par des valeurs de potentiel zeta légèrement positives (4 mV< ζ <19 mV). Ces résultats confirment que le PAA joue bien le rôle de « coating » de surface de l'Hybridosome lui apportant ainsi sa stabilité structurale et colloïdale.

En parallèle de cette étude, des suivis au cours du temps ont pu être menés par NTA ainsi qu'en microscopie TEM sur les mêmes échantillons à pH 4,0/pH 3,0/pH 2,0/pH 1,0, afin de déterminer l'impact de ces variations de pH sur la distribution de taille ainsi que la structure des Hybridosomes.

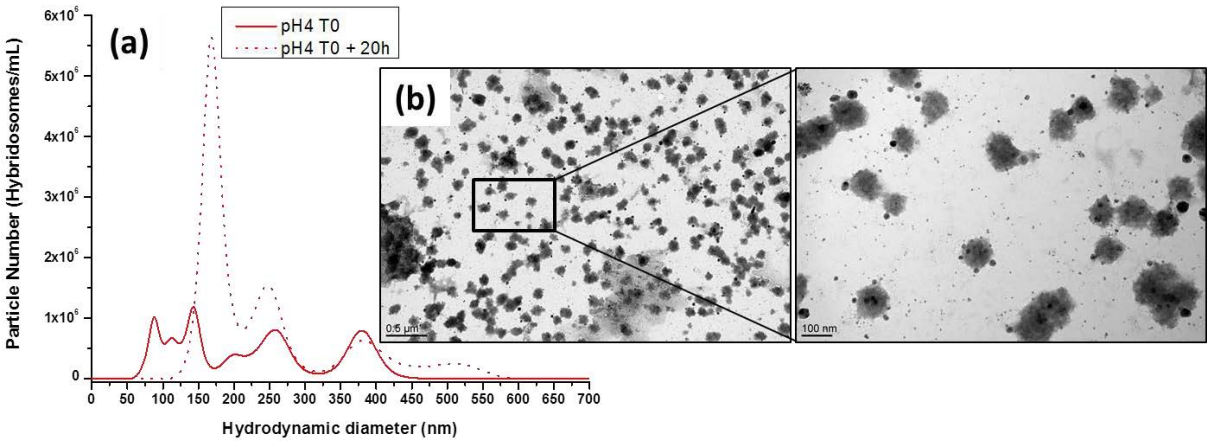

Figure 31: Stabilité des Hybridosomes IONP/PAA à pH 4,0 au cours du temps. (a) Distribution de taille des Hybridosomes obtenue par NTA à T_0 (ligne) et T_{0+20h} (pointillés), (b) Micrographies TEM à T_{0+20h}

La Figure 31a montre une légère évolution de la taille bien que les trois principales populations soient clairement identifiées à T_0 et T_{0+20h}. En revanche, le nombre d'objets comptés semble croître d'un facteur 5 ce qui pourrait indiquer une possible dissociation des Hybridosomes. De plus, les micrographies TEM à T_{0+20h} laissent apparaître des Hybridosomes détruits et deux observations sont faites. Premièrement la structure de capsule n'est plus du tout maintenue, puisque l'aspet des assemblages d'environ 150 nm ne resemblent plus à des Hybridosomes. De plus, les aggrégats apparaissent d'avantage collés. Deuxièmement, les IONP de 5 nm environ sont plus difficilement observables. Des structures de tailles entre 5 et 50nm avec de forts contrastes sont observées. Il pourrait s'agir de particules coalescées, ou encore de particules dissoutes puis recomplexées avec le PAA. Nous observons également des amas d'environ 200-300 nm qui sont probablement des agrégats de PAA et d'oxydes de fer coalescés ou dissouts. En effet les oxydes de Fer ne sont pas très stables à pH acide et l'observation montre qu'à 20 h, la majorité des IONP de 5 nm ont coalescées en particules d'environ 10-50 nm.

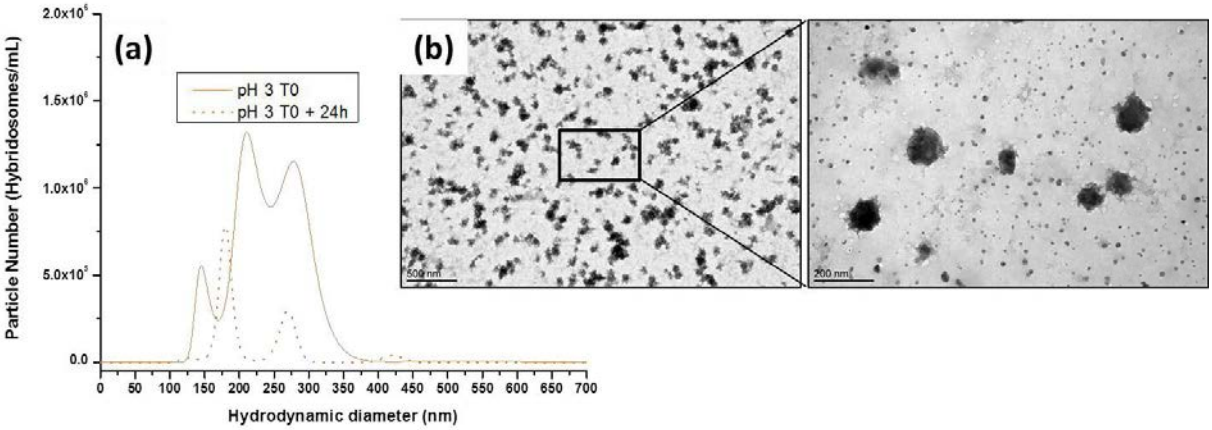

Figure 32 : Stabilité des Hybridosomes IONP/PAA à pH 3,0 au cours du temps, (a) Distribution obtenue par NTA à T_0 (ligne) et T_{0+24h} (pointillés), (b) Micrographies TEM à T_{0+24h}.

Au regard des résultats de l'étude menée à pH 3,0 Figure 32, on observe que la taille mesurée par NTA semble augmenter légèrement à T_{0+24h} avec une plus large distribution. Le nombre total d'objets semble décroitre sensiblement d'un facteur deux. Cependant la quantification du nombre d'objets varie déjà d'un facteur deux entre deux mesures faites à un intervalle de temps de l'ordre de la minute. L'information quantitative donnée par le Nanosight est à investiguer d'avantage afin d'être prise en compte. De plus, les objets observés en TEM sont similaires à ceux observés à pH 4,0.

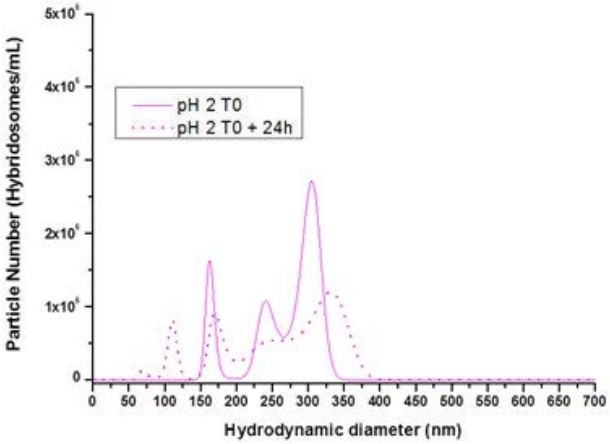

Figure 33: pH 2 T_0, T_{0+24h}, (a) Distribution de la taille des Hybridosomes obtenue par NTA à T_0 (ligne) et T_{0+24h} (pointillés)

A pH 2,0 (Figure 33), on observe des distributions de taille assez proches à T_0 et T_{0+24h}. On note deux petites populations apparaitrent entre 50 nm et 100 nm après 24h à pH 2,0.

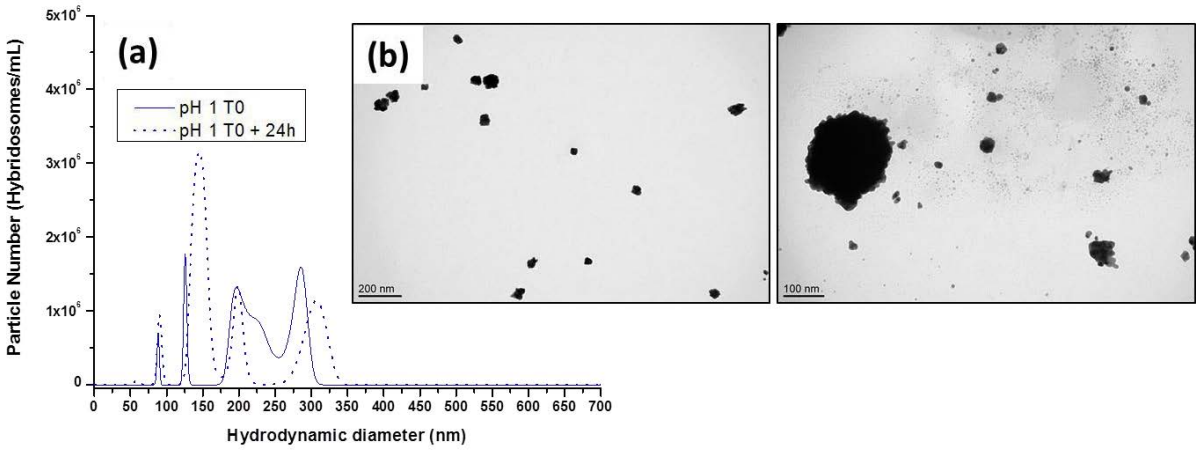

Figure 34: pH 1 T_0, T_{0+24h}, (a) Distribution de la taille des Hybridosomes obtenue par NTA à T_0 (ligne) et T_{0+24h} (pointillés), (b) Micrographie TEM à T_{0+24h}

Enfin l'étude à pH 1,0 confirme également les résultats précédents avec une légère variation de taille après 24 h, et les capsules apparaissent également complètement détruites et les IONP probablement coalescées (Figure 34).

Concernant les Hybridosomes IONP/Bodipy/PAA, les observations faites à pH 4,0/pH 3,0/pH 2,0/pH 1,0 par NTA sont similaires. La taille des objets en solution n'évoluent pas beaucoup après 24h (Figure 35a et b). De plus, l'observation au microscope électronique à transmission montre pour les Hybridosomes à pH 1 T_{0+20h} sont détruits et que les particules d'oxyde de fer sont détruites, certainement cloalescées ou dissoutes (Figure 35c). Ce point de stabilité en fonction du pH sera discuté également dans le chapitre 3.

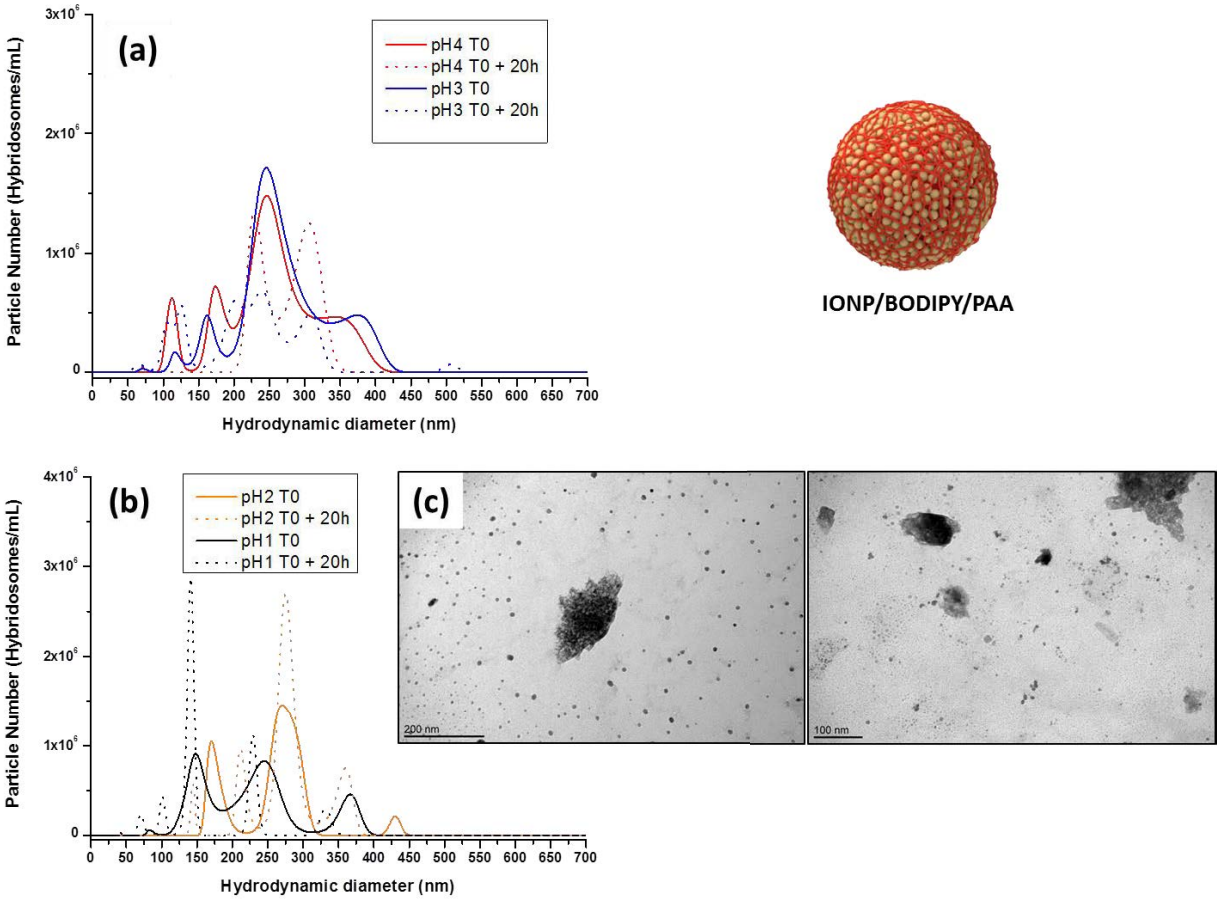

Figure 35: (a,b) Suivi de la taille des Hybridosomes IONP/Bodipy/PAA à différents pH et en fonction du temps. (c) Micrographies TEM à pH 1 T_{0+20h}.

4.4 Localisation des Hybridosomes *ex vivo*

Les résultats présentés dans l'article 1 ont confirmés l'efficacité des Hybridosomes *in vivo* comme agent de contrastes négatifs en IRM. Cependant, la question du devenir des Hybridosomes *in vivo*, de leur pharmacocinétique et de leur biodistribution est primordiale et doit être adressée dans le développement des Hybridosomes pour de telles applications.

Dans le cas présenté dans l'article 1, nous avons observé une extinction massive du signal principalement dans le foie et la rate, ce qui est en accord avec le devenir d'objets de d'environ 100 nm, lorsqu'ils circulent par voie sanguine. A la suite des injections et mesures en IRM effectuées en collaboration avec Dr. P.A. Elliat (UMS CNRS 3480 – INSERM 018 Biosit, plateforme BioScan PRISM), Dr. M.B. Troadec et H. Jakobzyck (équipe GEO, IGDR), les souris ont été sacrifiées afin de prélever et analyser ces deux tissus d'intérêt. Des études histopathologiques ont été réalisées au sein de la plateforme H^2P^2 (Biosit) à Rennes, sur les deux organes afin de vérifier l'intégrité des tissus par la méthode HES (Hémalun Eosine Safran) et de déterminer la présence de fer par la méthode de coloration de Perls.

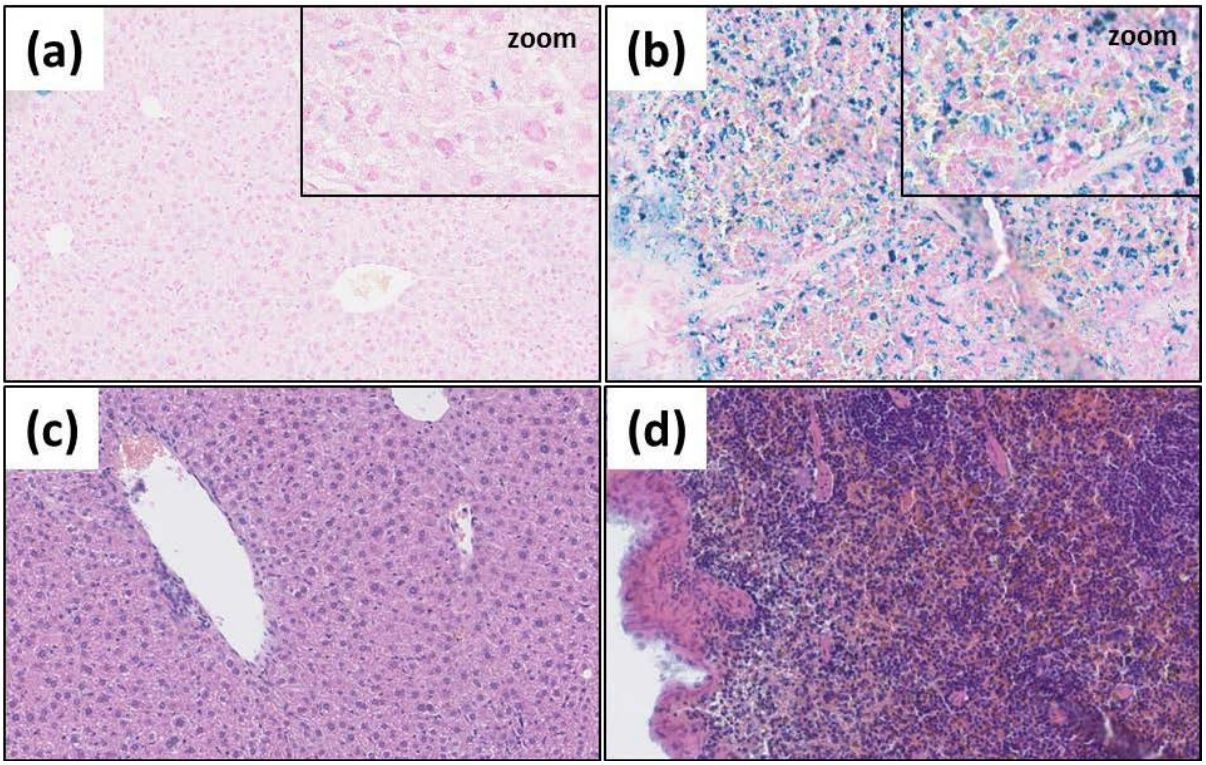

Figure 36 : Coloration Perls et HES des tissus *ex vivo* 24 h après injection des Hybridosomes. (a) Perls du foie et (b) de la rate, (c) HES du foie et (d) de la rate.

La méthode HES colore les noyaux en violet (hémalun), les cytoplasmes en rose (éosine) et la matrice extracellulaire, notamment le collagène, en jaune (safran). Cette coloration permet donc de visualiser l'architecture des tissus.

La coloration de Perls quant à elle permet la mise en évidence des ions Fe(III) par complexation avec le ferrocyanure de potassium et formation du bleu de Prusse. La Figure 36 montre les images de microscopie optique en coloration de Perls du foie et de la rate (Figure 36a et 17b) respectivement, ainsi que la coloration HES du foie et de la rate (Figure 36c et 17d) respectivement. Sur les coupes de foie, on discerne des hépatocytes sains dont le noyau circulaire apparait légèrement plus contrasté en rose (Figure 36a). L'architecture générale du foie est homogène et ne laisse pas suspecter d'altération particulière. Selon le Dr. B. Turlin, anatomopathologiste au CHU de Rennes, la coloration de Perls montre une charge en fer faible. C'est manifestement au niveau des cellules sinusoïdales que l'on visualise les dépôts. Le fait que les dépôts ne soient pas continus le long des sinusoïdes plaiderait plutôt pour des dépôts au sein des cellules de Kupffer. Ainsi la coloration bleue pourrait correspondre aux macrophages, c'est-à-dire aux cellules de Kuppfer responsables de la métabolisation des déchets et débris cellulaires et qui pourraient avoir phagocyté les Hybridosomes. On remarque également la présence de taches brunes de petite taille, observées dans certains cas, qui reste pour l'instant sans explication.

La Figure 36b de la rate est nettement plus colorée par le Perls et indique une présence plus importante de fer, issu de la dégradation par les macrophages de l'hémoglobine des globules rouges vieillissants dégradés dans la rate. Cependant, nous ne pouvons pas conclure à la présence d'Hybridosomes dans la rate. Les observations de la Figure 36 ne montrent aucune altération des tissus 24h après injection des Hybridosomes, ce qui est en accord avec les résultats de viabilité cellulaire présentés dans l'article 1. Ainsi, il semblerait que les Hybridosomes *in vivo* soient principalement phagocytés par les macrophages du foie et possiblement de la rate.

Afin de d'interroger l'internalisation et la structure des Hybridosomes in vivo et de préciser leur localisation après 24h, nous avons réalisé l'étude des tissus par microscopie électronique à transmission grâce à une technique de micro coupes des tissus après fixation dans une résine époxy. Les micrographies présentées ci-après sont le fruit d'une collaboration avec Dr. A. Burel au sein de la plateforme MRic-TEM (UMS Biosit). La Figure 37 présente des micrographies de ces coupes à différents grandissements. On discerne dans l'image du contrôle externe sans injection d'Hybridosomes, un hépatocyte (Figure 37a), cellule majoritaires du foie, le réticulum endoplasmique (re) ainsi que des gouttes cytoplasmiques contenant des lipides (l).

La Figure 37b, présente une image de la coupe de foie après injection des Hybridosomes in vivo. On observe également les compartiments lipidiques (l). La cellule visible au centre du cliché Figure 37b n'est pas clairement identifiable et il pourrait s'agir d'une cellule de Kuppfer, macrophages du foie. On y observe des zones aux densités de contraste remarquables. A un grandissement plus important Figure 37c, l'aspect de capsules déformées permet d'identifier les Hybridosomes. Les Hybridosomes semblent internalisés dans des vésicules, très probablement des lysosomes, vésicules typiques à l'aspect contrasté.

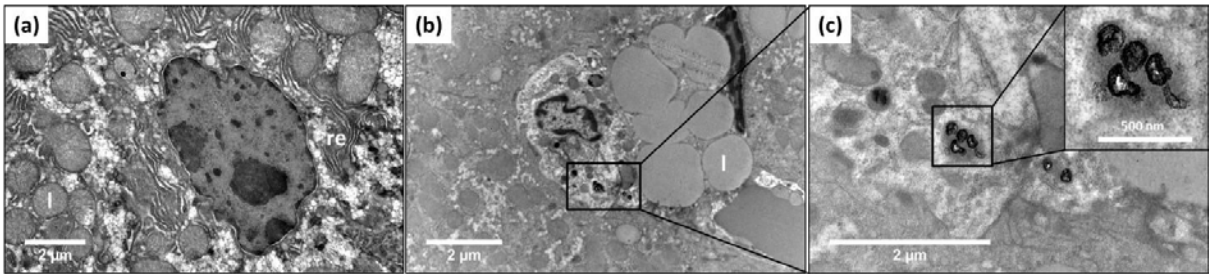

Figure 37: Micrographie TEM de coupe de foie de souris à différents grandissements. (a) sans injection d'Hybridosomes (b,c) après injection des Hybridosomes

La Figure 38 présente les observations de la rate après et sans injection d'Hybridosomes. Les observations des coupes montrent les cellules, probablement de la pulpe, dont le noyau apparait plus sombre. Cette observation a été réalisée dans le cas du contrôle externe (souris non injectée) et interne (souris injectée), ce qui confirme que l'injection des Hybridosomes, dans les conditions de l'expérience décrite dans l'article 1, et leur accumulation au sein des tissus ne modifie en aucun cas l'intégrité du tissu dans lequel ils s'accumulent. De plus, la Figure 38b met en évidence la présence de zones très contrastées comme précédemment dans le foie. Comme pour le foie, la présence des intra-cytoplasmique des Hybridosomes semble être confirmée avec la présence dans les lysosomes de structures anormalement contrastées, semblables à des capsules dégonflées, caractéristiques des Hybridosomes. Cependant, le type cellulaire n'est également pas clairement identifié. Les Hybridosomes apparaissent regroupés par « paquets » à l'intérieur de lysosomes et leur structure semble être conservée au sein de ces vésicules lysosomales.

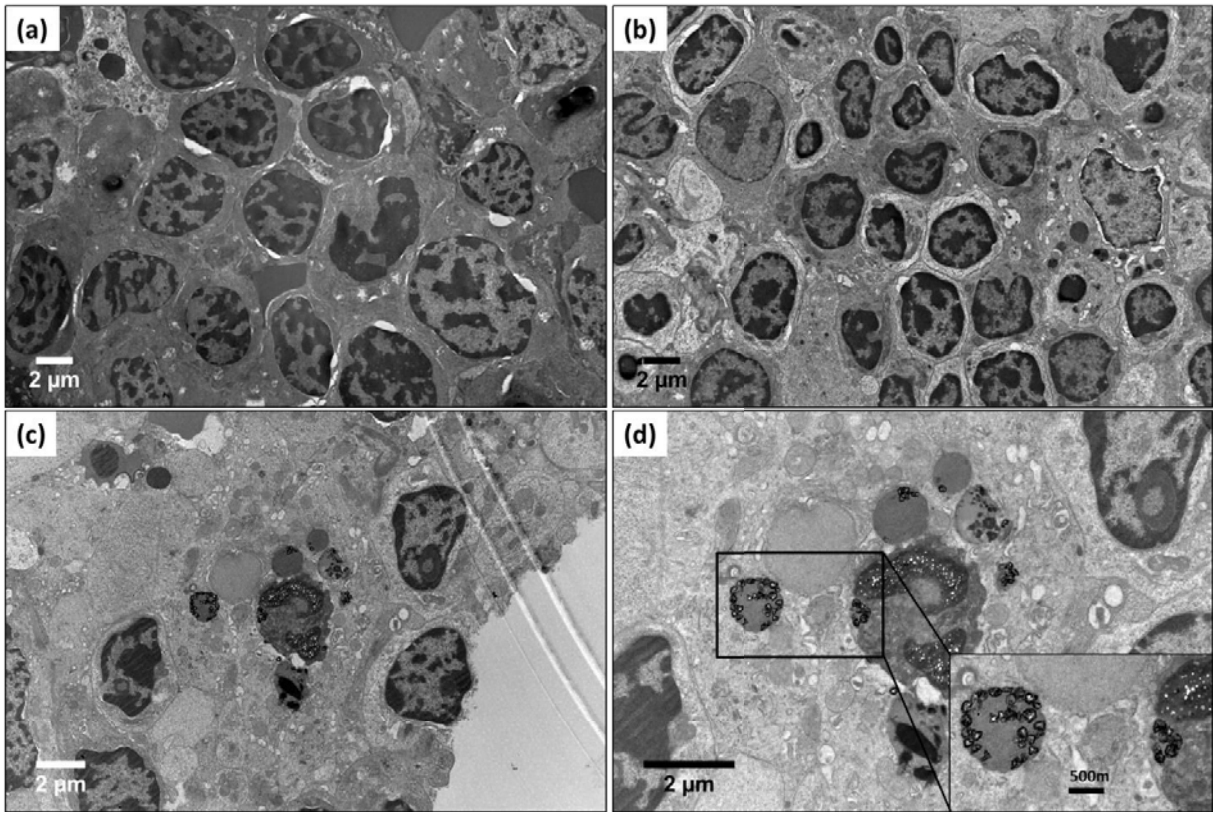

Figure 38: Micrographie TEM de coupe de rate à différents grandissements. (a) Image contrôle externe d'une rate sans injection d'Hybridosomes. (b,c,d) Micrographies de la rate d'une souris après injection des Hybridosomes.

Pour résumé, ces études Histopathologiques et de microscopies dans le foie et dans la rate confirme l'internalisation des Hybridosomes dans les deux tissus. Dans le foie, il pourrait s'agir des cellules de Kupffer, macrophages du foie mais une accumulation extra hépatocytaire n'est pas exclue. A partir des observations TEM, nous pouvons suspecter la présence des Hybridosomes sans pouvoir discerner les organites cellulaires dans lesquels ils sont localisés. D'autres études de microscopie confocal sont en cours afin de confirmer l'internalisation des capsules et de déterminer dans quels compartiments cellulaires a-t-elle lieu. Les résultats confirment également la stabilité structurale des Hybridosomes *in vivo* 24 h après injection. Ils apparaissent intègres, et la structure de capsule est facilement discernable comme lors des autres observations au TEM + SEM après dépôt de suspension.

5. Conclusion

Nous avons vu dans ce chapitre le principe d'élaboration de nouvelles capsules hybrides à partir de nanoparticules inorganiques recouvertes d'une couche de polymère appelées Hybridosomes®.

Concernant la présence d'un template liquide au sein du mélange THF/eau, le mécanisme d'assemblage n'est pas encore totalement compris et l'origine de ces « gouttes » est encore à éclaircir. En effet, le solvant utilisé n'est pas totalement pur et contient à minima quelques ppm de stabilisant de type butylhydroxytoluène (BHT) utilisé comme antioxydant. La présence du BHT peut suggérer que l'effet Ouzo pourrait être à l'origine des structures de gouttes. La présence de petites molécules hydrophobes en faible quantité est parfois négligée selon les domaines scientifiques et les différentes communautés. La compréhension de ces mélanges pose encore un grand nombre de questions et continue de faire l'objet d'études à différentes échelles.

La pluralité de la méthode d'assemblage a conduit à l'élaboration d'une grande variété d'Hybridosomes. Nous avons montré que les Hybridosomes pouvaient être constitués de plusieurs types de nanoparticules et les mélanges IONP/AuNP, IONP/QD, IONP/QD/AuNP, UCNP/QD, UCNP/AuNP ont été étudiés. Dans la plupart de ces assemblages, seule la preuve de concept a été apportée, avec des mesures de taille (DLS, NTA) et de fluorescences dans le cas des IONP/QD-Hybridosomes. Des études approfondies concernant d'éventuelles synergies, effet d'extinction/augmentation de fluorescence, couplage magnétique/plasmonique interparticules, sont en cours sur différents types d'Hybridosomes.

Par ailleurs, le procédé d'assemblage a permis de piéger un fluorophore de la famille des Bodipy et ainsi de conférer à l'Hybridosome une modalité de fluorescence *via* un fluorophore moléculaire. Nous reviendrons sur ce point un peu plus en détail dans le chapitre suivant.

Une étude plus profonde a été réalisée sur les Hybridosomes uniquement composé d'IONP et de PAA afin de montrer l'influence de la quantité des deux composants majeurs. Il a été montré qu'en faisant varier ce ratio il est possible de modifier la taille des Hybridosomes formés de 80 à 150 nm tout en gardant une très bonne monodispersité. Les résultats présentés dans l'article 2 démontrent que la paroi de l'Hybridosome peut être constituée d'une seule couche de particule. Nous n'avons à ce jour pas réussi à mettre en évidence la relation entre la quantité de nanoparticules lors de l'assemblage et le nombre d'Hybridosomes formés, ainsi que l'épaisseur de la paroi. D'après les observations Figure 23, il semble évident que la paroi peut aussi être composée de plusieurs couches de particules, jusqu'à même former des sphères pleines d'environ 600 - 800 nm, qui pourraient également présenter de nouvelles propriétés en fonction des particules utilisées

(magnétiques, plasmoniques etc). Si un couplage plasmonique avec un déplacement batochrome du pic d'absorption a parfois pu être observé avec des assemblages composé d'AuNP, nous n'avons pas réussi à les stabiliser. Ce point reste à adresser.

La structure des Hybridosomes a été observée et caractérisée à différents niveaux (échelle d'une capsule et en suspension à l'échelle bulk) ainsi que dans différents états (secs et en solution). Les propriétés d'élasticité et de porosité de la paroi ont été mesurées. La capsule présente une très bonne élasticité avec un module d'Young de l'ordre du MPa. Le PAA confère, quant à lui une certaine porosité estimée à quelques nanomètres environ. Il fait des Hybridosomes des capsules déformables et très stables dans le temps. Leur stabilité chimique dans le temps est également très bonne entre pH 5 et 8 et donc à pH physiologique. En dehors de cette gamme, la stabilité de l'interaction entre le PAA et les hydroxyles de surface est difficile à appréhender et l'aspect cinétique est primordial car la stabilité des nanoparticules est aussi à prendre en compte. Là encore, ce point reste à adresser plus particulièrement, par exemple en travaillant avec d'autres polymères.

Les Hybridosomes ont montré des propriétés intéressantes en tant qu'agent de contraste négatif en IRM, présentant des relaxivités transmersales comparables *in vitro* et *in vivo* à celles d'un agent commercial pré clinique (FeraSpin™, Myltenyi).[14] Les relaxivités *in vitro* des Hybridosomes IONP/PAA et IONP/AuNP/PAA sont de r_2 = 257±15 $mM^{-1}.s^{-1}$ et r_2 = 274±35 $mM^{-1}.s^{-1}$ comparée à une valeur de r_2 = 307±80 $mM^{-1}.s^{-1}$ dans le cas du FeraSpin™. Les observations *in vivo* ont également permis de calculer un contraste négatif (« enhanced contrast ratio ») de 63 % et 43 % pour les Hybridosomes IONP/PAA et IONP/AuNP/PAA respectivement.

Plus que leurs propriétés de relaxivités, la structure et la grande modularité des Hybridosomes ouvrent de nombreuses voies vers des applications en imagerie multimodale pour les Hybridosomes comportant deux types de particules ou plus. Ainsi, la voie de la radiothérapie est également envisageable avec les particules d'or, celle de l'hyperthermie avec les particules de fer. La combinaison de ces deux types d'applications permettrait d'envisager des solutions en theranostic

La potentialité du cœur de la capsule est aussi un atout dans le transport de « charge » et la protection de molécules hydrophobes (drogues, chromophores, biomolécules etc). Le polymère de surface est lui aussi fonctionnalisable et la conjugaison d'un fluorophore, d'un groupement fonctionnel ou encore d'une biomolécule a aussi été envisagée. Nous aborderons ces points plus en détails dans les chapitres 3 et 4.

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Chapitre 3

Auto-construction de films nanostructurés à base d'Hybridosomes® et par chimie électro-click.

1. Contexte initial : des nanocapsules aux films nanostructurés

Ce chapitre présente le projet que j'ai eu la chance de développer au sein du National Institue for Materials Science (NIMS, Tsukuba), dans l'équipe du Pr. K. Ariga. Ce projet a été réalisable grâce à une bourse de mobilité de trois mois, octroyée par l'université de Rennes 1 ainsi que l'ambassade de France au Japon par l'intermédiaire du Laboratory for INnovatives Key materials and structures (LINK), représenté par Dr. F. Grasset. Ce projet a été initié avec Dr. G. Rydzek, qui a développé une technique de fonctionnalisation de surface par chimie électro-click. Depuis plusieurs années il a développé ce procédé qui consiste à générer le Cu(I) qui est le catalyseur de la réaction de 1,3-cycloaddition de Huisgen par un stimulus électrique. La génération de cette espèce au voisinage d'une électrode permet de confiner la réaction chimique entre espèces porteuses de groupements azide (RN_3) et alcyne (RCCH) à l'électrode. Rydzek *et al.* ont ainsi fait croître des films de polyélectrolytes et de polymères et ont démontré le contrôle à la fois de la morphologie et de l'épaisseur de tels films. Plus récemment ils ont incorporé aux films des nanoparticules magnétiques d'oxydes de Fer préalablement fonctionnalisées par un groupement azide ou alcyne apportant des propriétés hybrides aux films. C'est dans ce contexte que nous avons proposé de fonctionnaliser les Hybridosomes afin de les engager dans une réaction électro-click et ainsi faire croître les premiers films nanostructurés en combinant les avantages des deux techniques. Grâce aux propriétés des Hybridosomes, nous avons pu élaborer des films multifonctionnels et étudier leur réponse à différents stimuli. Ce travail a conduit à la rédaction d'un article soumis en juin 2017 (ACS Nano nn-2017-039354) que nous présentons ci-après.

2. Article 3: Self-Construction of Hybridosome® Nanocapsule Films Covalently Reticulated by Electro-Click Chemistry

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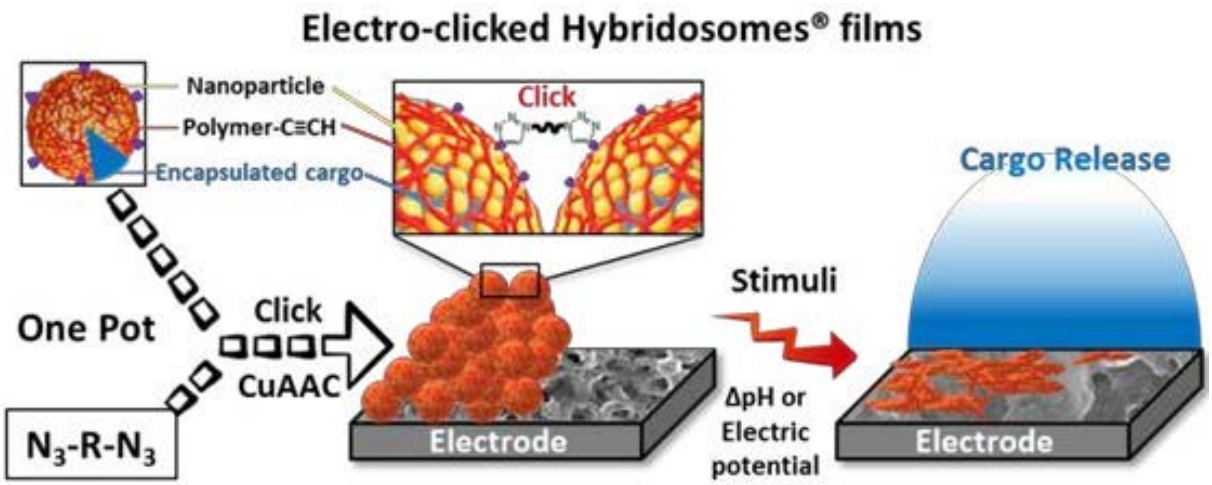

Self-Construction of Hybridosome® Nanocapsule Films Covalently Reticulated by Electro-Click Chemistry

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KEYWORDS: Nanostructured coating, hollow nanoparticles, hybrid colloids, stimulus-responsive, controlled release, self-assembly.

ABSTRACT

Simple preparation of versatile organic/inorganic coatings combining a defined and reliable nanostructure with stimuli-responsive features remains a critical challenge for designing devices in fields including sensors, catalysis, energy storage and controlled release. Here, hollow nanocapsules (named Hybridosomes®) possessing a polymer/nanoparticles shell were used to covalently self-construct hybrid films in one-pot. Alkyne bearing organic/inorganic Hybridosomes® were reticulated with azide bearing homobifunctional polyethyleneglycol (PEG) linkers, by using an electro-click reaction on F-SnO_2 (FTO) electrodes. The coatings were obtained by promoting the Cu(I)-catalyzed click reaction between alkyne and azide moieties in the vicinity of the electrode by the electrochemical generation of Cu(I) ions. The covalently reticulated hybrid films obtained were studied by SEM, AFM, UV-Vis and fluorescence spectroscopy. The one-pot covalent click reaction between the nanocapsules and the PEG linkers in the film did not affect the desirable features of the Hybridosomes® *i.e.* their hollow nanostructure their chemical versatility and their pH-sensitivity. Consequently, both the composition and the cargo-loading of Hybridosomes® films could be tuned, demonstrating the versatility of these hybrid coatings. As an example, Hybridosome® films were used to encapsulate and release a bodipy fluorescent probe in response to either a pH drop or the application of an oxidative +1V potential (*vs* Ag/AgCl) at the substrate. These results open considerable perspectives for designing multifunctional coatings where tunable chemical composition, controllable stability and hollow nanostructure are required.

Over the past several decades, the continuous development of smart and active interfaces has highlighted the requirement for multifunctional coatings. Several strategies have been developed to address this, including Layer-by-Layer films,[1] self-assembled monolayers,[2,3] and various electro-synthesis technics.[4–6] Coatings with increasing complexity have thus been prepared, culminating in the development of one-pot self-constructed coatings[7–9] and the control of their micro/nano-structure.[10–13] For instance, films composed of hollow nanostructured building blocks have attracted a significant interest. The resulting surface platforms triggered applications in several fields including biomaterials,[14,15] sensors,[16] energy storage,[17,18] electrocatalysis[19] and hydrophobic surfaces.[20] In this regard, the development of these applications requires multifunctional films that can be assembled in a simple manner. These coatings ought to be assembled in one-pot from both organic and inorganic building blocks, while possessing a versatile chemical composition, a controlled morphology and a defined nanostructure. Here we report on the one-pot self-construction of hollow nanocapsules-based films, composed of organic/inorganic components, and whose thickness and stability can be controlled. To synthesize such a coating, our strategy Hybridosomes represent a new class of tunable hollow nanocarriers with several levels of versatility arising from the individual characteristics of their organic and inorganic components and their hollow structure. For instance, these versatile nanocapsules can be prepared from iron oxide nanoparticles (IONPs), gold nanoparticles, Quantum Dots (QDs) and their mixtures. Their soft shell exhibits interesting deformable and tunable properties conferring to these nanocapsules outstanding mechanical properties.[21] To assemble the film, Hybridosomes bearing alkyne moieties (PAA-C≡CH) were reticulated by electro-click reaction with azide bearing homobifunctional polyethyleneglycol (N_3-PEG-N_3) linkers on F-SnO_2 (FTO) electrodes. A poly(ethyleneimine)-alkyne (PEI-C≡CH) pre-coating layer was used to promote the film buildup. This film self-construction process, illustrated Figure 1b, was performed by electrochemically generating Cu(I) ions, allowing the occurrence of a Cu(I)-catalyzed Alkyne-Azide Click reaction (CuAAC) between the building blocks.[22–24] Such an "electro-click" reaction allows the self-construction of polymeric,[24] organic[25] and inorganic[26] films on electrodes, including with supramolecular interactions[27] and with pattern control.[28,29] In this context, we have investigated the properties of electro-clicked Hybridosomes coatings and assess their potential as smart multifunctional surface platforms with respect to molecular encapsulation and subsequent stimuli-responsive release

Proof of concept

Both Hybridosome® nanocapsules and electro-click chemistry are emergent concepts useful for providing functional yet versatile materials.[24,30] Hybridosomes are a new class of hybrid nanocapsules that combine the features of both the inorganic nanoparticles and the polymer chains contained in their shells. Here Hybridosomes based on IONPs and PAA-C≡CH have been used as clickable building blocks.

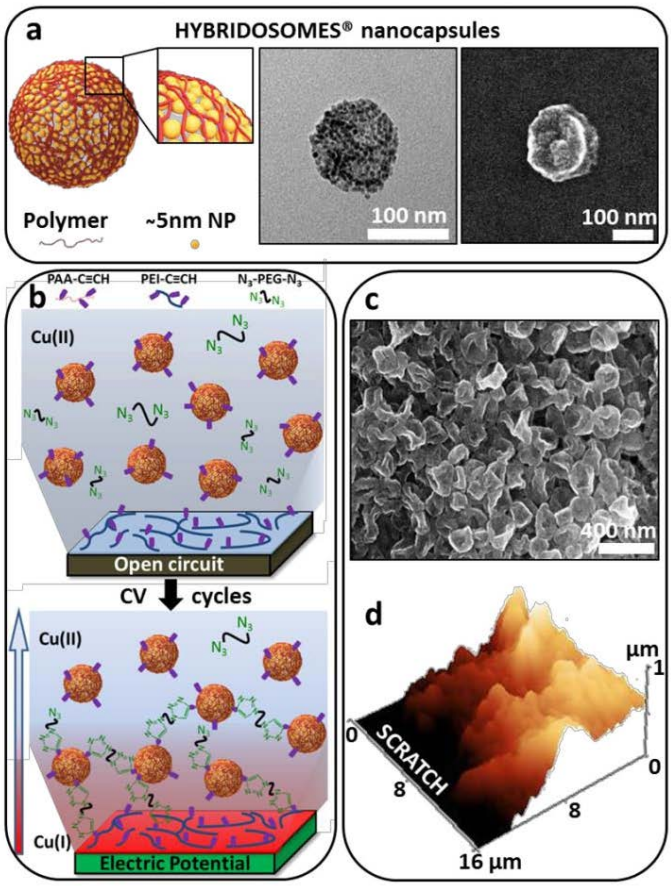

Figure 1: Concept of Hybridosomes® films covalently self-constructed by using electro-click chemistry. a) Schematic depiction, TEM and SEM of deflated Hybridosome nanocapsules composed of iron oxide nanoparticles (IONPs) and polyacrylic acid. b) Schematic representation of the electro-click process, allowing the self-construction of films on PEI-C≡CH pre-coated electrodes, by using alkyne-functionalized Hybridosomes and azide homobifunctionnal PEG linkers as building blocks in the presence of electrogenerated Cu(I) catalyst. c) SEM micrograph of a typical Hybridosomes film in the dried state. The film was self-constructed by electro-click after 800 CV cycles (-0.2 V to 0.6V *vs* Ag/AgCl, 50 mV/s) in the presence of $4.5x10^9$ Hybridosomes/mL, 0.1 mg/mL N_3-PEG-N_3 and 0.6 mM $CuSO_4$ at pH 3.5. d) Typical 3D view of the corresponding scratched film measured by AFM in the dry state and contact mode.

Electro-click chemistry is now a recognized method for achieving a spatially confined covalent reaction between both organic and inorganic functional building blocks, resulting in the formation of engineered

interfaces.[26,31–34] The concept introduced here is based on the assembly of Hybridosome nanocapsules into covalent coatings by using the electro-click approach, while preserving the remarkable features of Hybridosomes. A 6% alkyne functionalized polyacrylic acid (PAA-C≡CH) (synthesis and characterization of functional polymers is described in Schemes S-1, S-2 and Figure S-2) was used to decorate Hybridosomes with click-suitable moieties. The resulting nanocapsules were covalently coupled with azide homobifunctionnal PEG linkers (N_3-PEG-N_3) in the vicinity of a PEI-C≡CH pre-coated FTO electrode (Figure 1b). The local catalysis of the alkyne-azide Huisgen cycloaddition reaction was made possible by locally generating Cu(I) ions from Cu(II) in solution, by using cyclic voltammetry (CV). This process resulted in the self-construction of films composed of Hybridosome nanocapsules covalently reticulated with PEG linkers. Scanning electronic microscopy (SEM) investigations on the resulting electrodes reveal the stacking of several nanocapsules, forming a film (Figure 1c) whose thickness, measured by atomic force microscopy (AFM) in the dried state, exceeded 850 nm after 800 CV cycles (Figure 1d). Such a thickness value corresponds approximately to the stacking of 10 layers of Hybridosomes since dried nanocapsules, as seen by AFM, are approximately 70 nm thick. To demonstrate that this film construction can be attributed to the occurrence of the alkyne-azide Huisgen cycloaddition between the functional building blocks, control experiments were performed (Figure S-3). When one component required for the click reaction was removed, *i.e.* either the copper ions, the N_3-PEG-N_3 linkers or the CV required to generate the Cu(I) catalyst, no film self-construction was observed. This implies that the coating growth originates from the covalent "click" reaction between functional Hybridosomes and N_3-PEG-N_3linkers.[24]

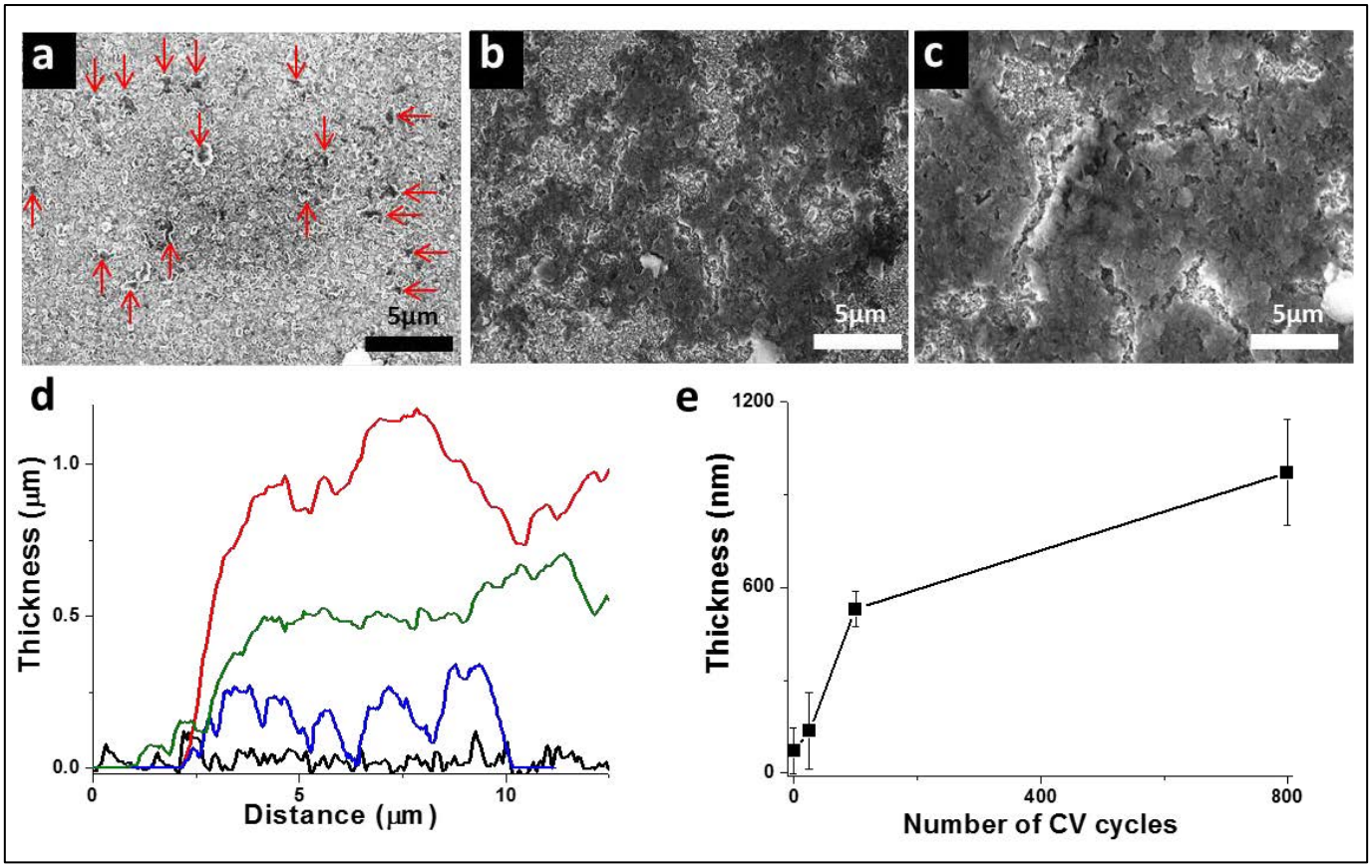

Figure 2: Germination and growth of covalently reticulated Hybridosomes® films. SEM micrographs of self-constructed Hybridosome films on FTO after 25 (a), 100 (b) and 800 (c) CV cycles (-0.2 V to 0.6V *vs* Ag/AgCl, 50 mV/s) in the presence of 4.5×10^9 Hybridosomes/mL, 0.1 mg/mL N_3-PEG-N_3 and 0.6 mM $CuSO_4$ at pH 3.5. d) Cross-sectional profiles of Hybridosome films, measured by AFM in the dry state and contact mode, after 0 (black line), 25 (blue line), 100 (green line) and 800 (red line) CV cycles. e) Evolution of the thickness of the corresponding films in the dry state.

Total Reflectance Fourier-Transform InfraRed spectroscopy (ATR-FTIR) was performed on the film and compared with the spectrum of its organic components, *i.e.* the N_3-PEG-N_3 linkers, the PAA-C≡CH from clickable Hybridosomes and the PEI-C≡CH from the pre-coating layer. The spectrum of electro-clicked Hybridosome coatings exhibited typical absorption bands of all these components, confirming their inclusion in the film (Figure S-4). Interestingly, the intense absorption peak of azide groups, visible on the spectrum of N_3-PEG-N_3 at 2100 cm^{-1},was absent from the spectrum of the film, contrary to the absorption peaks of ethylene oxide group of PEG at 1060 cm^{-1}, further confirming the occurrence of the click reaction.[23,35]

Hybridosome® films self-construction and growth mechanism.

The growth mechanism of IONP-based Hybridosome films was investigated by SEM microscopy after self-constructing for 25, 100 and 800 CV cycles (Figure 2). At each stage, a rough evaluation of the film surface coverage was performed by thresholding the micrographs. Several germination points emerged at early growth stages, achieving a surface coverage of around 11%, as calculated from Figure 2 a. When the self-construction was allowed to proceed further, the film growth achieved percolation, reaching a surface coverage of 75% after 100 CV cycles (calculated from Figure 2b) and above 90 % after 800 cycles (calculated from Figure 2 c). In addition to this topological evolution, the thickness of the film increased to 850 nm after 800 CV cycles (Figure 2 d, e and S-5). When Hybridosomes were adsorbed on the PEI-C≡CH coated electrode (0 CV cycles), the surface topography exhibited nodes around 70 nm thick corresponding to the thickness previously measured by AFM for single dried Hybridosomes (Figure 2d, black line). Single Hybridosomes seem thus able to adsorb on the PEI-C≡CH-coated electrode in the absence of electro-click reaction. to any significant surface coverage nor to the stacking of several nanocapsules (Figure S-3c). In contrast, when electro-click self-construction was performed for 25 CV cycles, the emergence of 200-300 nm thick structures was observed, suggesting that stacks of to three to four layers of Hybridosomes already formed (Figure 2d, blue line). After a larger number of electro-click CV cycles, both the surface coverage and the thickness of the coating increased, reaching respectively 90% and 850 nm after 800 CV cycles in the dried state (Figure 2d, green and red lines). These results confirm that the electro-click process with Hybridosomes leads to both the germination and growth of films based on multiple layers of covalently reticulated nanocapsules with both a tunable surface coverage and thickness.

Preservation of Hybridosomes® properties in the film.

Hybridosomes are promising building blocks for designing materials due to their chemical versatility[32], their hybrid polymer/NP composition and their hollow structure. Preserving these features when Hybridosomes are integrated in electro-clicked coatings is thus desirable. The nanocapsules' stability against the electrochemical conditions used for self-constructing the films was investigated by applying a CV to an IONPs-based Hybridosomes dispersion. The absence of any faradic current measured in the (-0.2V to 0.6V) CV window indicated the electrochemical stability of Hybridosomes (Figure S-6a). The structural stability of these building blocks was investigated by measuring the size-distribution of nanocapsules by SEM and scanning transmission electronic microscopy (STEM), both before and after their incorporation in self-constructed films (Figure S-6b). The size distribution of Hybridosomes did not significantly change after their incorporation in electro-clicked coatings with an average diameter increasing from 114 nm (+/- 22 nm) to 122 nm (+/- 21 nm). Hybridosomes building blocks thus exhibit good structural tolerance to the electro-click synthesis conditions, leading to hybrid films with a hollow nanostructure. The chemical composition of self-constructed IONPs-based Hybridosome films was determined by using Energy Dispersive X-ray (EDX) spectroscopy (Figure S-7). Detection of oxygen, carbon, nitrogen and iron indicate the presence of the polymeric (PAA-C≡CH, PEI-C≡CH, N_3-PEG-N_3) and inorganic (IONPs) components in the film. The hybrid nature of Hybridosomes self-constructed films was thus confirmed. Since Hybridosomes can be synthesized by using a large range of NPs,[30] the transferability of this chemical versatility to electro-clicked films was investigated. The possibility to assemble nanocapsule films based on a different choice of NPs would indeed open perspectives in several fields of applications including catalysis,[36–38] energy storage,[11,39]optoelectronic,[40] sensing[16] and controlled release[14,30]. As an example, another type of Hybridosome has been synthesized by using CdSe@ZnS QD, IONPs and PAA-C≡CH as building blocks. The resulting Hybridosomes exhibited the same morphology as nanocapsules entirely composed of IONPs. However, as expected, their EDX analysis indicates the presence of Zn, Se, Cd and S elements contained in the QDs (Figure 3a). When such Hybridosomes were used as building-blocks in the electro-click process, the coatings obtained presented similar morphological properties as films self-constructed from IONPs-based Hybridosomes (Figure 3b). Chemical analysis of these films, by using EDX, revealed the presence of Fe, O, Zn, Se, S confirming the inclusion of both IONPs and CdSe@ZnS QDs in the coating. These results demonstrated how self-constructed films can inherit the chemical versatility of Hybridosome and their unique nanostructural features.

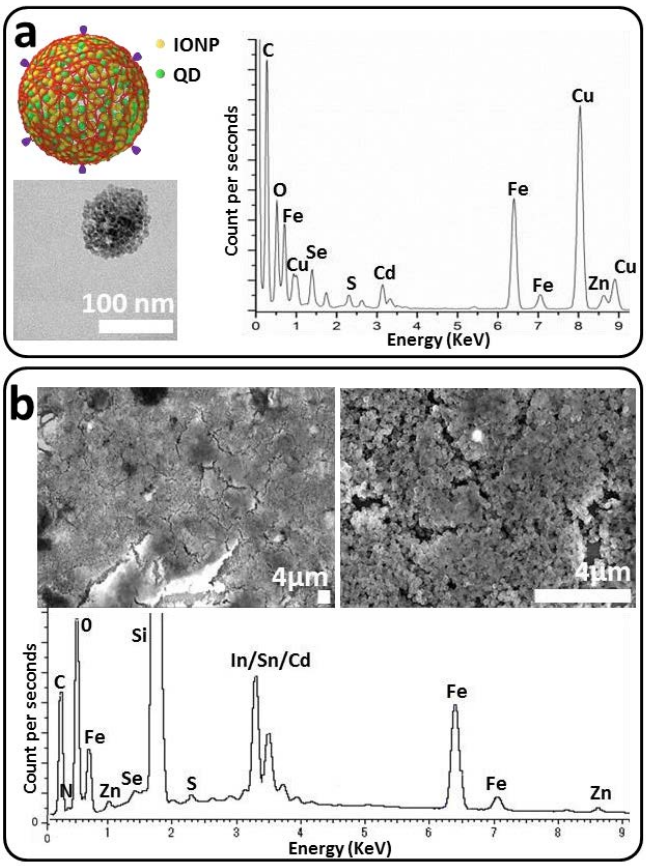

Figure 3: Chemical composition of self-constructed films based on QD-containing Hybridosomes®. a) Schematic depiction, TEM micrograph and corresponding EDX analysis of Hybridosome nanocapsules composed of CdSe@ZnS quantum dots (green), IONPs (yellow) and PAA-C≡CH (red). b) SEM micrographs and EDX analysis of the corresponding self-constructed films obtained after 800 CV cycles (-0.2 V to 0.6V vs Ag/AgCl, 50 mV/s) in the presence of 4.5x109 Hybridosomes/mL, 0.1 mg/mL N_3-PEG-N_3 and 0.6 mM CuSO4 at pH 3.5.

Encapsulation abilities of Hybridosomes®.

The preservation of the Hybridosomes nanostructure at the interior of electro-clicked films suggests applications in several fields including encapsulation and release of hydrophobic compounds.[30] As a proof of concept, a fluorescent bodipy probe was encapsulated into Hybridosomes during their synthesis. The UV-visible absorbance spectrum of the resulting bodipy-loaded Hybridosomes dispersion was compared with the spectrum of both bodipy and empty Hybridosomes dispersions in water (Figure S-8a). Both bodipy-containing samples exhibited a similar UV-Vis absorption spectrum marked by the emergence of two prominent absorbance bands localized at 502 nm and 532 nm. These peaks were absent from the spectrum of empty Hybridosomes dispersions, suggesting that the encapsulation process was successful and did not significantly modify the chemical properties of the probe. The fluorescence properties of bodipy-loaded Hybridosomes dispersions were compared with the spectra of bodipy in a good solvent (Tetrahydrofuran, THF) and in water where bodipy is poorly soluble (Figure S-8b). A single fluorescence peak localized at 540 nm was observed for well-dissolved bodipy in THF. On the contrary, the spectrum of bodipy in water was marked by the emergence of 2 broad emission peaks at 560 nm and 625 nm, indicating the formation of J-aggregates.[41] Interestingly the spectrum of bodipy-loaded Hybridosomes dispersions exhibited a main peak around 540 nm and a shoulder around 565 nm, demonstrating that the encapsulation process largely inhibits aggregation of bodipy (Figure S-8b).Features of bodipy-encapsulated Hybridosome® films. By using bodipy-loaded Hybridosomes for self-constructing films, coatings with a similar morphology as the one exhibited by the previous "empty" nanocapsules films were obtained (Figure 4a). To probe further the integrity of the Hybridosomes integrated in electro-clicked films, the spectral features of bodipy-loaded Hybridosomes coatings were investigated in the dry state and compared with bodipy and bodipy-loaded nanocapsules drop-casted on FTO (Figure 4b and 4c). On the one hand, the UV-Vis absorbance spectra of all films exhibited similar characteristics with two prominent peaks at 502 nm and 532 nm, confirming the inclusion of bodipy in the films. Probe aggregation during drying was signaled by the appearance of a new absorption band at 680 nm (Figure 4b). On the other hand, the fluorescence spectra of these films exhibited significant differences (Figure 4c). The spectrum of drop-casted bodipy films was comparable to the emission spectrum of bodipy in water, confirming that aggregation already was occurring in solution. However, bodipy-loaded Hybridosomes films, either drop-casted or electro-clicked, exhibited a broad emission band around 565 nm, corresponding to the aggregate component already observed on the spectrum of bodipy-loaded Hybridosomes dispersions (Figures 4c and S-8b). Free bodipy was thus absent from Hybridosomes films, suggesting the absence of bodipy leaking from the nanocapsules during the electro-click process. These results confirm that the electro-click process with bodipy-loaded Hybridosomes not only preserves the nanocapsules integrity but also its cargo content.

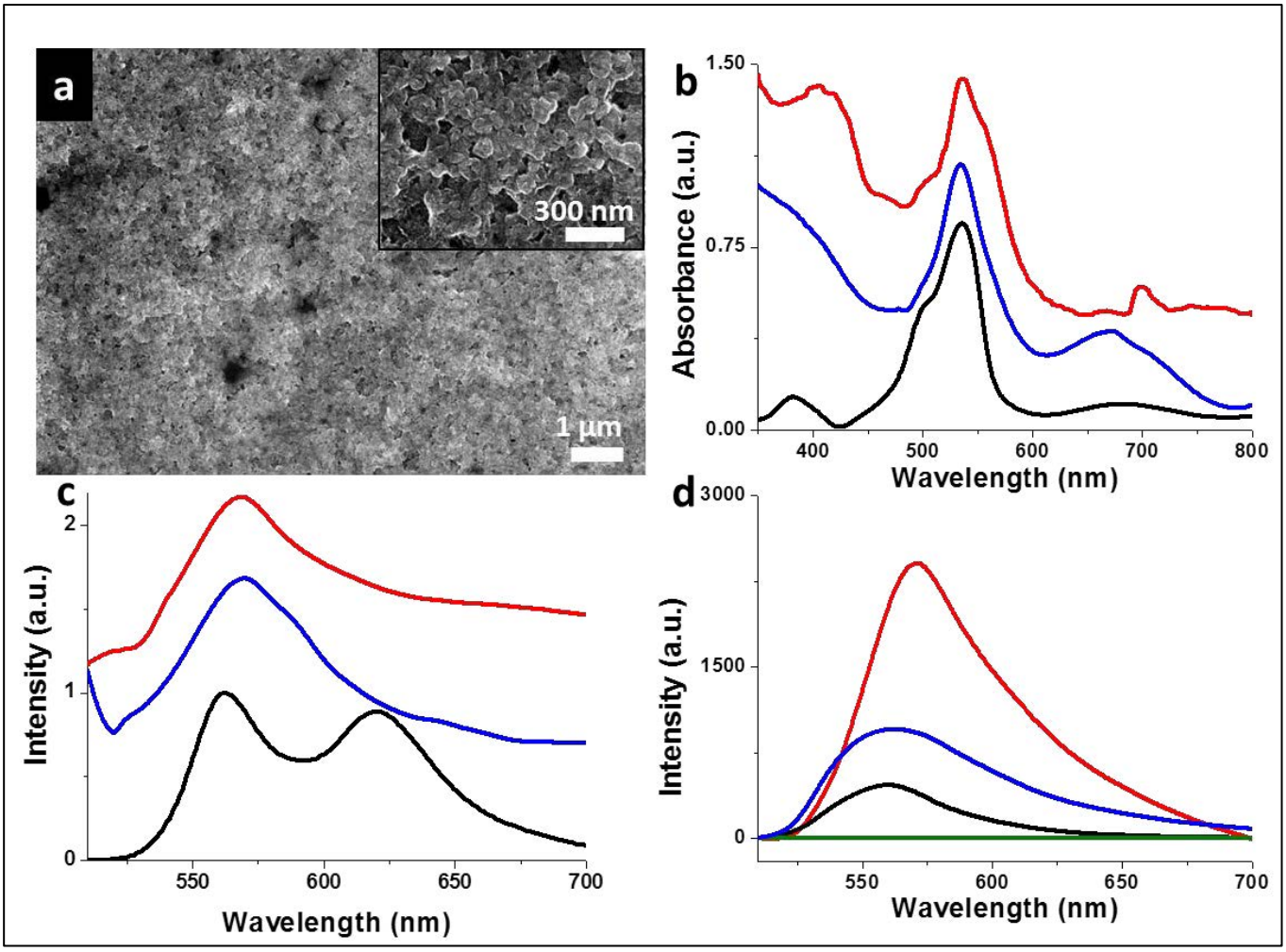

Figure 4: Bodipy-encapsulated Hybridosome® films. a) Typical SEM micrographs of bodipy-loaded Hybridosome films after 800 self-construction CV cycles. (b) UV-Visible absorbance spectra and (c) fluorescence spectra in the dry state (λ_{exc} at 480 nm) of bodipy (black line), drop-casted bodipy-loaded Hybridosome (blue line) and an electro-clicked film based on bodipy-loaded Hybridosomes (red line). d) Fluorescence spectra in the dry state (λ_{exc} at 480 nm) of electro-clicked Hybridosome films obtained from building mixtures containing 0% (green line), 10% (black line), 50 % (blue line) and 90 % (red line) of bodipy-loaded Hybridosomes.

The relationship between the composition of the building mixture and the resulting electro-clicked self-constructed films was investigated by mixing both empty (E) and bodipy-loaded (L) Hybridosome batches at different ratios. When the L/E ratio increased from 0 % (pure empty Hybridosomes) to 10 %, 50 % and 90 %, a fluorescence emission peak emerged and increased in intensity between 660 and 670 nm, demonstrating the presence of increasing quantities of bodipy in the films (Figure 4 d). The area of the corresponding peaks increased accordingly, suggesting the absence of quenching when the L/E ratio was increased (Figure S-9). The interaction of the fluorophore with itself seems thus to remain constant, supporting the hypothesis that the probe is encapsulated inside separated compartments within the film.[42] The simultaneous incorporation of several types of nanocapsules in the films, with a tunable ratio, seems thus possible. Hybridosome electro-clicked films constitute therefore a promising candidate for designing multiply-loaded coatings and multifunctional interfaces.

Stimulus-induced destabilization of Hybridosomes® films.

The stability of electro-clicked nanocapsules was probed by using pH drops as an external stimulus. The effect of decreasing the pH from pH 4 to pH 1 was first studied on Hybridosomes dispersions and resulted in a two-step destabilization. At pH 3, many nanocapsules were destabilized and were reorganized into larger structures up to several microns in size (Figure S-10). pH 3 coincides with the full protonation point of carboxylic groups of PAA chains, causing decreased polymer solubility in water, and reducing its ability to act as a colloidal stabilizer. This effect was recently reported as being responsible for the aggregation of PAA-coated silver and TiO_2 nanoparticles at acidic pH.[43,44] This destabilization of Hybridosomes dispersions at pH 3 illustrates how these nanocapsules inherit the properties of their polymer component.[30]

When the pH of Hybridosomes dispersions was decreased further to pH 1, nanocapsules could no longer be observed and the morphology of the dispersed material dramatically changed (Figure S-10). Although IONPs composing the Hybridosomes are thermodynamically unstable at both pH 1 and pH 3, their dissolution kinetic is faster at pH 1.

This illustrates how Hybridosomes also inherit properties of their inorganic components.[45,46] The pH-sensitivity of self-constructed Hybridosome films was also investigated at pH 3, 2.5 and 1, confirming the trend observed in dispersion (Figure S-11). Interestingly, the coatings were destabilized after soaking 15 min in a pH 2.5 solution instead of pH 3 for nanocapsule dispersions. This suggests that the nanocapsules are better stabilized in the film environment. However, aggregated Hybridosomes were still clearly visible in the film at this pH value. In contrast, at pH 1, the morphology of Hybridosome films observed by SEM was marked by the absence of nanocapsules, in agreement with results obtained with Hybridosomes dispersions.

Destabilization of bodipy-loaded Hybridosome® films. When bodipy-loaded nanocapsule films were brought into contact with a pH 1 HCl solution, the partial dissolution of the coating was also observed (Figure S-12a and b). As a result, bodipy encapsulated in the film was released into the supernatant, which exhibited a fluorescence emission peak around 560 nm while the fluorescence of the film dramatically decreased (Figure S-12 c and d). This result demonstrates the ability of Hybridosome films to encapsulate and release molecules upon a direct pH change of the environment. Since these films are electro-clicked on conducting substrates, the possibility of destabilizing the coating by an electrochemically-induced reduction of local pH was also investigated (Figure 5).

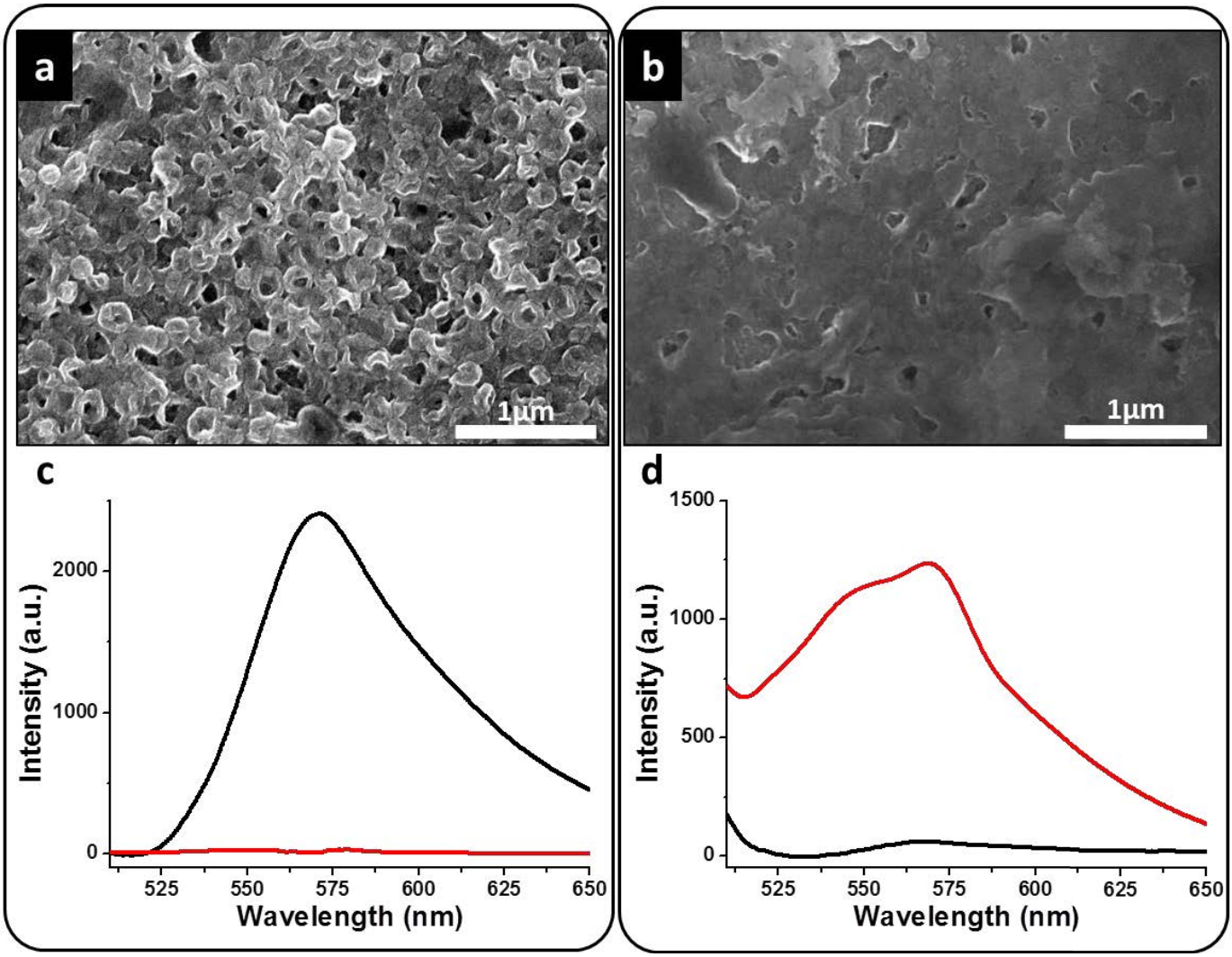

Figure 5: Electro-triggered release abilities of self-constructed Hybridosomes® films. SEM micrographs (a, b) and fluorescence spectra (λ_{exc} at 480 nm) in the dry state (c, d) of bodipy-loaded Hybridosome films self-constructed on FTO (black line) and of the corresponding supernatant (red line) before (a,c) and after (b,d) application of +1 V potential (*vs* Ag/AgCl) for 15 minutes in a 0.1M NaCl solution.

This approach, which consists of generating a proton gradient near the electrode by water electrolysis, has been reportedly used for assembly and dissolution of pH-sensitive polymer films.[47–49] Such a localized and easily controllable dissolution process is expected to trigger applications in the field of localized release of medical drugs, for instance by using implantable microelectrodes.[50,51] A potential of +1 V (*vs* Ag/AgCl, 0.1M NaCl) was applied for 15 min to an electro-clicked Hybridosome film self-constructed on FTO in order to generate a proton gradient at the electrode.[47] This treatment resulted in the destabilization of the coating as testified by the disappearance of nanocapsules from the surface at low SEM magnification (Figure 5a and b). When higher magnifications were used, severely disorganized and fused Hybridosomes were visible on SEM micrographs, leading to the loss of their nanostructure (Figure S-13). This result was similar to the one obtained with Hybridosomes dispersions at pH 3 and films soaked at pH 2.5 (Figure S-10 and S-11). Both the coated electrodes and their supernatant were studied by fluorescence spectroscopy (λ_{exc} at 480 nm) prior to and following the electrochemical stimulus. Before water electrolysis, no fluorescence was measured in the supernatant while the coated electrode exhibited an emission peak centered at 568 nm, indicating the presence of encapsulated bodipy (Figure 5c, black line). After 15 min of water electrolysis, the fluorescence intensity of the electrode dramatically decreased while the supernatant exhibited two broad emission peaks centered at 550 and 570 nm indicating the release of aggregated bodipy in the aqueous supernatant (Figure 5d). These two methods for destabilizing electro-click Hybridosome films suggest their application in the directed release of encapsulated molecules in response to either a global pH change or localized water electrolysis.

Conclusion

One-pot self-constructed films were assembled on FTO electrodes by electro-click cross-linking of nanostructured organic/inorganic Hybridosome® nanocapsules. By varying the self-construction parameters, both the surface coverage (calculated from 520 μm^2 area micrographs) and the thickness of the films were tuned, exceeding 90% and 850 nm respectively after 800 CV cycles. The resulting hybrid coatings, composed of hollow PAA/IONPs nanocapsules and PEG linkers, maintained the chemical and structural features of Hybridosomes. Consequently, both the composition and the loading of these nanocapsules could be changed, demonstrating the versatility and encapsulation abilities of Hybridosome films. As an example, bodipy-loaded nanocapsules were used for self-constructing films without altering the properties of the probe. The corresponding coatings could be destabilized by using either a pH or an electrochemical stimulus, releasing their fluorescent content. These results open considerable perspectives for multifunctional coatings where a chemical versatility, a controllable stability and a hollow nanostructure are required.

Method

Materials. Copper sulfate pentahydrate ($CuSO_4$×5 H_2O, *M* = 249.7 g/mol, CAS 7758-99-8), branched polyethyleneimine (*M*= 25kDa, CAS 9002-98-6), Polyacrylic acid (*M*= 450kDa, CAS 9003-01-4), amino-EG_4-alkyne (*M* = 231.3 g/mol, CAS 1013921-36-2), 10-undecynoic acid (*M* = 182.3 g/mol, CAS 2777-65-3), polyoxyethylene bis(azide) (*M* = 2050g/mol, CAS 82055-94-5), Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, *M* = 442.28g/mol, CAS 56602-33-6), N,N-Diisopropylethylamine (DIEA, *M* = 129.24g/mol, CAS 7087-68-5), N,N-Dimethylformamide (DMF, *M* = 73.09g/mol, CAS 68-12-2), octadecylamine-coated CdSe@ZnS (product number 790192) were purchased from Sigma-aldrich and used as received. Tetrahydrofuran (THF, *M* = 72.10g/mol, CAS 109-99-9) was purchased from VWR. Hydrochloric acid (HCl, *M* = 36.46g/mol, CAS 7647-01-0), Sodium Hydroxide (NaOH, *M* =39.99 g/mol, CAS 122000-64-5) were purchased from Wako. 4,4-Difluoro-8-(4'-trimethylsilylethynylphenyl)-1,3,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacene (Bodipy, *M* = 476.2g/mol) was kindly provided by O. Mongin (ISCR) and synthesized following a reported procedure.[52] FTO electrodes were purchased from ALS, Japan. All aqueous solutions were prepared with MilliQ water (18.2 MΩ.cm^{-1}) purified using a Purelab Prima system.

Synthesis of functional polymers. Synthesis of PAA-C≡CH was adapted from our previous work and entails grafting amino-EG_4-alkyne on the polyacrylic acid backbone.[53] PAA (2 mmol) was dissolved in DMF (7 mL) with BOP (142 µmol) under stirring for 10 m. Amino-EG_4-alkyne (108 µmol) was dissolved in DMF (5 mL) and DIEA (2 mmol) was added. After 90 minutes, DMF was evaporated under reduced pressure. The residue was dissolved in milliQ water, dialyzed (Spectra/Por, MWCO 12kDa) against milliQ water for 48 h and recovered by evaporation under reduced pressure and freeze drying. Synthesis of PAA-C≡CH was performed by using an EDC/NHS approach. 10-Undecynoic acid (0.2 mmol) was dissolved in dichloromethane (7 mL) with 3-fold molar excess of (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-Hydroxysuccinimide. After 10 minutes stirring, PEI (2 mmol) dissolved in dichloromethane (7 mL) were added and the reaction was allowed to proceed for 90 minutes. Dichloromethane was evaporated under reduced pressure. The residue was dissolved in milliQ

water, dialyzed (Spectra/Por, MWCO 12kDa) against milliQ water for 48h and recovered by evaporation under reduced pressure and freeze drying. The functionalization degree of the obtained polymers was evaluated by using NMR spectroscopy (Supporting Information).

Preparation of Iron Oxide based Hybridosomes® and encapsulation step. Hybridosomes were elaborated as previously reported[30] by using PAA-C≡CH and superparamagnetic iron oxide nanoparticles previously synthesized.[54] In a typical process, THF (100 µL) was added to a dispersion of iron oxide nanoparticles (m_{Fe} = 52.6 µg) followed by water (800 µL) then stirred. After 24h, PAA-C≡CH (2,1mM) was added to the mixture before solvent evaporation for 15 h at 40°C. The resulting precipitate was magnetically attracted and dispersed in milliQ water (930 µL). The same procedure was used to synthesize mixed iron oxide (IONPs)/ quantum dots (QDs) Hybridosomes by initially mixing 50µL (m_{Fe} = 26.3µg) of iron oxide (IONPs) dispersion with 50µL (m_{QD} = 25µg) of quantum dots (QDs) dispersion in 100µL of THF. Encapsulation of bodipy in Hybridosomes was performed by adding 100µL of a 1mM bodipy in THF into 50µL (m_{Fe} = 26,3µg) of the initial iron oxide (IONPs) suspension.

Film self-construction. ITO and FTO electrodes were cleaned by dipping in 0.1M NaOH and 0.1M HCl baths for 15 minutes followed by rinsing. A PEI-C≡CH pre-coating layer was deposited by dipping (10 mg/mL) for 15 minutes and subsequent rinsing. The film was self-constructed by applying a cyclic voltammetric current (-200 mV to +600 mV *vs* Ag/AgCl at 50mV.s^{-1} under stirring) to the electrode in contact with a pH 3.5 solution containing typically 4.5 × 10 Hybridosomes/mL, 0.6 mM $CuSO_4$ and 0.1 mg/mL N_3-PEG-N_3.

Film destabilization. Bodipy-loaded Hybridosomes films were first self-constructed by using 800 CV cycles. The resulting coated electrodes were either dipped in an HCl solution at desired pH or subjected to a +1 V potential (*vs* Ag/AgCl, in a 0.1 M NaCl buffer) for 15 minutes. The supernatant was analyzed directly after the process. The electrodes were rinsed with Milli-Q water and dried before analysis.

Cyclic Voltametry: A CHI model 613B potentiostat was used with a three-electrode apparatus based on an ITO and FTO coated quartz as working electrode, a platinum wire as counter electrode, and an RE1S Ag/AgCl-based reference electrode. The electrodes were purchased from ALS.

Fluorescence spectroscopy was performed using a JASCO FP8500 spectrofluorometer.

NMR analysis of functionalized polymers PEI-C≡CH and PAA-C≡CH were performed in D_2O on a Bruker Avance III HD 500MHz spectrometer fitted with a Dual ^1H /^{13}C probehead.

Atomic force microscopy (AFM) was performed by using an AFM SPA400-SPI4000 (Seiko Instruments Inc., Chiba, Japan) in contact mode and in the dried state with silicon nitride cantilevers, spring constant 0.08 N/m (model SN-AF01S-NTK-W10200326 from Seiko Instruments). Height images were scanned at a fixed scan rate of 1 Hz. The thickness of PEM films was measured by imaging the coatings after scratching. When possible, the AFM scanning direction was perpendicular to the scratch. Data evaluation was performed by using the Gwyddion software. A plane-fit treatment was applied to the scratched area and its minimum height set to z = 0.

Scanning electron microscopy (SEM): scanning transmission electron microscopy (STEM) and energy-dispersive X-ray spectroscopy (EDX) were performed using a Hitachi S-4800 at accelerating voltages of 30 kV. The samples were observed directly after 15 min drying under vacuum. Calculation of Hybridosomes size distribution analysis and film surface coverage was performed from SEM and STEM data by using the ImageJ software.

Transmission electron microscopy (TEM), energy-dispersive X-ray spectroscopy (EDS) and tomography were performed using a JEM-2100 (JEOL) transmission electron microscope (accelerating voltage 200kV) equipped with a CCD camera.

UV-visible spectroscopy was performed using a Shimadzu (Japan) UV visible NIR spectrophotometer (model UV-3600).

Attenuated total reflection infrared spectroscopy (ATR-FTIR) on Hybridosomes films and functionalized polymers was performed by using a Thermo Scientific Nicolet 4700 apparatus (USA).

Nanoparticle Tracker Analysis (NTA) was performed using a Nanosight LM10 (Malvern instruments) equipped with a sample chamber with a 405nm laser and a CCD camera.

Competing financial interest: The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by the International Center for Young Scientists (ICYS), JSPS KAKENHI Grant Number JP16H06518 (Coordination Asymmetry) and

CREST, JST. F. S. warmly thanks the Embassy of France at Tokyo and MAEDI for travel financial support and University Bretagne Loire and the Brittany region for daily allowance financial support. G.R. thanks Dr. Loic Jierry for fruitful discussions. The authors wish to acknowledge the financial support of the Centre National de la Recherche Scientifique (CNRS).

ASSOCIATED CONTENT

Supporting Information TEM Tomography, NTA scattering, NMR, ATR-FT IR spectroscopy, UV-Visible spectroscopy, S(T)EM, EDX, CV, AFM and fluorescence spectroscopy analysis are available as supporting information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Supporting Information

Figure S-1: Structure of Hybridosomes®.

Video S-1: TEM tomography of a single Hybridosome®.

Scheme S-1: Grafting degree of PAA-C≡CH.

Scheme S-2: Grafting degree of PEI-C≡C.

Figure S-2: ATR-FTIR Spectroscopy of functionalized polymers.

Figure S-3: Self-construction of the film.

Figure S-4: Click reaction occurrence.

Figure S-5: Film growth, thickness evolution.

Figure S-6: Chemical and structural stability of Hybridosomes® along the process of film construction.

Figure S-7: Chemical composition of films self-constructed with IONPs based Hybridosomes®.

Figure S-8: Bodipy encapsulation, UV/Vis and Fluorescence spectra.

Figure S-9: Surface area of fluorescence emission bands.

Figure S-10: pH sensitivity of functionalized clickable Hybridosomes® dispersions.

Figure S-11: pH sensitivity of electro-clicked Hybridosome®-based films.

Figure S-12: pH-triggered release abilities of self-constructed Hybridosomes®-based films.

Figure S-13: Cargo release under electrochemical stimulus.

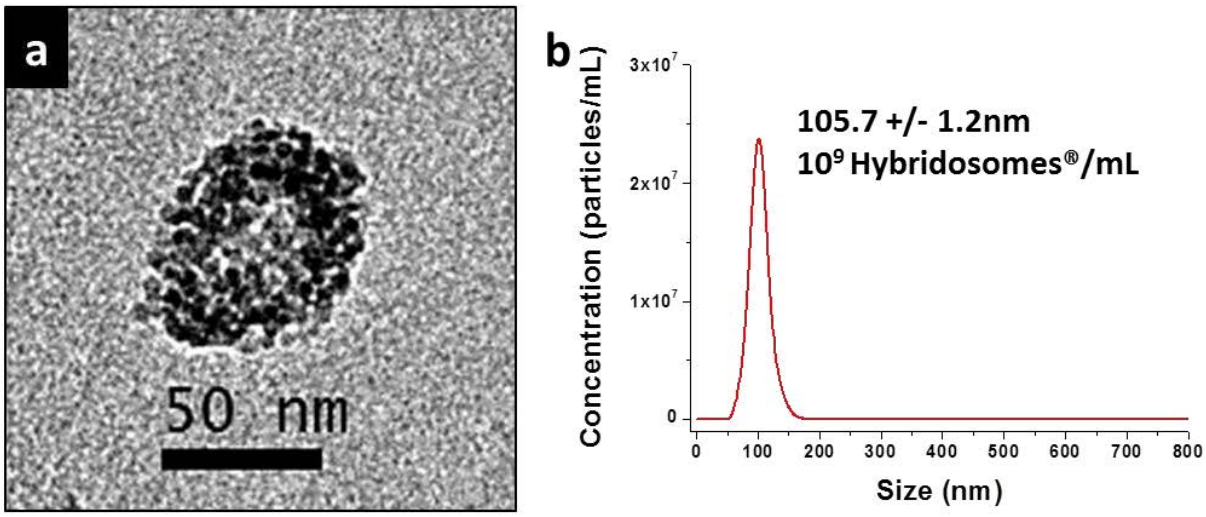


Figure S-1: Structure of Hybridosomes®. a) TEM micrograph of a single Hybridosome corresponding to the first slice of the S-1 video. b) Size distribution of a Hybridosome in aqueous dispersion, measured by Nanoparticles Tracker Analysis (NTA), which allows the mean-size and the concentration of Hybridosomes suspensions to be calculated**.**

Video S-1: TEM tomography of a single Hybridosome®.

Reconstructed video obtained from 76 tomography-slices measured from –60° to +15° with a 1° increment.

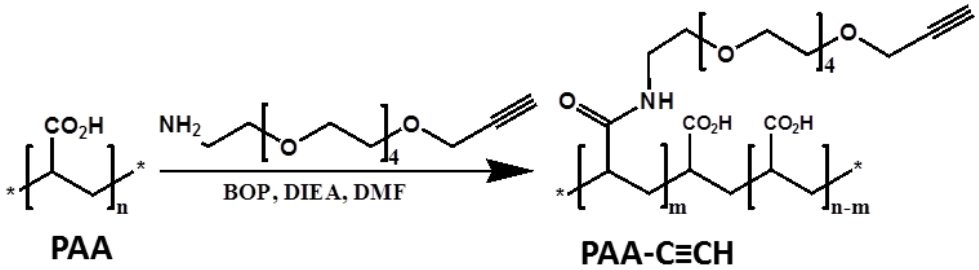
https://mycore.core-cloud.net/public.php?service=files&t=10205a71e3d5fd4d80dc4370f2b0812c

Polymer grafting degrees.

- Estimation of the grafting degree of PAA-C≡CH by ^{1}H NMR.

We arbitrarily fixed the integration value of the alkyne CC*H* signal of PEG (2.83 ppm) at 1. From a comparison of the integration values of signals between 3.72-3.16 ppm (NHCH_2, CH_2NH, CH_2OCH_2 of PEG) and those between 2.52 -1.37 ppm (CH_2CHCO of PAA and $CH_2CH_2CH_2$CCH of PEG), the effective degree of modification was estimated to be 6%.

^{1}HNMR assignment in ppm (500 MHz, 10% D_2O): 8,07 (b, CON*H*CH2), 4,16 (s, OCH_2CCH), 3.67 (bm, CONHCH$_2CH_2$O), 3.62 (bs, OCH_2CH_2O), 3.52 (bm, CONHCH_2CH$_2$O), 2.83 (s, OCH$_2$CH$_2$CC*H*), 2.29 (b, C*H*(COOH)CH$_2$), 1.82 (b, CHCH_2), 1.64 (b, CH(COOH)CH_2),1.53 (b, CH_3CH).

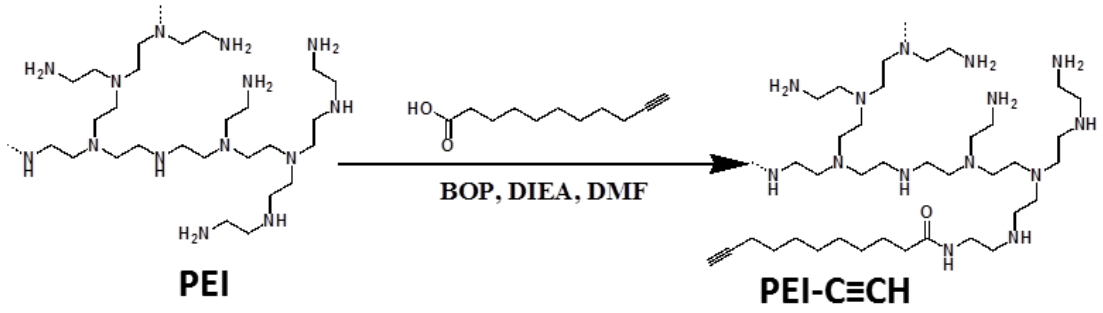


Scheme S-1: Synthesis of PAA-C≡CH.

- Estimation of the grafting degree of PEI-C≡CH by ^{1}H NMR.

We arbitrarily fixed the integration value of the signals resonating between 1.66 ppm and 0.97 ppm at 12 (according to the corresponding number of protons contained in the repetition unit of the polymer). From a comparison of the integration values of signals between 3.1 and 2.3 ppm (N*CH$_2$CH$_2$*N of the grafted 10-undecynoic acid), the effective degree of modification was estimated to be 12 %.

^{1}HNMR assignment in ppm (500 MHz, 10% D_2O): 3.35 (bs, NH_2*CH$_2$*), 3.18 (s, CONH*CH$_2$* CH_2), 2.92/2.76/2.63/2.55 (b, N*CH$_2$CH$_2$*N), 2.07 (bs, NHCO*CH$_2$*CH_2), 1.41/1.28/1.18 (b, CH_2*CH$_2$*CH_2).



Scheme S-2: Synthesis of PEI-C≡CH.

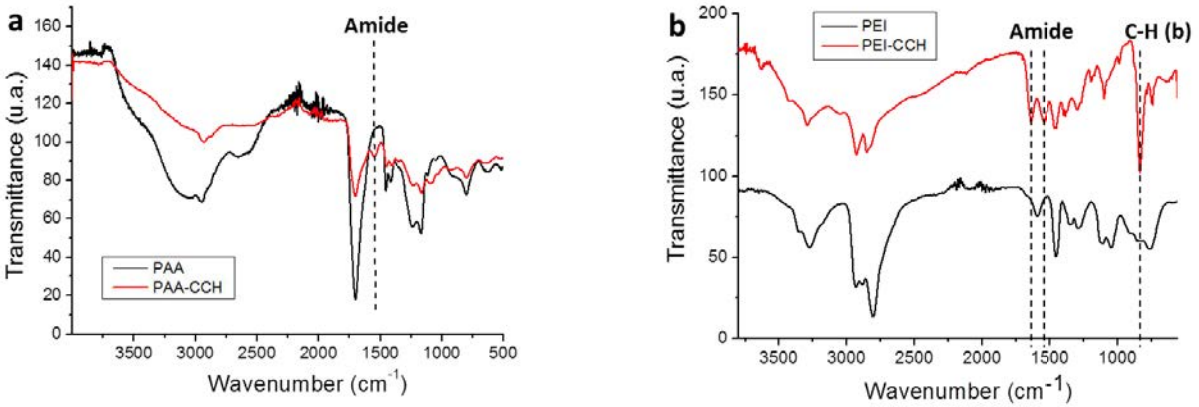


Figure S-2: ATR-FTIR Spectroscopy of functionalized polymers. a) ATR-FTIR spectra of PAA (black line) and PAA-C≡CH (red line). b) Typical ATR-FTIR spectra of PEI (black line) and PEI-C≡CH (red line) with characteristic bands of Amide 1540cm^{-1} and 1640 cm^{-1}, and characteristic peak of the bending vibration C-H from the undecynoic acid grafted pendant chain.

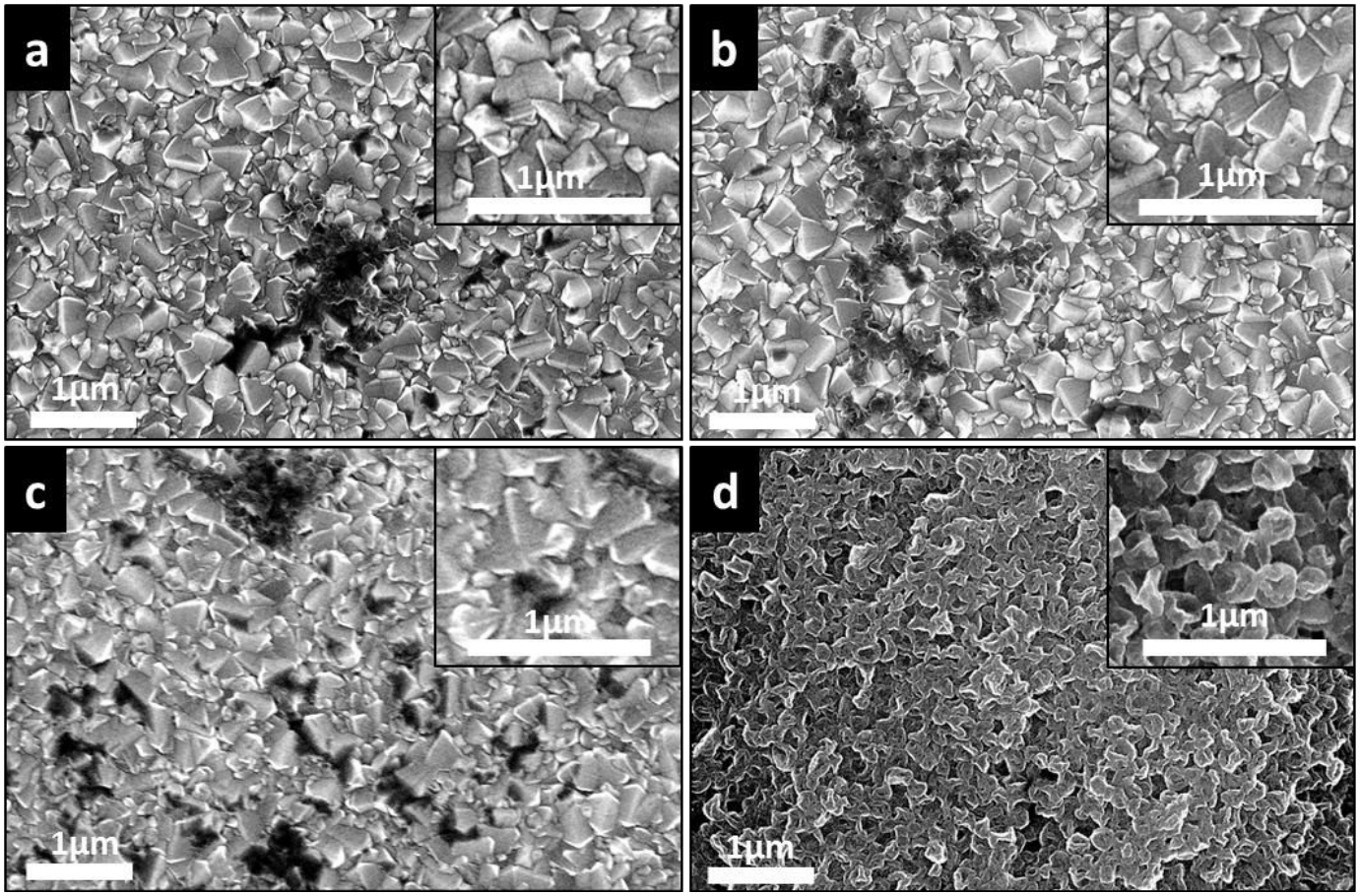
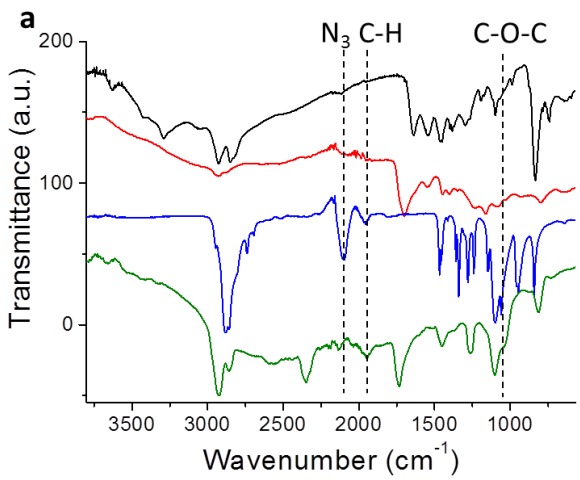


Figure S-3: Self-construction of the film. Typical SEM micrographs of the deposited material on FTO (a) in absence of $CuSO_4$, (b) in absence of N_3-PEG-N_3 linker, and (c) in absence of any applied potential. (d) Electro-clicked nanocapsule film obtained by using typical conditions (-0.2 V to 0.6 V vs Ag/AgCl, 50 mV/s in the presence of 4.5×10^9 Hybridosomes/mL 0.1 mg/mL N_3-PEG-N_3 and 0.6 mM $CuSO_4$ at pH 3.5).



b

Peak (cm^{-1})	Assignment	PEI-CC	PAA-CC	N_3-PEG-N_3	Film
3630 - 3300	N-H (s)	■			■
2930 - 2850	N-H (s) O-H (s) C-H (s)	■	■	■	■
2340	CO_2 (s)				■
2100	N_3 (s)			■	
1954	C-H (b)			■	■
1730	C=O (s)		■		■
1638	N-H (b)	■			
1543	Amide (b)	■	■		
1455	C-H (sc)	■	■	■	■
1400 1350 1335	C-H (b) O-H (b)	■	■	■	■
1297	C-H (b)	■		■	
1280	C-O (s)		■	■	■
1236	C-O-C (s)			■	
1190	C-N (s)	■			
1160		■	■		■
1146	C-O-C (s)			■	
1100	C-O (s) C-N (s)	■	■	■	■
1060	C-O-C (s)			■	■
1030	C-O (s)		■		
986 - 950	C-H (b)	■		■	
830-750	$C-H_2$ (b) N-H (b)	■	■	■	■

Figure S-4: Click reaction occurrence. (a) ATR-FTIR spectra of PEI-C≡CH (black line), PAA-C≡CH (red line), N_3-PEG-N_3 linker (blue line) and of the film (green line) constructed under typical conditions (-0.2 V to 0.6 V vs Ag/AgCl, 50 mV/s in the presence of 4.5×10^9 Hybridosomes/mL, 0.1 mg/mL N_3-PEG-N_3 and 0.6 mM $CuSO_4$ at pH 3.5). (b) Corresponding peak assignments.

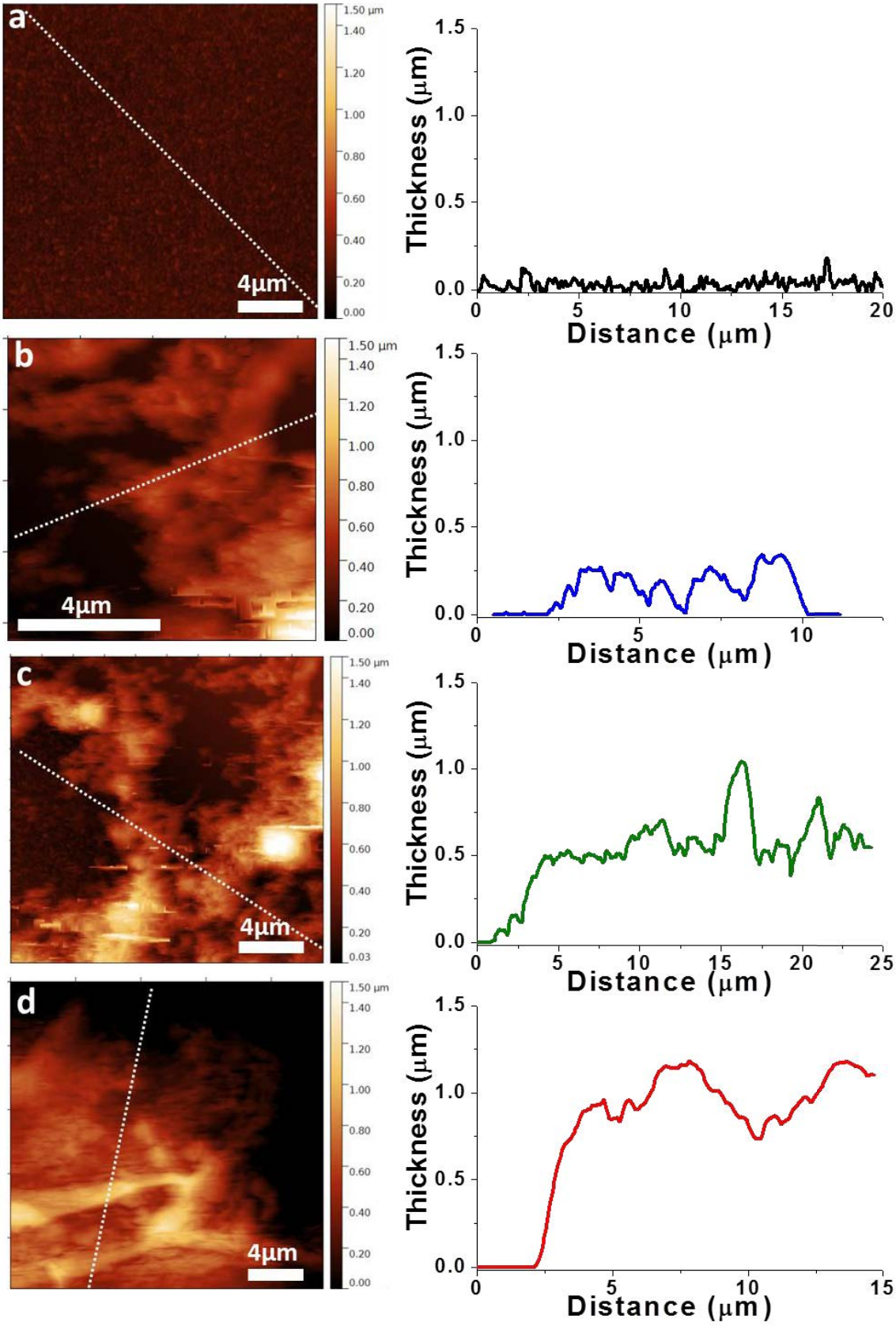


Figure S-5: Film growth, thickness evolution. AFM images and corresponding thickness profiles of (a) adsorbed Hybridosomes and self-constructed films obtained under typical conditions after (b) 25, (c) 100 and (d) 800 CV cycles.

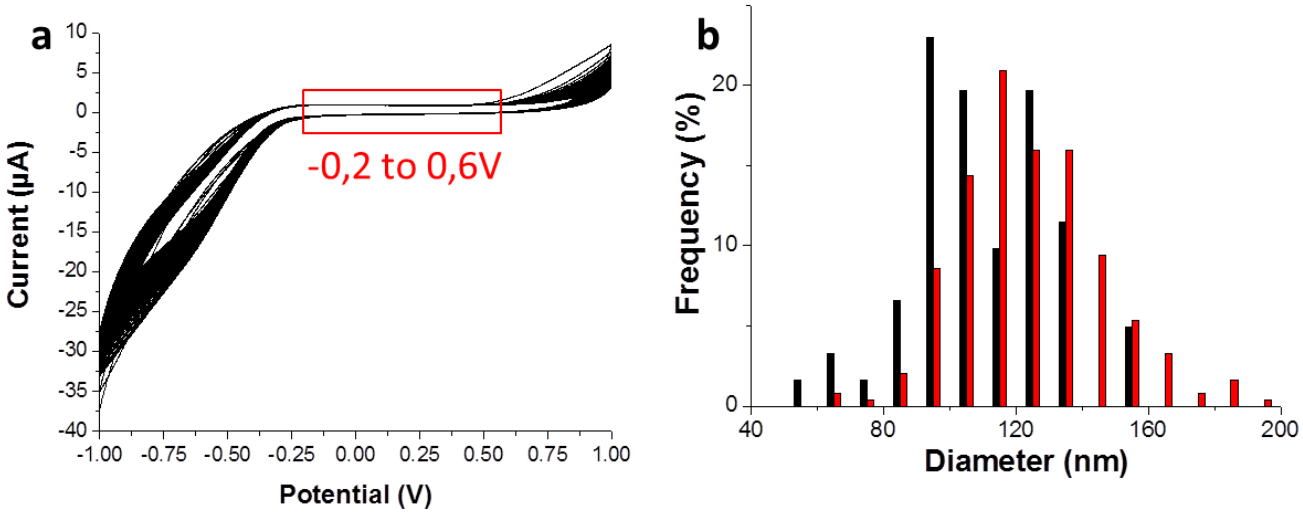
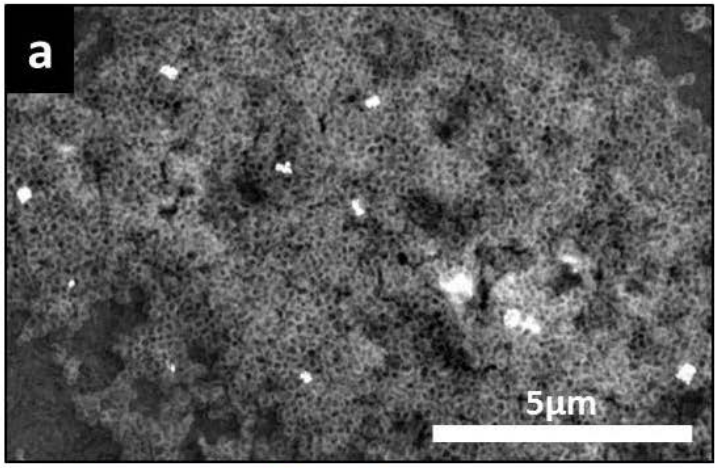


Figure S-6: Chemical and structural stability of Hybridosomes® along the film self-construction process. a) Voltamogram of 50 cycles from -1V to 1V (*vs* Ag/AgCl) at pH 3.5 of a Hybridosome dispersion. (b) Size distribution of Hybridosomes**,** calculated from low-magnification SEM micrographs with ImageJ, of adsorbed nanocapsules on a STEM grid (black bars) and after inclusion of the nanocapsules in the electro-clicked film (red bars).



c

Element	Atomic %
C	**16.80**
N	**11.22**
O	**46.67**
Si (substrate)	20.43
Fe	**1.32**
In (substrate)	3.30
Sn (substrate)	0.25

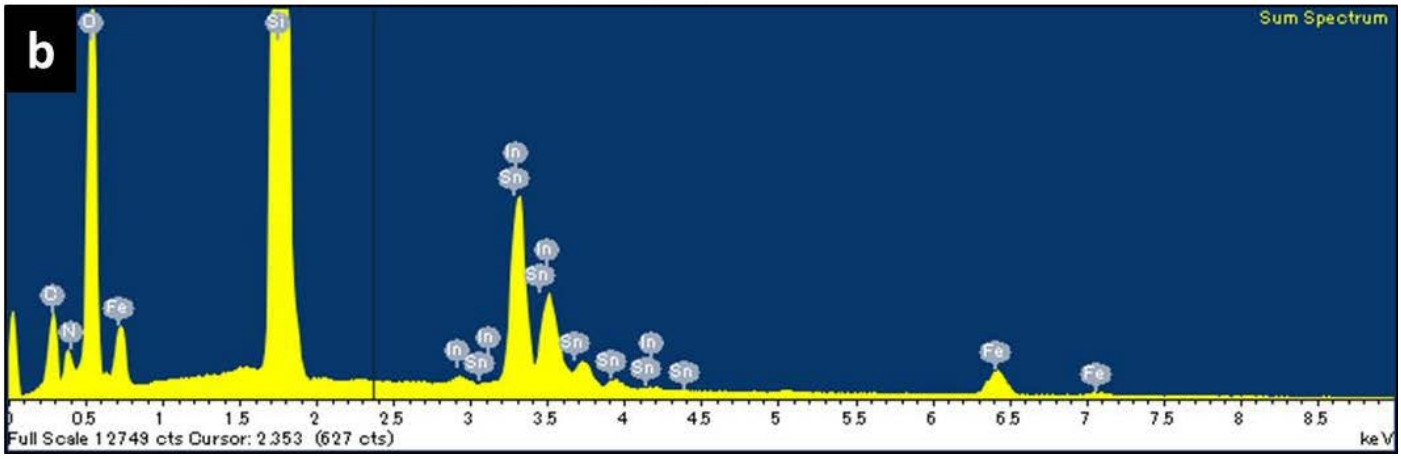


Figure S-7: Chemical composition of films self-constructed with IONPs based Hybridosomes®. a) SEM micrograph of a film self-constructed in typical electro-click conditions after 800 CV cycles. (b) Corresponding EDX analysis of the film. c) Element analysis of the corresponding zone of the film.

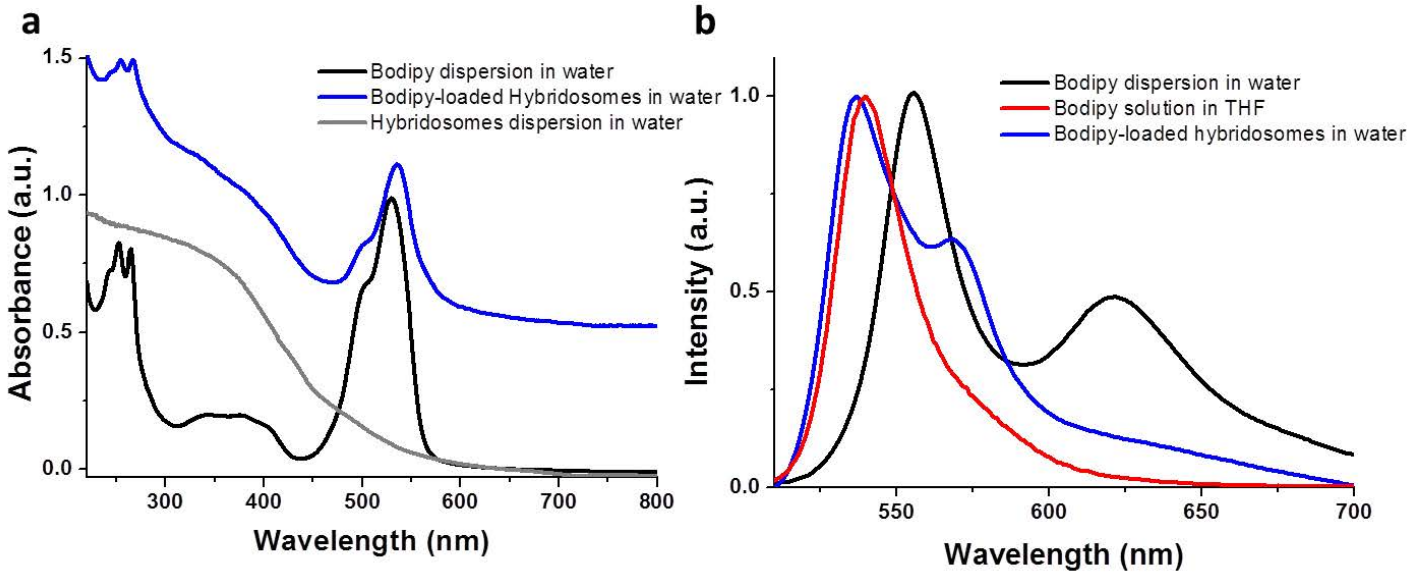


Figure S-8: Bodipy encapsulation, UV/Vis and Fluorescence spectra. a) UV/Vis absorbance spectra of aqueous dispersions of bodipy (black line), bodipy-loaded Hybridosomes (blue line) and « empty » Hybridosomes (grey line). (b) Fluorescence spectra (λ_{EX} at 480 nm) of bodipy dissolved in THF (red line), and aqueous dispersions of bodipy (black line) and bodipy-loaded Hybridosomes (blue line).

L/E ratio (%)	Peak area (a.u.)
0	0
10	27 000
50	82 000
90	167 000

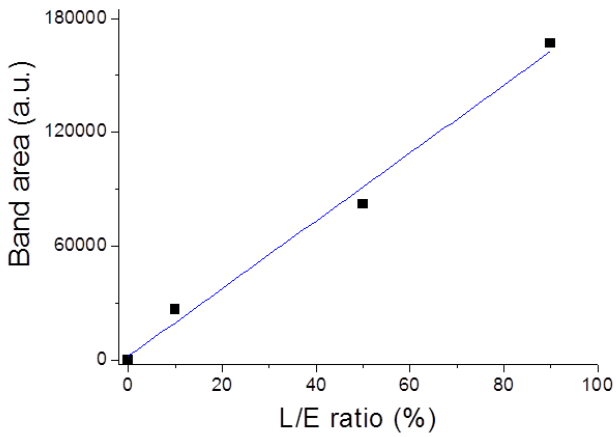


Figure S-9: Surface area of fluorescence emission bands (a) calculated by integration of the corresponding peaks between 510 nm and 700 nm. (b) Linear regression (y = 1783.x + 2159; R^2=0.99) of the peak areas in function of the "loading/empty" nanocapsules ratio in the building solution.

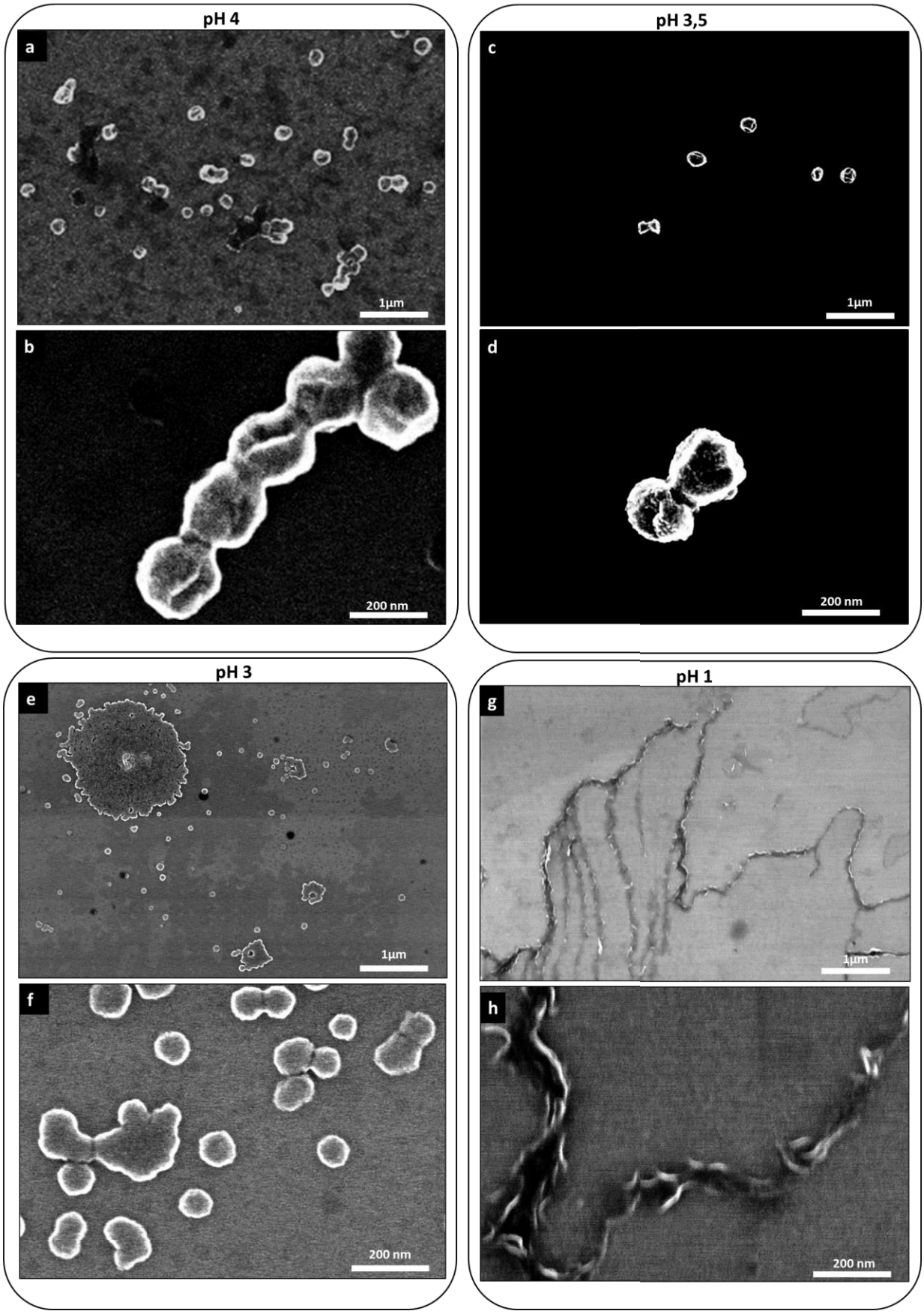


Figure S-10: pH sensitivity of functionalized "clickable" Hybridosomes® dispersions. Multiscale SEM analysis of Hybridosomes dispersions after 15 min of incubation with HCl solutions of pH 4 (a,b) pH 3.5 (c,d), pH 3 (e,f) and pH 1 (g,h).

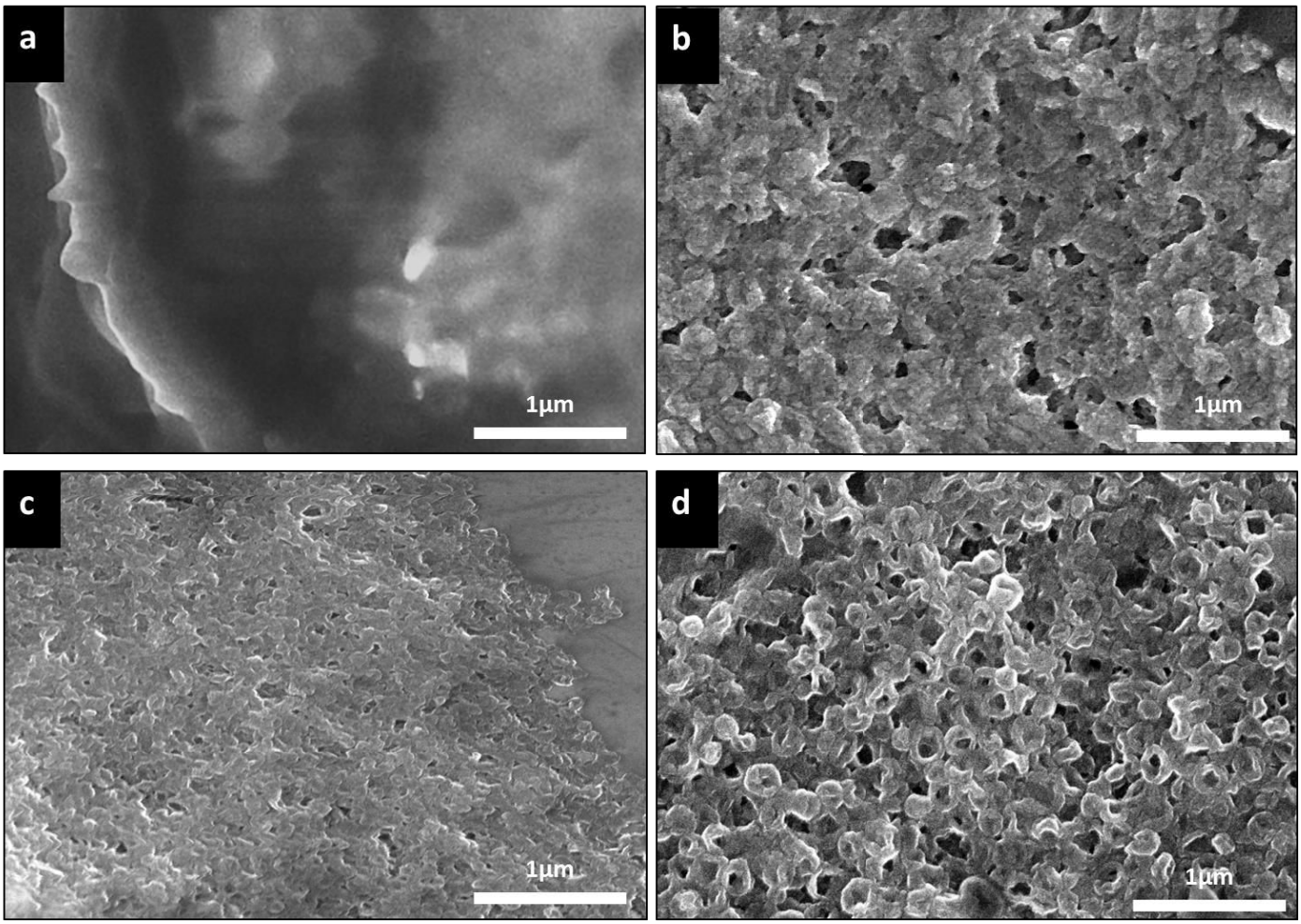


Figure S-11: pH sensitivity of electro-clicked Hybridosome®-based films. SEM micrographs of self-constructed Hybridosome films obtained after 800 CV cycles and incubated 15 min in HCl solutions of pH 1 (a), pH 2.5 (b), pH 3 (c) and pH 3.5 (d).

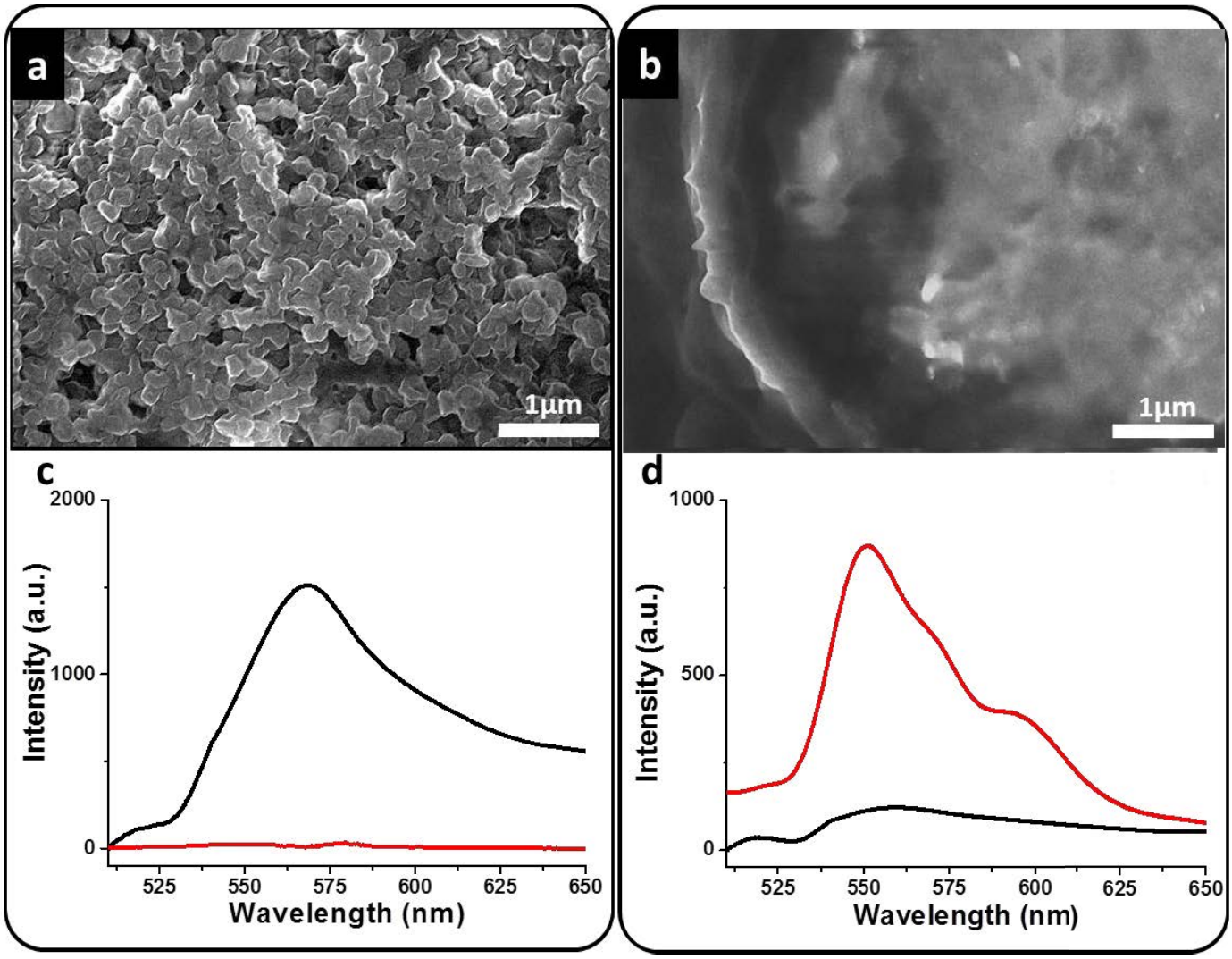


Figure S-12: pH-triggered release abilities of self-constructed Hybridosomes®-based films. SEM micrographs (a, b) and fluorescence spectra (λ_{exc} at 480 nm) in the dry state (c, d) of bodipy-loaded Hybridosome films self-constructed on ITO (black line) and of the corresponding supernatant (red line) before (a,c) and after (b,d) application contact with a 0.1M HCl solution for 15min.

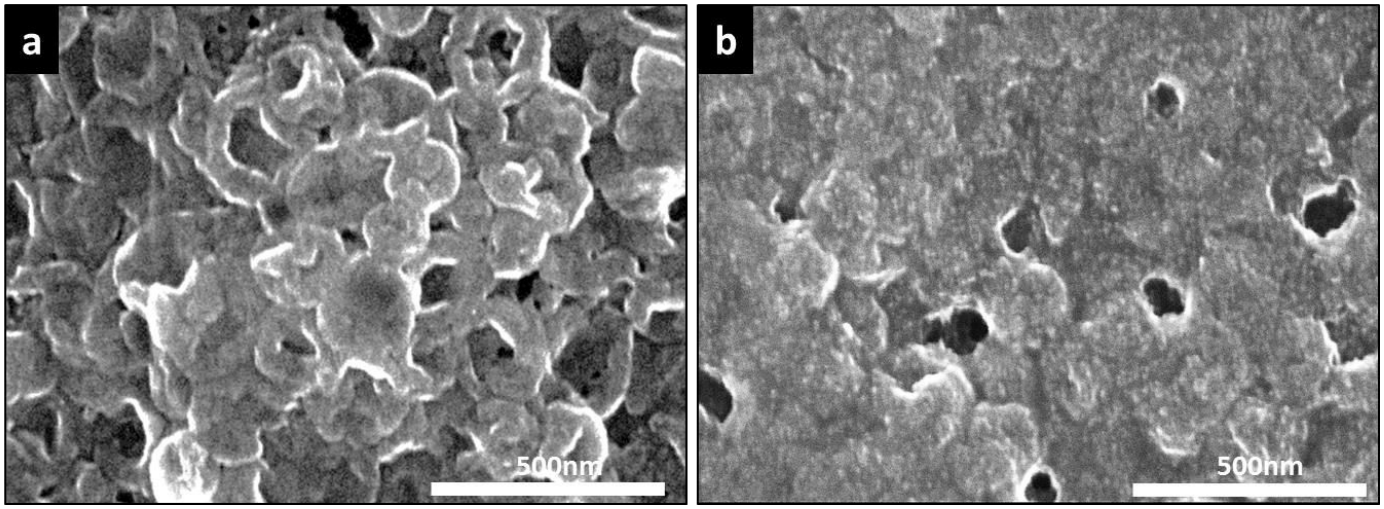


Figure S-13: Cargo release under electrochemical stimulus. SEM micrographs with higher magnification before (a) and after (b) applying +1 V potential *vs* Ag/AgCl for 15min in a 0.1M NaCl solution.

3. Résultats et discussions complémentaires

3.1 Cas des Hybridosomes IONP/Bodipy/PAA

Nous avons montré au chapitre 2 qu'il est possible de fonctionnaliser des Hybridosomes nottament via le piégeage d'un fluorophore moléculaire. Une question primordiale, peu discutée jusqu'à présent, est la quantité de fluorophore piégé avec l'Hybridosome. Du fait du choix de la bodipy (4,4-Difluoro-8-(4'-trimethylsilylethynylphenyl)-1,3,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacene) comme fluorophore (Figure 39), certains points restent encore à éclaircir.

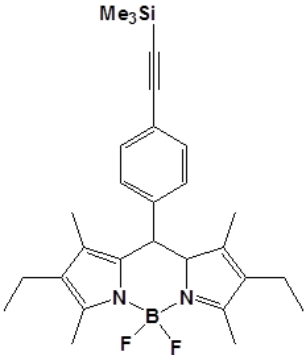


Figure 39: Structure du 4,4-Difluoro-8-(4'-trimethylsilylethynylphenyl)-1,3,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacene = Bodipy

En effet, il se trouve que la Bodipy utilisée présente des comportements particuliers dans nos conditions d'assemblage. La Bodipy est soluble dans le THF et partiellement soluble dans l'eau. De ce fait, son comportement dans un mélange THF/eau pourrait conduire à des phénomènes de nanoprécipitation. De nombreux travaux s'intéressent aux propriétés luminescentes de fluorochromes dans des milieux de type solvant/eau et plus particulièrement les mélanges THF/eau. Deux principaux phénomènes sont mis en évidence : le quenching causé par l'agrégation (aggregation-caused quenching ACQ) et son opposé, l'émission induite par l'agrégation (aggregation induced enhancement AIE)[55]. Ces phénomènes sont décrits pour de très nombreux systèmes π-conjugués polycycliques comme les dérivés multi-phényl perylèniques,[56] coronenes,[57] triphénylamines,[58] porphyrines,[59] et cette liste est très loin d'être exhaustive. Tous ces composés font l'objet d'études d'assemblages en des nanostructures de multiples formes (fibres, tubes, sphères, disques etc) et des tailles variables. On entend par nanostructure 0D une structure dans laquelle les toutes les dimensions sont à l'échelle nanométrique.

Plusieurs études discutent des mécanismes mis en jeu en fonction de la structure planaire ou des degrés de liberté de la molécule, et des interactions intermoléculaires et intramoléculaires liées à leur confinement (π-π stacking, dipolaires, électrostatiques).

Ces assemblages sont parfois appelés « fluorescent organic nanoparticles » (FON), « nanoagrégats » ou même « nanocristaux », et trouvent des intérêts dans les domaines de l'optique (linéaire et non linéaire), optoélectronique, bioimagerie (à un ou plusieurs photons), détecteurs.[60,61] Leurs stratégies d'assemblage sont proches des méthodes mentionnées au chapitre 1. Ainsi, on retrouve entre autres, les stratégies dites « soft template »[62] parfois en présence de micelles ou copolymeres, les méthodes s'appuyant sur un template solide (dites « hard template »)[63], ainsi que les méthodes basées sur l'évaporation d'un solvant.[64] Dans ce contexte, l'étude du comportement de la bodipy vis-à-vis du procédé d'assemblage des Hybridosomes semble donc primordiale.

Dans l'article 3, nous avons comparé l'émission de fluorescence de la Bodipy dans le THF ainsi que dans l'eau et observé des différences au niveau de l'émission entre 500 et 700nm. Ce déplacement bathochrome s'explique par la formation de « J-agrégats ». Les J-agrégats sont des assemblages supramoléculaires qui présentent des propriétés d'émissions de fluorescence différents de l'état moléculaire.[65] Les phénomènes électroniques entres molécules adjacentes conduisent à l'apparition d'une nouvelle bande d'émission à plus grande longueur d'onde. Inversement, lorsque les interactions électroniques conduisent à un déplacement vers de plus basses longueurs d'ondes (déplacement hypsochrome), on parle de « H-agrégats ».

D'autres résultats nous ont permis de mettre en évidence la présence d'agrégats de l'ordre de 200 nm lorsque seule la bodipy suit le procédé d'assemblage sans nanoparticules ni polymère, avant et après évaporation du solvant (Figure 40).

Les distributions de taille présentées Figure 40a confirment bien que des structures de 150-200 nm sont présentes avant et après évaporation du solvant. Les micrographies TEM des suspensions après évaporation du solvant et à l'état sec semblent montrer plusieurs type de structures : (i) des structures certainement cristallines de l'ordre de plusieurs centaines de microns Figure 40b, (ii) une sorte de matrice délimitant des zones circulaires de 500 nm environ Figure 40c, (iii) des grains de 10-50 nm Figure 40d, (iv) des sphères et capsules d'environ 300 nm Figure 40e et f. Bien que faites à l'état sec, ces observations semblent montrer la présence de structures sphériques de l'ordre de 300 nm et sont en accord avec les observations faites en DLS.

L'étude des propriétés de luminescence dans les mélanges THF/eau n'a pas été priorisée jusque-là, mais elle est actuellement en cours. Ces études sont délicates puisque la tendance à l'agrégation et à la sédimentation rendent la quantification de telles suspensions plus aléatoire. Cependant, les résultats préliminaires présentés dans cette partie ont permis de mettre en évidence la présence de plusieurs types d'agrégats amorphes et probablement cristallins, de différentes tailles et même de voir des assemblages sphériques et sous forme de capsules. Ces observations sont encourageantes pour la compréhension du procédé d'assemblage.

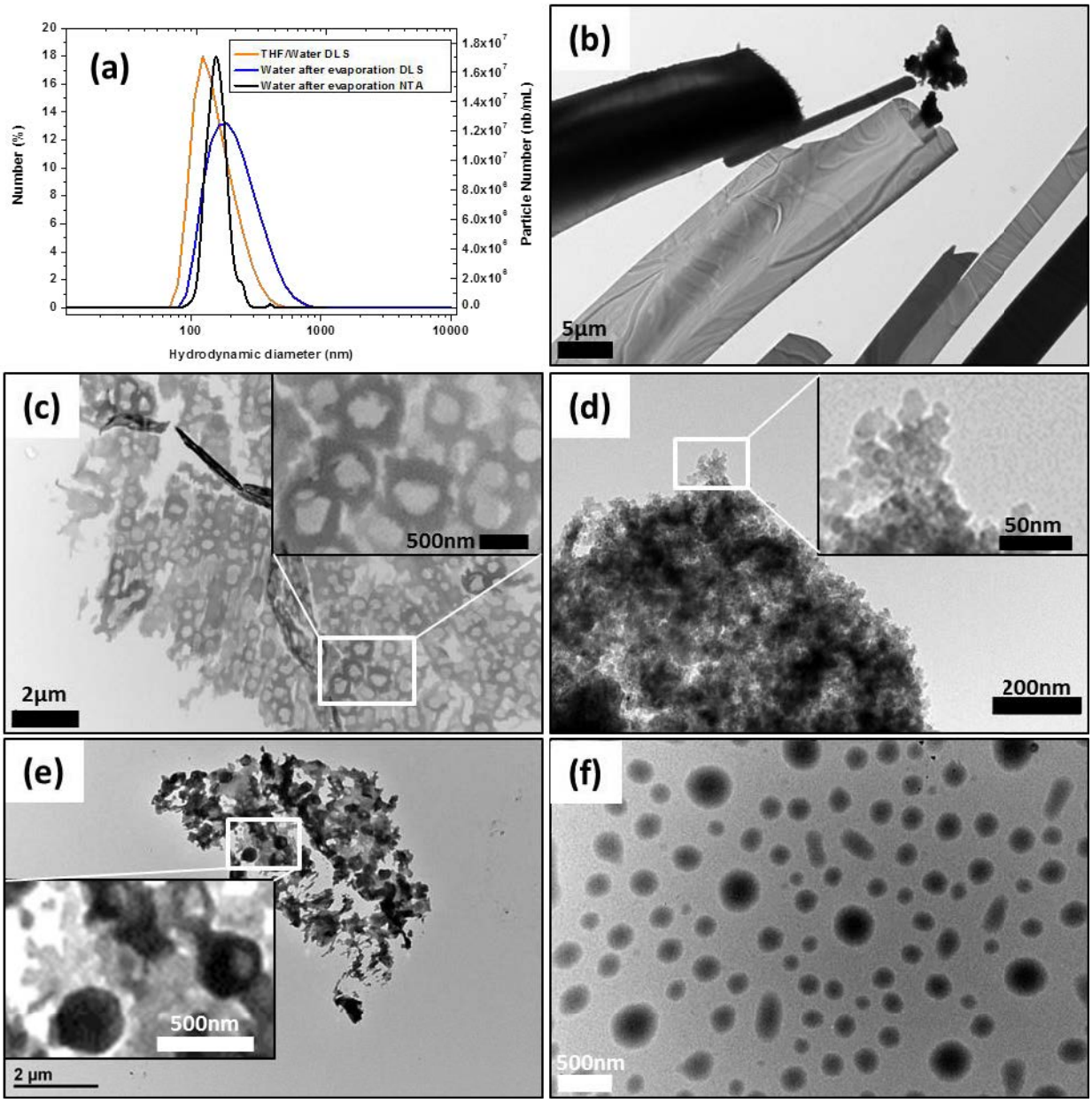


Figure 40: Caractérisation de la suspension de Bodipy. (a) Distribution de taille des suspensions de Bodipy avant évaporation (orange) et après évaporation (DLS bleu, NTA noir). (b - f) Micrographies TEM des suspensions de Bodipy après le procédé d'assemblage.

3.2 Réponse des films après un stimulus de pH

Dans l'article précédent, nous avons étudié la déstabilisation du film construit avec les Hybridosomes IONP/Bodipy/PAA en milieu acide ou par application d'un potentiel électrique. En parallèle de cette étude, le surnageant issu de l'étape de déstabilisation a été imagée en TEM (Figure 41). Les micrographies laissent apparaître de gros objets de l'ordre du micron qui restent non identifiés (Figure 41a). Il s'agit très probablement d'agrégats de particules d'oxydes de fer et de PAA du fait de leur contraste important. A plus fort grandissement, on observe des particules sphériques de taille hétérogène (10-50nm) qui semblent être les particules d'oxydes de fer coalescées (Figure 41c).

Remarque : Nous avons abordé dans le chapitre 2 le point de la cinétique de dégradation des oxydes de fer aux valeurs de pH < 4. Nous avons montré qu'après 24h à pH < 4 les IONP avaient complètement coalescé. Il est important de noter ici que la préparation des grilles de TEM a été réalisée plusieurs jours plus tard. Les oxydes de fer sont donc à un stade encore plus avancé de dégradation.

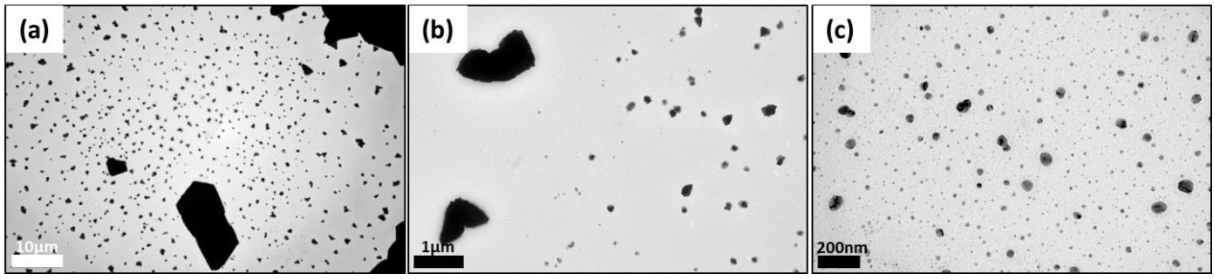


Figure 41: Micrographies TEM du surnageant après déstabilisation d'un film d'Hybridosomes IONP/Bodipy/PAA par trempage dans une solution à pH 1.

4. Conclusion

Nous avons montré dans ce chapitre que les Hybridosomes pouvaient également être fonctionnalisés en surface, grâce au polymère qui les réticule. Le PAA a été muni d'un groupement alcyne et les Hybridosomes-Alcyne ont pu être engagés en tant que « building blocs » dans une réaction de chimie électro-click. Des films de l'échelle de plusieurs centaines de microns ont été ainsi construits et les différentes propriétés des Hybridosomes ont été transposées aux films. Ainsi, la composition des films hybrides et nanostructurés a pu être variée par ses constituants organiques et inorganiques. Les films ont également montré des propriétés de relargage d'un fluorophore par des stimuli électrique et de pH. Ces résultats nous ont aussi permis d'évaluer la réponse des

hybridosomes aux variations de conditions de pH et ainsi appréhender les effets de relargage. Ces résultats sont un bon point de départ vers le développement de matériaux stimuli-réactifs.

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Chapitre 4

Potentialités des Hybridosomes en nanomedecine

1. Introduction et context

Dans l'article 1 nous avons établi une première preuve d'efficacité des Hybridosomes comme agent de contraste en IRM. Pour cela, nous avons d'abord évalué la toxicité *in vitro* des Hybridosomes et conclue que la viabilité des cellules Hela est maintenue après 24h de mise en contact avec les nanocapsules. Ensuite, l'efficacité des Hybridosomes a été comparée à un agent de contraste négatif préclinique (FeraSpin™, Myltenyi) et jugée équivalente *in vitro* et *in vivo*. Ce projet appliqué au développement de l'IRM du petit animal est le fruit d'une collaboration avec Dr. M.B. Troadec et H. Jacobzyck de l'équipe Expression de Gênes et Oncogenèse (GEO) de l'Institut de Génétique et de Développement de Rennes (IGDR, UMR CNRS 6290), ainsi qu'avec Dr. P.A. Eliat au sein de la plateforme PRISM (Plateforme Rennaise d'Imagerie et Spectroscopie Multi-modales). Le développement d'agents précliniques et cliniques soulèvent de nombreuses questions sur le devenir des Hybridosomes *in vivo*, leur pharmacocinétique et biodistribution qui sont des questions à adresser impérativement en nanomédecine.

L'équipe GEO de l'IGDR concentre ses recherches sur les mécanismes et processus oncogéniques pour mieux comprendre les pathologies cancéreuses du sang et leur évolution vers les stades métastatiques. Un des modèles étudiés est celui des leucémies du lignage B qui sont parmi les cancers hématologiques les plus fréquents. Dans le cadre de notre collaboration, nous avons choisi de concentrer nos efforts sur le développement d'une application théranostique des Hybridosommes pour l'imagerie multimodale ciblée et le traitement des cancers exprimant la protéine membranaire CD19. Ce projet interdsciplinaire a été soutenu et en partie financée par l'UMS BIOSIT. L'anticorps anti-CD19 utilisé dans ce projet a été produit au sein de l'Etablissement Français du Sang par Dr. F. Vérité et Dr. Y.Danger.

Nous présentons ici un article de revue et de perspectives à propos des potentialités des nanoparticules ciblant CD19 vis-à-vis des seuls traitements actuels ciblant CD19, plus précisément en immunothérapie.

2. Article 4: Promises, Specificity and Limitations of Nanoparticles in the Era of Gene Therapy: Example with CD19-Targeting Chimeric Antigen Receptor (CAR)-Modified T Cells

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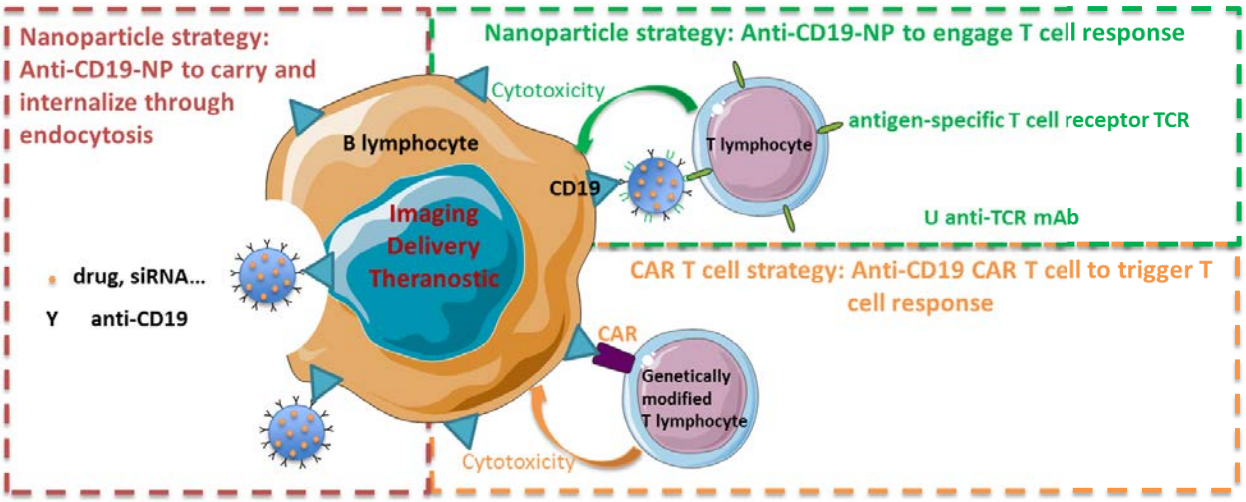

Promises and limitations of nanoparticles in the era of cell therapy: example with CD19-targeting chimeric antigen receptor (CAR)-modified T cells.

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ABSTRACT

A number of –organic, inorganic and even hybrid– nanoparticles have been developed by chemists for biomedical applications to meet imaging and targeting needs. In parallel, adoptive T therapy with chimeric antigen receptor engineered T cells (CAR T cells) has recently held great promise in B-cell malignancy treatments thanks to the development of anti-CD19 CAR T cells. Indeed, CD19 is a reliable B cell marker and a validated target protein for therapy. In this perspective article, we propose to discuss the advantages, limits and challenges of nanoparticles in the era of CAR T cells, focusing on CD19 targeting objects: anti-CD19 nanoparticles and anti-CD19 CAR T cells because those genetically-modified cells are the more widely developed in clinical setting. In the first part, we introduce B cell malignancies and the CD19 surface marker, and then we present an overview of the definition and application of nanoparticles, before exposing CAR T technology. Finally, we discuss the complementary approaches between nanoparticles and CAR T cells.

KEY WORDS

Nanoparticles, CD19, chimeric antigen receptor, T cell, B cell, cell therapy

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INTRODUCTION

A malignant cell is defined as a cell blocked in an early stage of differentiation and undergoing an uncontrolled proliferation. Despite huge progress in cancer treatment over the last 50 years, cancer is still a major cause of death worldwide. Until now, the progresses have been mainly obtained through to the identification of therapeutic drugs, better molecular comprehension of the onset and progression of malignancy, more sensitive detection of tumor cells, more efficient follow-up of the disease, better management of adverse-effects, optimization of protocol design… Still, challenges are to be undertaken. From the time a patient arrives to be diagnosed to the moment he is cured, physicians and medical staff would have encountered at least the following issues: tumor identification (*i.e*. is that a nodule of B cells?), tumor cells imaging (where are localized the malignant cells? Is that the primary tumor or a metastasis?), delivery of therapeutic drugs and avoidance of adverse effects on non-malignant cells (sometimes minimizing the risk of generation of a secondary cancer), and finally identification of residual cells that could be ultimately at the origin of refractory cancer or relapse.

The chimeric antigen receptor (CAR) T cell therapy is a revolutionary approach of targeted immunotherapy to treat cancer. In CAR T cell therapy, the therapeutic effector is a genetically-modified cell. If CAR T cells might not yet be poised to overtake chemotherapy as the standard of care, CAR T cell therapy is looking promising as treatment for certain patients with no other feasible options, such as in the cases of refractory patients or relapse. An alternative research approach for the treatment of cancer is the use of nanoparticles, which have been proposed as carriers for drug encapsulation in the 60's. Since then, a variety of organic and inorganic nanoparticles, with sizes ranging from *circa* 5 nm to 200 nm, have been designed for a wide range of applications including targeted drug delivery and imaging, boosting the activity of nanomedicine, with some remarkable results particularly in the field of cancer diagnosis and therapy.

In this perspective article, we propose to discuss the advantages, limits and challenges of nanoparticles in the era of CAR T cells. We will focus on CD19 targeting objects: anti-CD19 nanoparticles and anti-CD19 CAR T cells because those genetically-modified cells are the more widely developed in clinical setting.

In the first part, we will introduce B cell malignancies and their CD19 surface marker, then we will present an overview of the definition and applications of nanoparticles, before exposing CAR T technology. Finally, we will discuss the complementary approaches between nanoparticles and CAR T cells. From the biological point of view, anti-CD19-grafted nanoparticles and anti-CD19 CAR T cells target the same cells: B cell lineage. From the therapeutic perspective, nanoparticles and CAR T cells approaches share a common objective: the optimization of therapeutic effect on target cells and minimization of adverse effects. However, mechanisms of action are different as well as efficiency and production process **(see Table Of Contents)**. It seems reasonable to conceive that nanoparticles could play a significant role for the potentiation of, and the cooperation with CAR T cell therapy in the future.

1 CD19, A B CELL RESTRICTED SURFACE PROTEIN AND A RELIABLE MARKER OF B CELL MALIGNANCIES

1.1 THE FUNCTIONS OF B LYMPHOCYTES

B cells (also named B lymphocytes) achieve multiple functions that explain their central role in the immune system **(Figure 1)**. Their main role is the production of antibodies to identify and neutralize pathogens. The binding of a B lymphocyte to an antigen triggers an initial step of multiplication and differentiation either into plasma cell which secretes antibodies or into memory B cell. Besides their role in humoral immunity, B cells are implicated in cytokine production (*e.g.* IFNγ, IL6, IL10), antigen presentation to T cells, wound healing, cytokine balance for the differentiation between T lymphocytes (Th1 and Th2 cells), but also in the transplant rejection (review in [1]).

B cells undergo differentiation, from hematopoietic stem cells to plasma cells or memory B cells, through a series of stages characterized by the orderly rearrangement and expression of immunoglobulins genes including CD19 **(Figure 1)**. The development of B cells is also distinguished into different stages by the sequential expression of different transcription factors that induce immunoglobulin gene recombination and the expression of specific surface phenotypes. Until the immature stage B cells are located in the bone marrow, and from the mature stage, in the periphery [2].

1.2 B CELL MALIGNANCIES

B cell malignancies are hematopoietic cancer characterized by uncontrolled proliferation of B-lymphocytes blocked along their differentiation process. B cell malignancies are classified as leukemia (which develops in the bone marrow and disseminates into the body), lymphoma (a cancer of the lymphatic system characterized by the development of cancer cells in lymph nodes) and myeloma (cancer of mature B-lymphocytes in the bone marrow) (review in [3]). B cell malignancies represent 4% of all cancers in adults and 40% of all cancers in children. Clinical outcomes of these cancers under standard chemotherapy depend on the type of B cell malignancies. For instance, children with B-acute lymphoblastic leukemia (ALL) have an overall good prognosis, but some of them are refractory to chemotherapy or develop multiple relapses and have a poor prognosis (review in [4]). Adults with relapsed or refractory B cell ALL have a poor prognosis (review in [5])

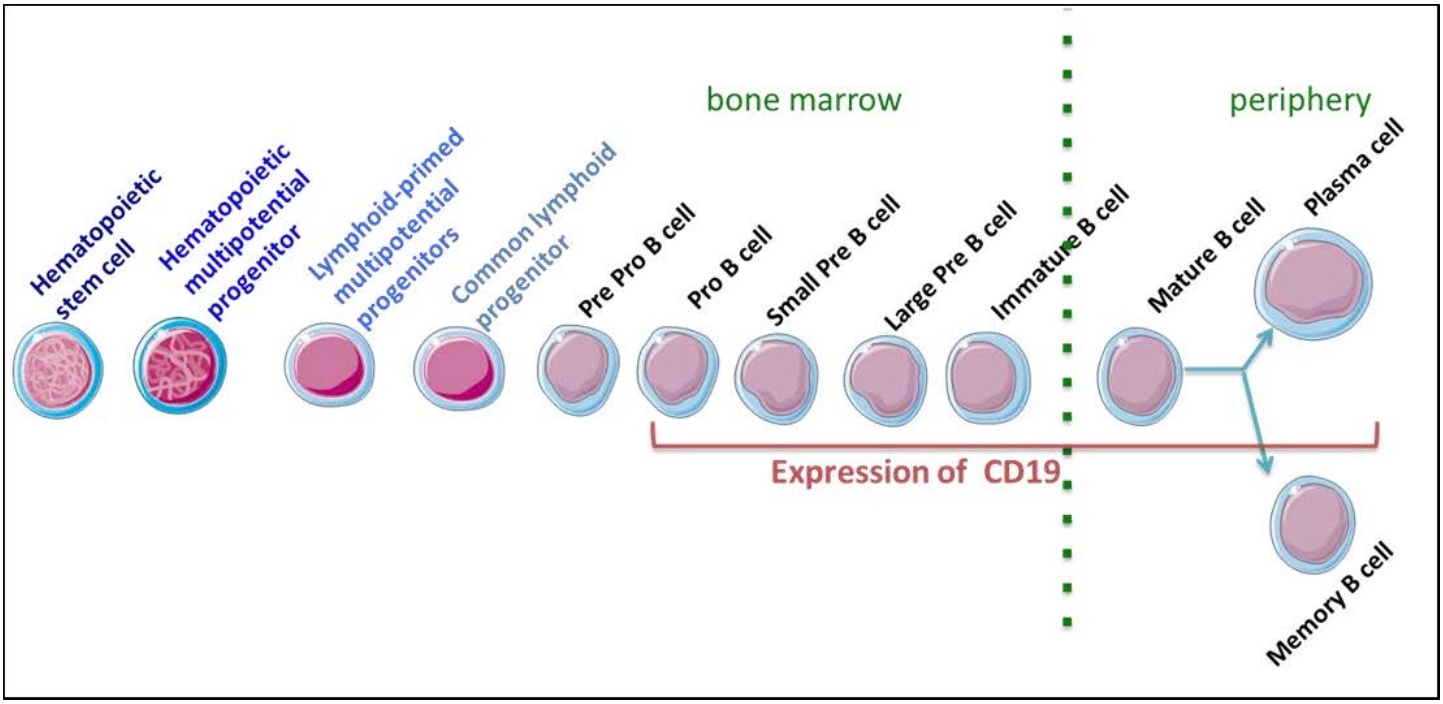


Figure 1: B cell development and differentiation B cell development begins in bone marrow and progresses through pre pro B cell, pro B cell, small pre B cell, large pre B cell and immature pre B cell. B cell locates within the circulatory system from mature B cell stage. The CD 19 protein is expressed from pro B cell stage.

1.3 THE SURFACE PROTEIN CD19 IS A VALIDATED TARGET PROTEIN FOR THERAPY

1.3.1 CD19 molecular description and function

CD19 is a 95 kDa transmembrane glycoprotein of the immunoglobulin superfamily composed of an extracellular domain, a single transmembrane domain, and a cytoplasmic domain [6]. CD19 belongs to the CD19 complex on the surface of B cells with CD21 and CD81 proteins **(Figure 2)**. CD19 activation induces two downstream pathways. The first cascade of activation is dependent on the B cell receptor (BCR): it is a co-receptor for BCR signal transduction (review in [3]). The BCR plays a role as antigen receptor. The BCR is composed of a membrane immunoglobulin and a signaling subunit composed of a heterodimer of immunoglobulin alpha and beta. The second pathway is independent of the BCR: the CD19 complex is able to bind activated complement fragment C3d and modulates BCR signaling (review in [3]).

1.3.2 Internalization of CD19 after binding to anti-CD19 antibody

CD19 molecules on the surface of each B lineage leukemia/lymphoma cells are rapidly internalized upon ligation with anti-CD19 antibodies or immunoconjugates[7,8] and end up in lysosomes [9 10,11]

1.3.3 In which type of cells is CD19 expressed?

CD19 is a B cell-specific protein expressed early in B cell ontogeny [6] **(Figure 1)**. CD19 transcripts are restricted to members of the B cell lineage and are not expressed on other hematological lineages including normal myeloid, erythroid, megakaryocytic, or multilineage bone marrow progenitor cells [12]. CD19 protein is found on the surface of B cells from the proB cell stage until plasma cell differentiation of the B lineage [13]. Several hundred thousand CD19 molecules can be found on the surface of each B-lineage leukemia/lymphoma [12]. All resting B cells display CD19 antigens, and CD19 expression persists upon activation, but is lost upon further differentiation to immunoglobulin-secreting plasma cells [6]. CD19 is also found most abundant in pre-B cell lines and least abundant in plasmacytomas [6]. Almost all early B cell malignancies show CD19 expression at normal to high levels: 80% of ALL, 88% of B cell lymphomas and 100% of B cell leukemias) (review in [3]). However its expression decrease in myeloma cases (review in [3])

1.3.4 CD19 as a target for therapy

Twenty years ago, CD19 was already proposed as a « suitable target for immunotoxin-mediated treatment of aggressive forms of B cell lymphomas and leukemia that respond poorly to conventional chemotherapy [12]». Currently, CD19 antibody-based therapy has become reality to treat B cells malignancy. In the 2010's, various strategies harnessing the potential of targeting B cells restricted to CD19 antigen were in development: antibody-drug conjugate, Fc-engineered human CD19 antibody with antibody-dependent cell-mediated cytotoxicity, chimeric antigen receptor, etc. [14]. The most advanced anti-CD19 therapy is the Blinatumomab (BLINCYTO®, Amgen) (review in [14]) [15], which is a bispecific CD19-directed CD3 T cell engager (BiTE) antibody construct that binds specifically to CD19, expressed on the surface of cells of B-lineage origin, and CD3 expressed on the surface of T cells. Blinatunomab brings nearer both cells so that the activated T cells can kill the B cells.

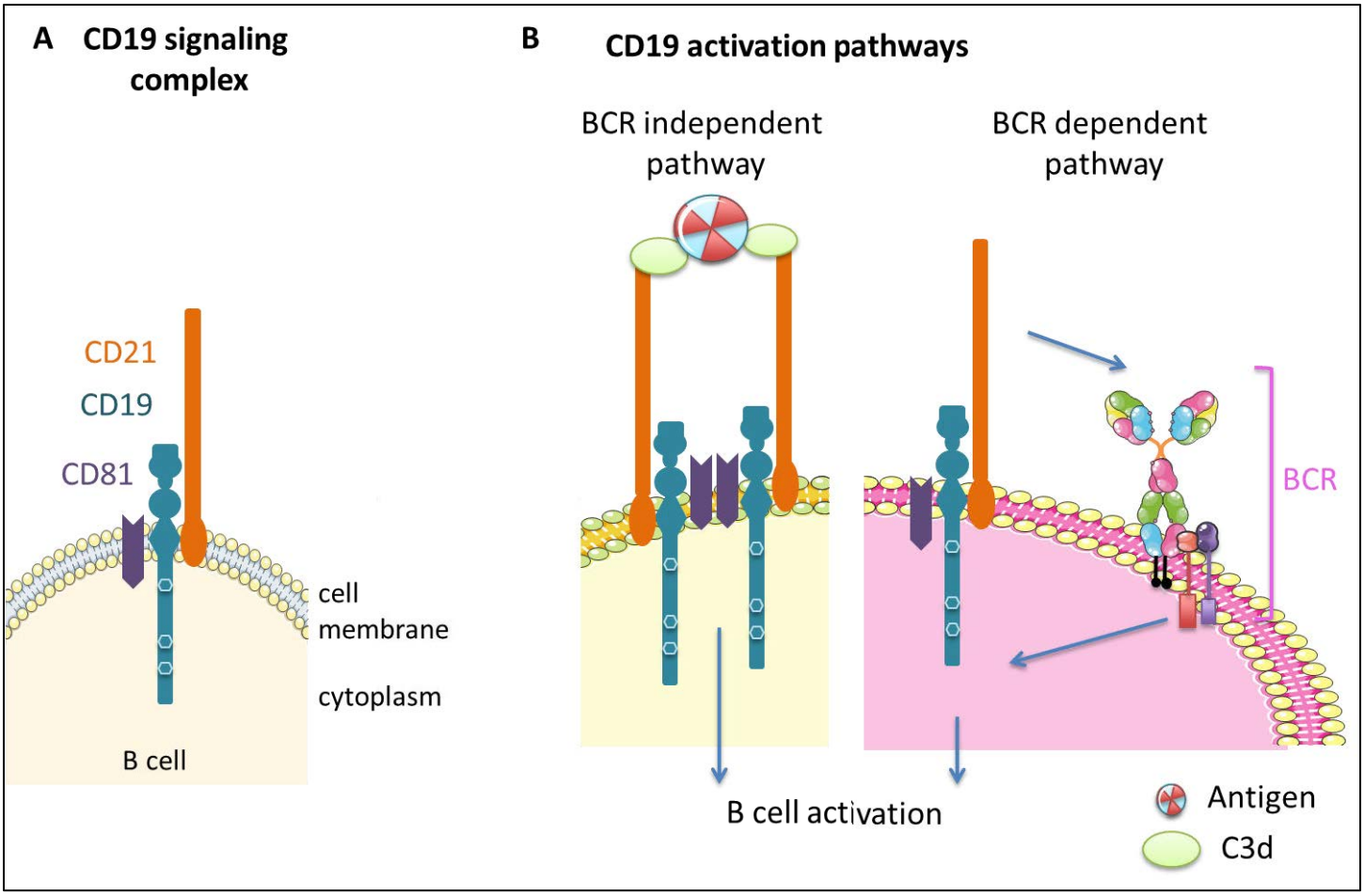


Figure 2: CD19 signaling complex and activation pathways (A) Schematic representation of the CD19 signaling complex. The CD19 complex is composed of CD21, CD81 and CD19 transmembrane proteins. CD19 possesses an intracellular tail with multiple tyrosine-kinase residues involved in signal transduction. (B) The first pathway of CD19 activation is dependent on the B cell receptor (BCR): it is a co-receptor for BCR signal transduction. The second pathway is independent of the BCR: the CD19 complex is able to bind activated complement fragment C3d and modulates BCR signaling (Figure adapted from [3]).

Blinatumomab is US Food-and-Drug-Administration (FDA) and European Commission (EC) approved for the treatment of Philadelphia chromosome-negative relapsed or refractory B-ALL, in adults in the USA and EC as well as in children in the USA. Additionally, in preclinical settings, anti-CD19 antibodies are also in development for radioimmunotherapy. ^{131}I-labeled anti-CD19 antibody has been largely explored for conventional ^{131}I radioimmunotherapy because antigen rapidly internalizes upon binding of antibody – resulting in catabolism and release of ^{131}I [16]. Moreover, ^{90}Y beta-particle-labeled anti-CD19 antibody has shown an efficacy comparable to ^{90}Y-labeled anti-CD20 antibody in the treatment of mice bearing human B lymphoma cell line xenografts [17].

2 NANOPARTICLES IN NANOMEDICINE

2.1 DIFFERENT TYPES OF NANOPARTICLES AND APPLICATIONS

A number of nanoparticles has been proposed by chemists for biomedical applications. These include organic nanoparticles such as liposomes, oil-in-water emulsions or polymeric particles, and inorganic nanoparticles such as superparamagnetic iron oxide nanocrystals, quantum dots, upconverting *nanoparticles (*UCNPs) **(Table 1)**. Historical developments of these two families -organic and inorganic nanoparticles- have followed separate but convergent paths. Indeed, organic nanoparticles were primary used as nanocontainers to encapsulate and deliver drugs and then developed to meet imaging and targeting needs. In contrast, inorganic nanocrystals were first considered as imaging contrast agents for their intrinsic optical or magnetic properties, and were further tailored to add therapeutic properties. More recently, hybrid liposomes or polymer particles containing nanocrystals appeared in the academic and patent literature.

Described first by Bangham in the 1960s [18], lipid vesicles -or "liposomes"- are the most ancient technology for encapsulation. The amphiphilic nature of most lipids enables them to self-assemble into bilayers which form the membrane of these vesicles. Liposomes can have one or several bilayers and their size span from a few tens of nanometer (small unilamellar vesicles or SUV) to microns (large unilamellar vesicles or LUV) [19]. Numerous polymers were designed for nanomedicine. Most of them combine a

hydrophobic and a hydrophilic component, and self-assemble in water or in water-solvent mixtures to form either nanoparticles or vesicles (polymersomes). Polymer nanoparticles like surfactant micelles have a hydrophobic/hydrophilic core-shell structure, and for this reason are sometimes improperly called polymer micelles [20]. The term "micelles" refers to a structure at thermodynamic equilibrium, which is usually not the case for polymer nanoparticles since the dynamics of exchange of the polymer chains with the bulk is generally very slow.

A few encapsulation systems have made their way to the market [19]. Commercialized liposomal formulations include anticancer drugs such as doxorubicin (Myocet, Doxil, Lipodox and Caelyx), antibiotics (amphotericin B in Ambisome, Abelcet and Amphotec), and vaccine formulations such as Inflexal V.

A few types of inorganic nanoparticles, such as silica nanoparticles, are also used for their encapsulation properties and the possibility to easily engineer their surface with targeting moieties. However, most of them are used for imaging or therapy because of their specific optical or magnetic properties but do not as such, have the capability to encapsulate. Gold nanoparticles have long been used in *ex vivo* transmission electron microscopy (TEM) imaging as immunomarkers. They are now considered for hyperthermia and photoacoustic imaging. Many other types of inorganic nanoparticles were developed mainly for imaging purposes. Among them, quantum dots (QD) are luminescent semi-conducting nanoparticles with a radius-dependent emission wavelength. Thus a single excitation wavelength can be used to generate two or more colors, allowing simultaneous detection of different markers. As compared with organic dyes, QD are generally much brighter and not submitted to photobleaching. However, most QD suffer from the presence of toxic elements such as Cd or Se and are not used in medical imaging. More recently, a family of luminescent nanoparticles -upconverting nanoparticles (UCNP)- featuring near infrared absorption and visible emission appeared in the literature. These specific optical properties enable to irradiate deeper in tissues. Superparamagnetic iron oxide nanoparticles were clinically used as MRI contrast agent until the late 80's. Despite their negligible toxicity, they have been substituted by soluble gadolinium complexes that have the interest of a better contrast and easier administration to the patient.

2.2 ADVANTAGES OF NANOPARTICLES

2.2.1 Heavy loading capacity and controlled release

Quantum dots as most other imaging nanoparticles are generally more efficient markers or contrast agents than their molecular counterparts, because of the concentration of material in a reduced volume. Similarly, nanocarriers and particularly polymer nanoparticles can transport high amounts of encapsulated drug which is an incomparable advantage if these can be delivered precisely to the sick cells. In addition, one of the most remarkable features of polymer nanoparticles and liposomes is their capacity to release their load over long periods of time. Sustained release is obtained by slowing down the diffusion of encapsulated drug using retardation barriers [21]. Alternatively, drug molecules can be released by erosion of the carrier itself (Bhattacharya et al., 2016). In this respect, the use of biodegradable polymers has been very successful [22]. With the ultimate goal of achieving spatial and temporal control of drug delivery, the nanoplatforms have evolved from the mere "sustained" release to "triggered" release, using endogenous or exogenous triggers **(Figure 3)**. In diseases such as cancer, abnormal local conditions such as pH, enzymatic activity or concentration in reactive oxygen species have been used to trigger responsive components of the delivery system. In addition to these endogenous signals, nanocarriers can also be designed to release their load on the effect of an external trigger, in general light, ultrasounds or a magnetic field. The energy absorbed by the nanoplatform is converted into thermal or chemical energy, yielding the destruction of the platform [22,23].

2.2.2 Multimodality

Another advantage of nanoparticles is their ability to anchor a large number of different molecules. Thus, nanoparticles have naturally appeared as ideal platforms to combine several functions simultaneously and a myriad of nano-objects customized to achieve complex tasks were designed, generally taking the form of hybrid structures combining organic and inorganic components. For instance, imaging contrast agents enabling multiple modalities such as magnetic resonance imaging/computerized tomography (MRI/CT) or magnetic resonance imaging/ positron emission tomography (MRI/PET) may offer useful complementary structural and functional information, or the ability to image with different resolutions and over different temporal and spatial scales. Similarly, nanoparticles can be used to combine multiple therapies, in a multimodal therapy approach. Thus the combination of thermotherapy or photothermal therapy with conventional chemotherapy holds great prospects in cancer treatment [24]. In addition, nanoparticles have been at the origin of a new concept, referred to as theranostic, associating diagnostic (MRI, fluorescence) and therapeutic (vectorization of therapeutics agents, hyperthermia, photothermal therapy…) non-invasive tools. Theranostic and the ability to combine over the same particle functions targeting various genetic and epigenetic markers meet the ambition of personalized medicine to identify the patients' molecular profiles and monitor their specific response to treatment in real-time.

NP type	Size (nm)	Organic/Inorganic	Principal application
Liposome	30-500	organic	encapsulation
Polymer NP	10-200	organic	encapsulation
Polymersome	50-1000	organic	encapsulation
Dendrimer	< 10	organic	encapsulation / imaging
Solid Lipid NP (and emulsion based particles)	> 100	organic	encapsulation
Silica NP	all range	inorganic	encapsulation / imaging
Quantum dot	5-20	inorganic	imaging
SPION	5-100	inorganic	imaging
Au NP	5-100	inorganic	imaging / therapy
Hybridosomes®	80-120	organic/inorganic	imaging / encapsulation / therapy

Table 1: Main chemical and physical properties of the different types of nanoparticles used in nanomedicine and their principal applications. Note that the given size corresponds to the primary nano-object. In the case of small nanoparticles (NP) such as dendrimers or quantum dots (QD), surface modification with PEG or other macromolecules result in larger dimension.

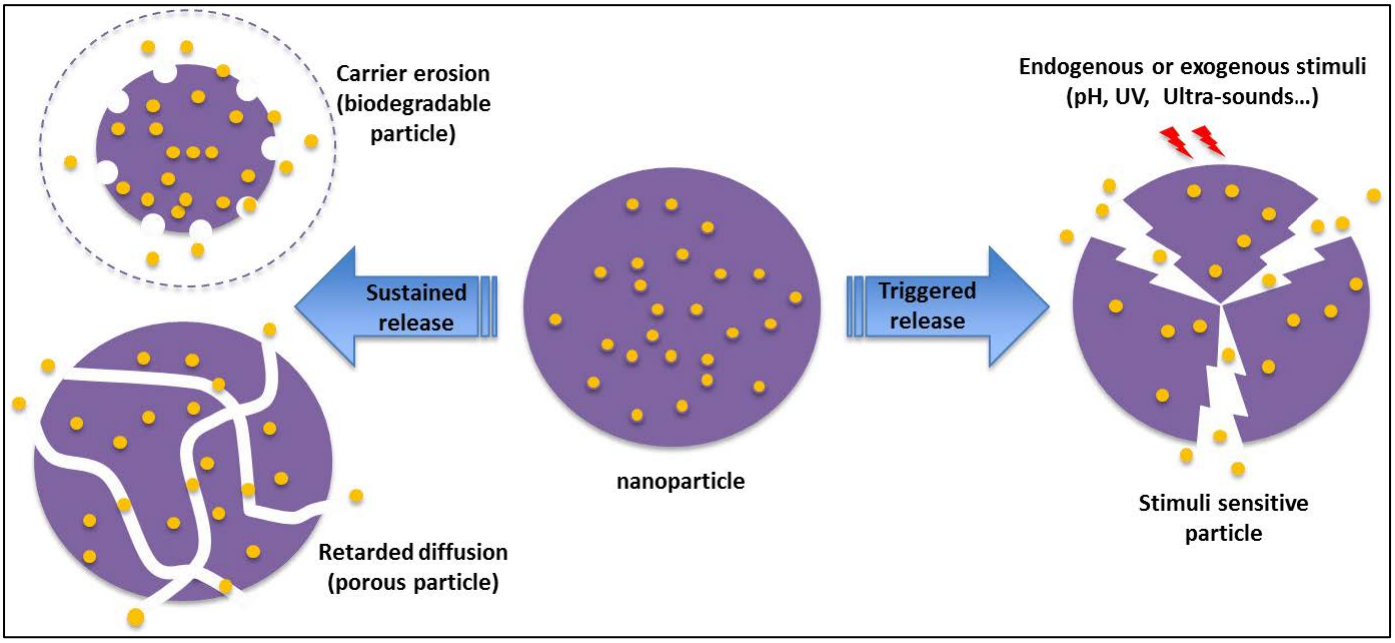


Figure 3: The two main modes of controlled release from carrier nanoparticles Sustained release can be operated by biodegradable carriers, most often polymeric, which are progressively eroded, or by porous (silica, polymer...) particles. Trigger-activated particles deliver their load at once, upon activation by an endogenous or exogenous trigger.

However, one has to keep in mind that complex formulations make complex toxicity and administrative regulation issues and until now, (almost) none of these "high-tech" nano-objects has made its way to the clinics.

2.2.3 Propensity to accumulate at tumor sites

One of the key advantages of using nanoparticles as compared to small molecular drugs or contrast agents is their propensity to accumulate at tumor sites, due to the abnormal properties of the vasculature at the interface between the tumor and the host. This phenomenon, known as the Enhanced Permeation and Retention (EPR) effect, has long been recognized and the use of nanoparticle formulations to enhance the accumulation of encapsulated drug into tumors is referred to as "passive" targeting [25–28]. It is generally admitted that tumor vessels exhibit intracellular gaps that are responsible for the extravasation of macromolecules and nanoparticles from the vasculature toward the tumor. However, other extravasation mechanisms such as intracellular vesicular transportation are also suspected but have not yet been investigated [29]. Other factors such as increased viscosity of the blood due to abnormal protein excretion and higher pressure in the blood vessels compared to the tumor tissues, favor the escape of the nanoparticles to the tumor intracellular medium. A recent review of the literature has demonstrated that tumor delivery is largely tumor dependent [29] but unfortunately, until now very few studies have focused on hematological malignancies, and there is a glaring lack of knowledge of nanomedicine penetration into such tumors.

2.3 LIMITS AND CHALLENGES OF CLINICAL APPLICATIONS OF NANOPARTICLES

A recent statistical review of the literature revealed that less than 1% (median value) of the nanoparticles injected systemically reaches their target, compromising their translation into clinical use [29]. Indeed, after intravenous injection, the nanoparticles have to pass several obstacles including clearance by the mononuclear phagocyte system (MPS, or reticuloendothelial system RES) and physiological barriers such as vascular membrane and tumor microenvironment. Indeed, once in the blood circulation, plasma proteins (opsonins) bind to nanoparticles triggering their uptake by phagocytic cells present in the blood, liver, spleen and bone marrow. This capture by the MPS not only competes with access to the nanoparticle target site, but also may lead to increased retention time with potentially toxic effects. If the particles circulate long enough to reach a solid tumor site, they may cross the vascular barrier, beneficiating from leaky vascularization. They will then have to migrate into the tumor microenvironment to reach the target malignant cells. Different tumor types, stages and sizes exhibit different tumor environments but it is generally admitted that smaller particles are able to explore the tumor, whereas the movement of larger ones is more restricted.

Except a few very specific circumstances, such as dysfunctions affecting the RES, where macrophage uptake is desirable, it is generally beneficial to apply camouflage strategies to avoid capture by the RES and increase the circulation time of particles. As a general rule, smaller particles, and particles with neutral surface charge show lower macrophage uptake [30]. The composition of the particles also affects their circulation time. Thus, several lipids such as sphingomyelin and phosphatidylinositol, limit liposomes uptake by macrophage [31]. The most ancient and still most common strategy is to coat the nanoparticles with neutral polymers, such as *polyethylene glycol* (PEG), polyvinylpyrrolidone (PVP) or more recently poly(oxozaline), with a direct correlation between the length and density of the polymer chains and circulation time [30]. Beyond mere "stealth" properties, many efforts were put on the development of particles with "active" targeting features, i.e. capable of selectively bind to specific cellular receptors. This could be a decisive advantage for the detection of small tumors not having a built up vasculature favoring the retention of nanoparticles through EPR, which is still one of the most challenging issues in the treatment of cancer. Particles grafted with targeting ligands, such as small molecules (folic acid, sugars…), peptides, aptamers, antibodies, generally show interaction with their target cells *in vitro*. However, the study of *in vivo* particle-cell interactions is still in his infancy and specific targeting effects still have to be demonstrated.

Thus, despite their numerous intrinsic advantages, the translation of nanoparticles into the clinical setting is still extremely challenging. One has to keep in mind all the aforementioned hurdles while designing new nanomedecine. In addition, besides mere clinical issues, the industrial and financial realities should not be forgotten. Nanomedicines should demonstrate clear advantages over existing molecular agents and their production price should meet the requirement of the market and of reimbursement policies [32].

3 NANOPARTICLES TARGETING CD19 SURFACE ANTIGEN

3.1 ANTIBODY-NANOPARTICULES CONJUGATES

Numerous molecules differing by their nature (peptides [33], aptamers [34], affibodies ((Blow, 2007 ; [35], etc.) and their size (antibodies, minibodies, diabodies, etc.) [36,37] have been proposed to achieve active targeted therapy or diagnosis and grafted to nanoparticles. [38,39] Among them, monoclonal antibodies (mAb) have long been considered as candidates of interest thanks to their high specificity and their long circulation time that can last several days. Studies have shown that the systemic clearance of antibody is inversely proportional to their size: the smaller the fragment, the faster it will be eliminated from the circulation,[37] process which is often associated with a higher renal toxicity. On the other hand, infiltration through the vascular tumor's network is inversely proportional to the size of the molecule: small molecules will often have lower penetrating-tumor rates. Thus, following the emergence of molecular engineering and the obtaining of different antibody formats, new specific

probes for the *in vivo* diagnosis and therapy of tumor have been developed, taking into account the size (pharmacokinetics and binding to nanoparticles strategy), the valency and the specificity (optimal signal/noise for imaging applications, and undesirable toxic effects) of the fragment conjugated to the nanocarrier and their profitability of production (low cost, high quantity, high stability). [36,37]

3.1.1 Choice of the fragment of antibody targeting the antigen

3.1.1.1 *Monoclonal antibody and its derivatives*

Monoclonal antibody (mAb) is composed of three fragments presented in Figure 4A: two identical variable fragments named Fab (VH and VL domains) and the constant fragment named Fc (CH and CL domains). Moreover, the advancement of molecular biology has allowed the engineering of fragments of antibodies of varying sizes and valencies, like single-chain fragment variable (scFv) fragments and chimeric constructions of scFv presented below and summarized in Figure 4A and 4B.

The variable fragment Fab (monomeric form of 55 kDa), its dimeric ($F(ab)_2$, 110 kDa) and its trimeric ($F(ab)_3$, 165 kDa) forms are easily produced by enzymatic digestion (papaïn, pepsin, trypsin, etc.) (Figure 4). They were the first antibody fragments used for *in vivo* diagnosis of tumors. The systemic clearance of the monomeric and the dimeric forms is reduced to about ten hours. The main disadvantage of the shortest monomeric format is its monovalency which diminishes its total affinity and thus reduces its tumor binding capacity. The $F(ab)_3$ format present a very short half-time (few hours) and a high specificity for its target because of its multivalency. Their lower cost of production constitutes a serious advantage.

The single-chain fragment variable (scFv) fragment of 25-30 kDa, consists of the light variable region and the heavy variable region. The two regions are connected by a peptide bond of 12 to 25 amino acids, generally with glycine and serine residues which provide flexibility and resistance to proteases. Representing one-sixth of the size of an entire antibody, scFv is the smallest functional unit among the different antibody formats (Figure 4), improving its tumor penetrating capacity compared to mAb. scFv retains the same specificity and affinity than the monoclonal antibody from which it is derived, but remains a monovalent fragment [40–42]. Because of its short systemic half-life <1 h, scFv fragments accumulate very little in tumors.[36]

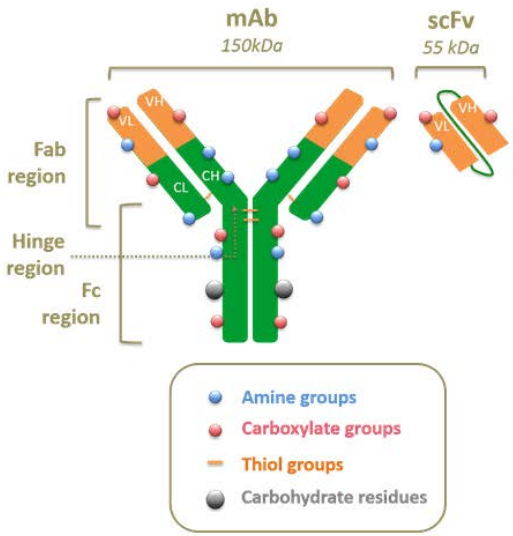
The Chimeras of scFv offer intermediate pharmacokinetics (Figure 4). Among them, two types of chimera were developed: A 75 kDa dimeric fragment, named Minibody (Quiocho F.A., 1993), corresponding to two monomeric scFv fragments connected *via* the third constant domain of a human immunoglobulin (scFv-CH3). *In vivo*, this format has a shorter systemic clearance and a faster tumor address. A 105 kDa dimeric fragment where the scFv fragment is bound to an entire Fc region of a human immunoglobulin. It behaves in the same way as whole mAb in terms of persistence in circulation and tumor penetration.

The Multimers of scFv*: diabody, triabody, tetrabody* (Figure 4) (Poljak 1994; Schoonjans et al., 2000; Zheng et al., 2017). A scFv molecule presenting a linkage of 3 to 12 residues associates with a second molecule to form a bivalent dimer named "diabody" (50 kDa). Reducing the linker length (< 3 residues) may force the association into trimers or tetramers forming a triabody (75 kDa) or a tetrabody (100 kDa) respectively. Being significantly larger than the monomer, these scFv multimers i) have an intermediate systemic clearance between that of the scFv and that of an entire mAb, ii) do not well penetrate tumors, and finally iii) have a great avidity for targeted cells, favoring their fixation. In addition to the mAb and their fragments, were discovered more recently the **Nanobody** proteins (about 15 kDa) in the serum of camels [43]. They were also identified later in all camelid species and in cartilaginous fish (Greenberg et al., 1995). These particular forms of antibody are devoid of light chain and of the constant domain (CH): their variable region is therefore reduced to a heavy variable region. One of the main advantages of this immunogenic protein is its low cost of production compared to mAb and its derivatives.

3.1.1.2 *Other alternative targeting molecules*

A new generation of probes for in vivo targeting relies on skeleton protein, which does not resemble to any conventional antibody or peptide, to which a binding function is added by molecular recombination. The two most studied examples are the affibodies and the DARPin.[44] The Affibody is a structure derived from protein A, the surface protein of bacteria Staphylococcus aureus which has the property of binding immunoglobulins. This small 7 kDa ligand is formed of three helices which possess an excellent binding property to a multitude of targets (Blow et al., 2007). This domain consists of 58 amino acids, 13 of which are randomized thereby generating a library of affibodies having varying binding specificities. The studies have demonstrated the potential of these ligands as an in vivo diagnostic probes for tumors. The injection of radiolabelled specific affibodies of a tumor biomarker makes it possible to localize the cancer mass with high contrast using SPECT or PET imaging.[45,46] The affibody format seems to be a promising in vivo tool, and is currently explored in preclinical and clinical settings. **The DARPin ligand** (Designed Ankyrin Repeat Protein) is a protein possessing a repetition of the ankyrin motif (Li et al., 2006). Specific DARPin ligands targeting a tumor biomarker have been efficiently produced for both *in vitro* diagnosis and *in vivo* tumor targeting (Tamaskovic et al., 2012). These molecules possess a great variability in terms of size, by multimerization or pegylation, and affinity, by point mutation.

A



B

Name	Size	Pharmacokinetics	Valency/ specificity		Strenghs & Weaknesses
mAb	~ 150 kDa			☺	High specificity/sensitivity towards their target
	up to 200 kDa depending on post-traductional modifications	long systemic clearance	Bivalent	☺	Approved by FDA
		half-time ~ few days to weeks	*monospecific*	☹	Costly to produce
				☹	Highly immunogenic
FAb	55 kDa			☺	Approved by FDA
		short systemic clearance	Monvalent	☺	Rapid tumor targeting
		half-time ~ 10 h	*monospecific*	☺	Less immunogenic than full mA
				☺	Improved tumor penetration comperd to the full mA
				☹	Low avidity (monovalency)
				☹	Renal toxicity (High renal uptake)
F(ab)$_2$	110 kDa			☺	Approved by FDA
		short systemic clearance	Bivalent	☺	Better avidity for the target than mAb
		half-time ~ 10 h	*bispecific*	☹	Renal toxicity (High renal uptake)
F(ab)$_3$	165 kDa			☺	High avidity for the target (multivalency)
		very short systemic clearance	Trivalent	☺	Improved tumor penetration
		half-time ~ 4-5 h	*trispecific*	☹	Renal toxicity (high uptake)
scFv	28 kDa			☺	Better tumor- penetrating capability
		ultra-short systemic clearance	Monovalent	☺	Less immunogenic than full mAb
		half-time > 1 h	*monospecific*	☹	Low functional avidity (monovalency)
				☹	Renal and hepatic toxicity (high uptake)
Minibody	75-105 kDa			☺	Shorter systemic clearance compared to mAb
		medium systemic clearance,	Bivalent	☺	Faster tumor addressing
		shorter than full mAb	*monospecific*	☺	Better therapeutic efficacy than mAb
				☺	High tumor uptake
				☹	Renal toxicity (high uptake)
Multimers of scFv			Monospecifc	☺	High avidity for the target (multivalency)
Diabody	50 kDa	intermediate systemic clearance, bet	*bivalent*	☺	Higher tumor uptake than full mAb
Triabody	75 kDa	*between that of the scFv and that of an entire mAb*	*trivalent*	☹	Do not well-penetrate tumors
Tetrabody	100 kDa		*tetravalent*	☹	Renal toxicity (high uptake)

Figure 4: Natural and engineered antibody formats, and functional groups available for covalent labeling or bioconjugation (A) Schematic representation of full monoclonal antibody (mAb) of 150 kDa and its scFv derivative of 55 kDa. Functional groups present on the antibodies and available for covalent labeling or bioconjugation are schematically represented (amine groups, carboxylate groups, thiol groups and carbohydrate residues). Fab: variable region; Fc region: constant region; VL: Variable Light chain; VH: Variable Heavy chain; CL: Constant Light chain; CH: Constant Heavy chain. (B) Comparison between mAb and its derivatives in terms of size, pharmacokinetics, valency/specificity and strengths/weaknesses

3.1.2 Chemical strategies for conjugation with antibodies

Briefly, three functional groups available on the antibodies or their fragment(s) and derivatives (Figure 4A) can be targeted for covalent labeling or bioconjugation, directly to nanoparticles and drugs, or indirectly *via* the use of linkers [47,48] which all present complementary functional groups at their surface:

(i) The primary amines $-NH_2$ of lysine residues, **or the carboxylate group** –COOH of aspartate and glutamate residues, which are abundant and well-distributed over the entire surface of the mAb (Figure 4A). The most popular conjugation method involves the use of NHS esters, like *N*-hydroxysuccinimide esters, and EDC (1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride for the formation of amide bonds. The complementary group $-NH_2$ or –COOH can be present or introduced to the surface of the nanoparticles. In the EDC/NHS coupling method, conjugation procedures will operate randomly to the antibody surface and inactivation can occur by the nonselective formation of amide bonds at the vicinity of the Fab region of antibody [49–51].

(ii) The thiol groups (-SH) found on cysteine residues and formed by selectively reducing S-S disulfide bonds in the hinge region of the antibody (Figure 4A). Free sulfohydril groups can also be obtained by maleimide activation of lysine residues using for instance SMCC (N-succinimidyl 4-(*N* maleimidomethyl)cyclohexane-1-carboxylate) or SPDP (N-succinimidyl 3-(2-pyridylthio)propionate).

(iii) The carbohydrate residues containing cis-diols, which can be activated by oxidation yielding aldehyde functions. Localized on the Fc region of the antibody (Figure 4A), they are usually quite abundant and often quite far from the epitope (but not always), lowering the risk of inactivation of the antibody after conjugation. As mentioned previously, the mAb and its fragments can be bioconjugated directly or indirectly to the nanoparticle surface, using the same chemistry succinctly described above. Thus, in order to prevent antibody inactivation, linkers can be introduced between the antibody and the nanoparticles.[47,52] Homobifunctional and heterobifunctional PEG derivatives are the most used linkers because of their biocompatibility, biodegradability, non-immunogenicity and non-toxicity (FDA-approved). Nguyen *et al.* compared two strategies to conjugate anti-CD19 to gold nanoparticles *via* activated PEG: The one step process using ortho pyridyldisulfide-polyethylene glycol-N-succinimidyl propionate (OPSS-PEG-NHS), was preferred to the two steps process where the antibodies were added to the purified nanoparticles (HS-PEG-COOH added to pegylated gold particles then exposed to PEG-SH. Indeed, the activation of the particles surface by ethyl dimethylaminopropyl carbodiimide and sulfoNHS chemistry) seemed to facilitate a well-protected and non-aggregating particle surface [53,11]. Finally, besides these classical methods, alternative strategies were proposed. For instance, the noncovalent conjugation of streptavidin/avidin coated nanoparticles and biotinylated antibodies can be used [51,54]. As an example, we can mention the work of Procko and co-workers [55] who produced a biotinylated-copolymer polyethylene glycol methacrylate copolymer for coupling to streptavidin-antiCD19.

3.2 IN VITRO AND IN VIVO IMAGING WITH ANTI-CD19 NANOPARTICLES

Few anti-CD19 grafted nanoparticles for *in vitro* imaging have been published so far. Nguyen *et al.* [53] designed pegylated SERS-gold (SERS= Surface Enhanced Raman Scattering) nanoparticles conjugated to human anti-CD19 antibody that target *in vitro* chronic lymphocytic leukemia (CLL) cells. The functional SERS nanoparticles were composed of a gold core onto which MGITC (Malachite Green Isothiocyanate), a reporter dye, was adsorbed. The signals were detected by dark-field microscopy and Raman spectrometry. The advantage of SERS nanoparticles over CD19 grafted fluorophores is their lack of interference with conventional fluorescent stains used in histology. Those patented pegylated SERS-gold nanoparticles conjugated to CD19 antibodies showed specific *in vitro* targeting CLL cells. [53,56] Another proof of Ramos B cells labeling through anti-CD19 mediator was demonstrated by Dong *et al.* by grafting an anti-CD19 antibody onto $Ag@SiO_2$ core-shell nanoparticles *via* non-covalent biotin-avidin binding [54]. In this study, the authors monitored the metal-enhanced fluorescence of a reporter (rhodamine B) adsorbed on the surface of the core-shell nanoparticles. To the best of our knowledge, anti-CD19-grafted-nanoparticles that would allow *in vivo* imaging (SERS, MRI, PET, CT, fluorescence tracking, etc.) have not been yet reported in mouse model or human.

3.3 THERAPY WITH ANTI-CD19 NANOPARTICLES

3.3.1 Chemotherapy: drug delivery

Nanoparticles decorated with anti-CD19 have already been reported as an efficient carrier for drug delivery (Table 2). Doxorubicin, an inhibitor of topoisomerase involved in DNA synthesis, is frequently the drug of choice for proof-of-concept as the cytotoxic effect of this drug is well demonstrated on B cells. An immunoliposome targeting B lymphocytes thanks to anti-CD19 antibody and containing doxorubicin showed, *in vitro*, a 6-fold more cytotoxic activity on B cells than non-targeted liposomes.[57] Similar results were observed *in vivo* with an improved survival of the mice injected with the anti-CD19-doxorubicin-liposome compared to non-targeted liposomes or free doxorubicin treatments.[57] Doxorubicin can also be encapsulated into block copolymer nanoparticles grafted with anti-CD19. The internalization of nanoparticles is mediated *via* the clathrin-dependent pathway suggesting that the grafting of anti-CD19 antibodies to nanoparticles did not alter the physiological internalization pathways of CD19. Doxorubicin loaded anti-CD19-grafted-nanoparticles induced more apoptosis of CD19 positive cells than the drug delivery alone *in vitro* and a better survival of the mice than those treated with saline or free doxorubicin *in vivo*.[58]

In 2007, Cheng *et al.* produced liposomal doxorubicin targeted *via* anti-CD19 monoclonal antibody fragments: either the single-chain variable fragment (scFv), or the variable fragment (Fab) or the monoclonal antibody (mAb). They compared the efficiency of the three targeting constructs and showed that the scFv single-chain variable fragment could be more suitable for development of immunotherapy for the following reasons: i) it contains less foreign peptides, ii) the production is easier, and ii) the cost of production is more economical thanks to the expression in bacterial systems.[59] *In vivo*, B cells xenografted mice exposed to anti-CD19-liposomes containing doxorubicin or vincristine were subjected to a higher cell cytotoxicity and showed a longer survival time than free drug mice.[60]. Anti-CD19-liposomes showed *in vitro* a greater binding, a more efficient internalization and an equivalent cytotoxicity on B cells compared to anti-CD20-liposomes.[60]

The encapsulation of other classes of drugs into CD-19 liposomial formulationa has been reported like the penta peptide mimic C61 (1,4-bis (9-*O*-dihydroquinidinyl) phthalazine/ hydroquinidine 1,4-phathalazinediyl diether) that was identified as a potent inhibitor of the cytoplasmic protein SYK (spleen tyrosine kinase): an important regulator of B cell apoptosis **(Table 2)**.

Myers *et al.* demonstrated that a liposomal nanoparticle formulation entrapping C61 and decorated with anti-CD19 caused *in vitro* the apoptosis of pre-B ALL cells, twice more than the non-decorated liposomes. [61] Immunocompromised NOD/SCID mice were xenografted with pre-B ALL cells, and injected with C61-liposome decorated with anti-CD19.
In those mice, tumor cells viability decreased and mice did not develop leukemic splenomegaly, showing a better therapeutic efficiency than irradiation with 2Gy γ-rays. In addition, the combination of C61 loaded anti-CD19-liposomal nanoparticles, with exposure to low dose of radiations, caused the abrogation of B leukemia in engrafted mice [61].

In another study, Yu *et al.* also proposed a dual ligand conjugation on immunoliposomes.[62] They first evaluated the level of expression of CD19, CD20 and CD37 antigens in several B cell lines and primary B-CLL cells and found comparable results for CD19 and CD37. They also calculated the internalization rate of the three antibodies in lymphoma cells (Raji cells) and confirmed the choice of anti-CD37 as the primary ligand for specific targeting of B cells. Then they measured by microarray assay, the binding efficiency of single or mixtures of anti-CD19, anti-CD20 and anti-CD37 B-CLL cells isolated from patients. At this point, the greater binding efficiency occurred with dual combinations of anti-CD19 and anti-CD20 with anti-CD37 antibody.

NP type	Composition	Targeting agent	Size (nm)	Application	Reference
Liposome	PEG-DSPE	anti-CD19	100-120	doxorubicin *carrier* *140-160 µg/µmol of phospholipid*	*Lopes de Menezes et al., 1998*
Liposome	HSPC/Chol/ mPEG-DSPE	anti-CD19	90-110	doxorubicin *carrier*	*Sapra and Allen, 2004*
Liposome	SM/Chol/ mPEG-DSPE	anti-CD19	110-130	vincristin *carrier*	*Sapra and Allen, 2004*
Liposome	$mPEG_{2000}$-DSPE	anti-CD19 hd37-cch fragment	80-120	doxorubicin *carrier*	*Cheng and Allen, 2008*
Liposome	EggPC/Chol/ PEG_{2000}-DSPE	anti-CD19 + anti-CD37 / anti-CD19 + anti-CD20 + anti-CD37	100	FTY720 *carrier*	*Yu et al.,2013*
Liposome	DSPE-PEG_{3400}-NHS	mouse anti-CD19	~135	C61 *carrier* *9,4 mg/mL*	*Myers et al., 2014*
Polymer NP	PEG-PLGA	anti-CD19 / anti-CD19 + anti-CD20	~300	hydroxychloroquine *carrier* *165 µg/mg of polymer*	*Mansilla et al., 2010*
Polymer NP	$EG_{113}CL_{152}TSU_{25}$	anti-CD19	~ 60	doxorubicin *carrier* *72,1+/-6,4 µg/mg of polymer*	*Krishnan et al., 2015*
Inorganic	Au@PEG	human anti-CD19	60-80	SERS *cell imaging* *MGITC = Raman tag*	*Nguyen et al., 2010*
Inorganic	Ag@SiO_2	anti-CD19	100-140	Fluorescence *cell imaging*	*Dong et al., 2014*
Inorganic	Iron oxide@dextran	pep-MHC-Ig dimer or anti-TCR-specific with anti-human CD19	~50	*Targeting* *Redirect T cells against tumor cells*	*Schütz et al., 2016*

Table 2: Nanoparticles (NP) grafted with anti-CD19 antibody and their applications. Abbreviation: Ag Silver; Au: Gold; Chol: Choline; DOTAP: 1,2-dioleoyl-3-trimethylammoniumpropane; DOPE: dioleoylphosphatidylethanolamine; DSPE: Distearoylphosphatidylethanolamine; EggPC: Egg yolk phosphatidylcholine; HD37-CCH: Hybridomas HD37-c-myc-Cys-His5 scFv; HSPC: hydrogenated soy phosphatidylcholine; LNP: liposomal nanoparticle ; MHC-Ig: Major Histocompatibility Complex-Immunoglobulin; NHS: N-hydroxysuccinimide; PEG: Polyethylene glycol; PLGA: poly(lactic-co-glycolic acid); SERS: Surface Enhanced Raman Scattering; SiO_2: Silicon dioxide ; SYK: Spleen Tyrosine Kinase; TCR: T cell receptor

The authors also optimized the antibodies ratio for the synthesis of immunoliposomes to increase the cellular uptake and take advantage of this synergetic effect. They chose a sphingosine analogue (FTY20) to determine the efficacy of payload mediated apoptosis induction and showed once again the better efficiency of double conjugated formulations. All multifunctional immunoliposomes exhibit higher selectivity as well as greater binding affinity ensuring enhanced apoptosis induction of different B-CLL cells.[63] The combination of several specific antibodies is also a promising strategy to overcome the variability in the expression of target antigens among patients.

In this context, hydroxychloroquine, an anti-malaria and anti-rheumatic drug has also been encapsulated in order to overcome pharmacokinetic obstacles and to deliver a larger amount of this apoptotic drug into B-CLL cells from patients. Mansilla *et al.* encapsulated hydroxychloroquine in commercial PEG-PLGA nanoparticles mono-functionalized by anti-CD19 antibody or bi-functionalized with anti-CD19 and anti-CD20 antibodies. [64] The authors measured a maximum apoptosis of B-CLL cells compared to normal B lymphocytes from healthy volunteers. At low concentrations the induction of apoptosis is respectively enhanced by a factor 4 and 5 in the case of mono and bi-functionalized nanoparticles in B-CLL cells compared to normal B lymphocytes. The authors showed a significant induction of apoptosis with mono- or bi-functionalized nanoparticles compared to non-functionalized nanoparticles and compared to non-drug loaded nanoparticles.

3.3.2 Gene therapy

Nanoparticles are excellent carriers to achieve gene delivery due to their loading capacity and properties facilitating cellular uptake. A variety of nucleic acids designed for different therapeutic strategies have been used in gene therapy: plasmid DNA, messenger RNA (mRNA), small interfering RNA (siRNA), microRNA (miRNA), and DNAzymes which can cleave mRNA molecules.[65] The siRNA, allowing downregulation of gene expression, are described as a new class of therapeutic agents which can be an alternative when drugs are inefficient. The siRNA is a short RNA of 20 to 25 nucleotide-long with a perfect complementarity to a target RNA which leads to its cleavage and degradation. Due to the enzymatic degradation of siRNA, its use in therapy remains challenging. Therefore, nanoparticles could protect siRNA from degradation and facilitate their cellular uptake.

To the best of our knowledge, anti-CD19-grafted-nanoparticles loaded with siRNA or with plasmid DNA have not been reported yet However, having shown the role of a splice variant of exon 12 in CD22 encoding CD22ΔE12 protein in aggressiveness of infant B-ALL cells, Uckun *et al.* designed a siRNA against CD22ΔE12 in a liposomal nanoformulation. The depletion of CD22ΔE12 mRNA and protein caused *in vitro* selective apoptotic cell death. [66] Their perspective was to use anti-CD19 on the nanoparticles to target B-precursor ALL cells.[66]7 Furthermore, nanoparticles allow the combination of gene materials and drugs to improve the anti-cancer effect. A combination of siRNA against CD22ΔE12 with a cocktail of four drugs (dexamethasone, pegaspargase, adriamycin and vincristine) at a dose conventionally used in therapy showed a better efficacy than the drugs alone on the reduction in colony count. [66,68]

3.3.3 Nanoparticle-based immunotherapy

An innovative strategy consists in using nanoparticles exposing antibodies to stimulate the production of lymphocytes or even bridge malignant cells to killer T cells **(see the graphical abstract)**. Thus, biocompatible iron-dextran paramagnetic particles (50–100 nm in diameter) and avidin-coated quantum dot nanocrystals, (~30 nm) coated with anti-CD28 (referred to as "artificial antigen presenting cells") were shown to activate the proliferation of cytotoxic T lymphocyte and enhance tumor rejection in a subcutaneous mouse melanoma model.[69] Also, Schütz *et al.* designed new nanoparticles termed antigen-specific T cells redirectors (ATR). The ATR nanoparticles were conjugated to two antibodies: an anti-TCR antibody and a human anti-CD19, and were able to provide a physical proximity between T cells and tumor cells. ATR were able to redirect T cells to kill tumor cells. [70] *In vivo* assays on mice with of lymphoma cells xenograft showed smaller tumors and an improved survival compared to control after intratumoral injection of T cells and ATR.[0]

4 CD19-TARGETED CHIMERIC ANTIGEN RECEPTOR (CAR) T CELLS IMMUNOTHERAPY

An alternative to nanoparticles for targeting tumor cells is to take advantage of other cells. For instance, for years, hematological neoplasms have been treated by hematopoietic stem cell transplantations. The goal is that transplanted allogeneic hematopoietic stem cells kill residual malignant cells by a graft-versus-tumor effect. This cell therapy approach, used to fight leukemia, lymphoma or myeloma, leads to remission or immune control of the malignancy; however, some patients relapse. On the other hand, many therapeutic approaches tend to modulate the immune response to eliminate tumor cells. Immunotherapy has marked the past years by generating extraordinary advances in clinical applications for cancer treatment. Cell immunotherapy harnesses the power of both cell therapy and immunotherapy. It has proven tremendous clinical progress in the past decade.[71] For the purpose of the review, we will focus on CD19 antibody-based cell immunotherapies that target B cell neoplasms.

4.1 IMMUNOTHERAPIES: ANTIBODY-BASED AND ADOPTIVE CELLULAR THERAPIES

4.1.1 The concept of chimeric antigen receptor T cells (CAR T): retargeting a cytolytic immune cell by genetic-modification to eliminate a tumor cell

T lymphocytes are cells that play a central role in cell-mediated immunity. Different subsets of T cells achieve cytolytic, regulatory or memory roles. Genetically retargeting T cells against tumor surface antigens to trigger cytotoxic mechanisms against malignant cells is one of the principles of adoptive cell therapy. Particularly, the engineering of T cells to express a chimeric antigen receptor (CAR) is the most common gene-modifying strategy that is being investigated. CARs are synthetic receptors that direct the genetically engineered T cells against tumor surface antigens, for instance CD19 antigen. Adoptive cell therapy using gene-modified T cells has emerged as an exciting therapeutic approach for the treatment of cancer [72,73,74].

4.1.2 Main biological challenges for an effective antibody-based adoptive cellular therapy

Conceptually, many challenges should be faced to achieve an *in vivo* therapeutic efficiency. The first one is that CAR T cells must be able to persist *in vivo*, and then undergo cellular expansion [75]. They also must be able to infiltrate tumor tissues (in case of solid tumors), then to engage their target antigen expressed on tumor cells, and finally, to exert their cytolytic, proliferative, and cytokine secretory activities within the tumor microenvironment to eliminate malignant cells (review in [76]). Adoptive T cell therapy with chimeric antigen receptor engineered T cells (CAR T cells) has shown substantial clinical results against B cell malignancies [72,73,74]. The fact that CAR T cell therapy approach has proven to be of some effectiveness across a range of hematological malignancies [77] may be partly explained by the choice of a relevant target antigen (for instance CD19) and by the fact that those malignancies reside in the natural sites that adoptively transferred T cells naturally invade (review in [78]).

4.1.3 The choice of a relevant target antigen: CD19 gene therapy

CD19 is a reliable target antigen for antibody-based therapy (review in [14]). It is a cell surface protein that is normally expressed from early pre-B cells until plasma cell differentiation and by many B cell malignancies (review in [79]). More than half of all CAR-modified T cell studies in hematological malignancies have targeted CD19 antigen (review in [76]). CD19-specific CAR T cells have demonstrated potent activity in B cell ALL and lymphomas including CLL and non-Hodgkin lymphoma [72,75,80,81,82,83] review in [76]).

4.1.4 The chimeric antigen receptor (CAR) confers to genetically-engineered T cell the ability to persist and expand in vivo and to exert cytolytic activity

4.1.4.1 *Design of chimeric antigen receptor*

The chimeric antigen receptor (CAR) is composed by two main modules: (i) an extracellular component that recognizes a cell surface protein (*e.g.* CD19) (this extracellular moiety is a single-chain variable fragment (scFv) derived from an antibody) linked to (ii) an intracellular component consisting in T cell signaling domains of the T cell receptor (e.g. CD37) including co-stimulatory domains (e.g. CD28, or 4-1BB) involved in T cell activation **(Figure 5)** (review in [76] and [5]). The extracellular component is responsible for redirecting T cell specifically to the human tumor antigen whereas the intracellular component sustains T cell activation supporting cell expansion and cytokine release resulting in cytolytic activity. Intense work is done to optimize each module: the extracellular component which acts as the target-binding domain of the CAR, the hinge region connecting extracellular and intracellular component [84], and the intracellular component for an effective T cell proliferation and differentiation to mature effector T cells. The successive generations of CD19 CAR T differ in the number and origin of intracellular co-stimulatory domains **(Figure 5)** (e.g. 4-1BB or CD28) [85,72,80,4].

4.1.4.2 *Mechanism of action of CAR T cells*

The binding of the anti-CD19 scFV to CD19 antigen of tumor cell surface (the resulting complex is named the immune synapse) sends a signal through the CAR to the effector T cell. This signal results in the activation of the T cell and in the release of soluble molecules, perforin, granzyme and pro-apoptotic ligands, that kill the tumor cells. Additionally, activated T cells secrete proinflammatory cytokines (*e.g.* interferon IFN-γ, and IL-2), amplifying the immune response,[86,5] and leading to CAR T cells expansion. The range of *in vivo* expansion of CAR-modified T cells has been reported between 100- to 10 000- fold.[75]

4.2 A PERSONALIZED MULTI-STEP PRODUCTION PROCESS

4.2.1 A multi-step manufacturing process

Because CAR T cells are autologous T cells (*i.e.* the T cells belong to the patient himself) and are genetically modified with a viral or non-viral vector to express a CAR, the production of CAR T cells is a complex clinical manufacturing process which is personalized for each patient. [87,76]

The multi-step production involves:

- leukapheresis where peripheral blood mononuclear cells are collected from patients
- lymphocyte separation where CD3 T cells are separated from other blood mononuclear cells
- T cell gene modification where collected T cells are transduced with retrovirus or lentivirus for CAR-encoding gene transfer [4]. Alternative non-viral gene transfers using the Sleeping Beauty system are in development [88]
- *in vitro* cell stimulation and expansion to obtain the required cell number for treatment
- quality control of CAR T cells prior to infusion assessing CAR expression, T cell purity, and sterility.

The manufacturing process fails in approximately 5–10% cases [81,82,72,76]. Manufacturing feasibility may be improved by enhancing expansion capacity [89]

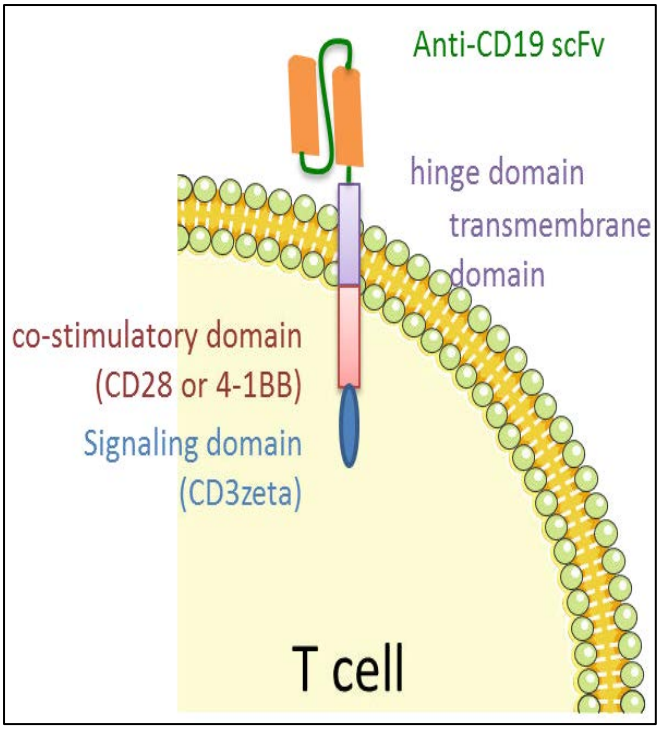


Figure 5: Chimeric antigen receptor (CAR). Chimeric antigen receptor (CAR) of second generation is composed of a targeting element (here the single chain variable fragment (scFv) of anti-CD19), a transmembrane domain, a co-stimulatory domain and a signaling domain

4.2.2 Infused doses

Infused doses to treat pediatric or adult B cell ALL, CLL and non-Hodgkin lymphoma range from 2.10^5 to 5.10^8 CAR T cells/kg [72; 75; 80; 81; 82; 83] (review in [4; 5]). Those studies have not identified a clear correlation between higher CAR T cell dose and greater efficacy or CAR T cell persistence. This may be explained by the fact that CAR T cells expand after infusion in response to their antigen, making the magnitude of their reactivity unpredictable [75]. The optimal CAR T cell dose to balance toxicity and efficacy remains under investigation [4].

4.2.3 Product composition

Different subsets of T cells achieve cytotoxic (CD8+ T cells), regulatory (CD4+ T cells), or memory roles (CD4+ T cells) and act synergistically. In CAR T therapy, CD4+ CAR T cells are responsible for cytokine production whereas CD8+ CAR T cells trigger direct antitumor effects. The ratio of CD4+/CD8+ CAR T cell subsets may be of importance in the balance between efficacy and toxicity.[4] In most reported trials, patients received T products comprising random compositions of CD4+ and CD8+ T cells. In contrast, Sommermeyer et al. and Turtle *et al.* showed that CAR T cell products generated from defined T cell subsets (1:1 ratio of CD4+ and CD8+ CAR T cells) can provide uniform potency compared with products derived from unselected T cells, even at the lower dose (2×10^5/kg) and induce complete remission without a high rate of toxicity in patients with a high tumor burden.[90,91] The optimal CAR T cell product composition to balance toxicity and efficacy remains under development.

4.3 CURRENT CLINICAL OUTCOMES, BENEFITS AND LIMITATIONS

4.3.1 Clinical outcomes

Many patients go into remission with standard chemotherapy for B cell malignancies. However, children and adults with relapsed or refractory B cell ALL have a poor prognosis. Substantial clinical efficacy has been demonstrated with a therapy based on CAR-modified T cells targeted to CD19. Approximately 70% of patients underwent complete or at least partial response to treatment with chimeric antigen receptor CAR-modified T cells targeted to CD19 [72-75,80-82]. Results are less impressive with CLL or with B cell non-Hodgkin lymphoma but still subsets of patients show significant benefits (review in [5]. Clinical trials are ongoing for multiple myeloma.

4.3.2 Advantages of CAR-modified T cells targeted to CD19 therapy to treat hematological malignancies

The following parameters have a positive effect on clinical outcomes of CD19 CAR T cell therapy in hematological malignancies.

(i) CAR T cells *in vivo* expansion and persistence is a clear determinant of clinical benefit [75,76].

(ii) The natural trafficking of CAR T cells within the blood, lymph nodes, and bone marrow where they encounter malignant cells also favors the efficacy of the therapy [76]. In those tissues, it appears that the inhibitory aspects of the tumor microenvironment are minor compared to solid tumors [5,78].

4.3.3 Limitations

Accumulation of data on patients treated from hematological malignancies with CD19 CAR T cell adoptive therapy enables the identification of the weaknesses and limitations of this approach.

4.3.3.1 *Genetic modification of autologous T cells*

First, each patient is infused with his own T cells. This specificity limits any large-scale manufacturing process and anticipated stocks. Then, autologous T cells are subjected to genetic modifications by retrovirus, lentivirus or non-viral gene transfer followed by *in vitro* stimulation. Currently, the complicated and individualized production of autologous CAR T cells may be one among others of the bottlenecks that reduce accessibility to this personalized therapy to many people. Suboptimal expression of the CAR at the surface of CAR T cells may also limit the benefit of CAR T cell therapies. Recently, Eyquem *et al.* have proposed that directing a CD19-specific CAR to the T cell receptor α constant (*TRAC*) locus not only results in uniform CAR expression in human peripheral blood T cells, but also enhances T cell potency, with edited cells vastly outperforming conventionally generated CAR T cells in a mouse model of ALL [92].

4.3.3.2 *Still under question: the need of lymphodepletion*

The purpose of chemotherapy, whose objective is to achieve lymphodepletion prior to CAR T cells infusion, is to create a more favorable environment for CAR T cells. Most studies

corroborated the notion that host lymphopenia (*i.e.* a low number of lymphocytes in the blood) facilitates expansion of adoptively transferred T cells. Whether lymphodepletion might further enhance the activity of allogeneic CAR T cells in this setting remains unclear [83,91]. To date, induction of lymphodepletion prior to infusion of CAR T cells continues to be often incorporated in clinical trials using CAR T cells.

4.3.3.3 *Clinical challenges: toxicity*

Then, the medical community will have to overcome clinical challenges related to CD19-targeted CAR T cells [5,4]. Major side-effects, particularly cytokine release syndrome, neurological toxicities, and B cell aplasia have been reported in all clinical trials using CD19-targeted CAR T cells. The cytokine release syndrome is a severe inflammatory response syndrome that appears within the hours to days following CAR T cell infusion. Clinical features include fevers, muscle pain, malaise, and, in more severe cases, hypoxia, hypotension, and occasionally renal dysfunction and coagulopathy. The cytokine release syndrome is characterized by elevation of pro-inflammatory cytokines (e.g. IL-6) and T cell activation and expansion. Tumor burden is positively correlated with the risks of severe cytokine release syndrome and neurotoxicity [74,91]. The cytokine release syndrome can be life-threatening and requires intensive supportive care. Mitigating strategies to reduce cytokine release syndrome frequency and severity comprise anti-IL-6 receptor antibody, steroids, and a protocol-specified algorithm to potentially start pre-emptive treatments [80,93,91,94]. Reversible neurologic toxicity has been observed after CAR T cell infusion, including delirium, seizure-like activity, confusion, word-finding difficulty, or aphasia. CD19-targeted CAR T cells therapy shows "on-target, off-tumor" toxicity that generates B cell aplasia [72,75,80]. Limiting B cell aplasia for CD19-targeted CAR T cells has been successfully managed with intravenous immunoglobulin replacement therapy [95]. Novel approaches to limit B cell aplasia are under investigation as the use of antigen-specific inhibitory CAR to protect normal B cells (Fedorov et al., 2013).

4.3.3.4 *Clinical challenges: CD19-antigen escape*

Loss of expression of the CD19-target antigen resulting in an antigen escape (e.g., CD19-negative relapse) may limit the benefit of CD19 CAR T cells therapy [75]. Tumor antigen escape has emerged as a main challenge for the long-term disease control (review in [96,97]). Studies are going on to understand the mechanism of loss of CD19 expression and overcome this difficulty. Braig *et al.* reported emergence of CD19-relapses due to CD19 mRNA splice variants [98]. Zah *et al.* reported the design of bispecific CARs that trigger robust cytotoxicity against target cells expressing either CD19 or CD20 and control both wild-type B cell lymphoma and CD19 mutants with equal *in vivo* efficiency [99].

4.3.3.5 *Other clinical challenges: controlling CAR T cell expansion and CAR T cell function*

CAR T cells have limitations associated with the lack of control over their activation and expansion *in vivo*. CAR T cells that target the CD19 receptor have been shown to proliferate in excess of 100,000-fold in some patients, ultimately accounting for over 50% of circulating lymphocytes. Poor T cell expansion may be limited by the use of defined CD4+/CD8+ CAR T cell subsets. On the contrary, approaches to limit expansion and activation are underway. Rodgers *et al.* propose a method to control CAR T cells using peptide-engrafted antibody-based molecular switches that act as a bridge between the target cell and CAR T cells [100].

5 PERSPECTIVES/DISCUSSION: HOW NANOPARTICLES AND CAR T CELL THERAPY COULD BE COMPLEMENTARY?

It is reasonable to conceive that nanoparticles could play a significant role for the potentiation of, and the cooperation with CAR T cell therapy. Their complementarity (in terms of function, distribution and time of administration) can be envisioned to fulfill at least three objectives: to track malignant and CAR T cells to monitor their biodistribution and expansion, to increase tumor accessibility and to manage CAR T cell toxicity.

5.1 TO TRACK MALIGNANT AND CAR T CELLS

Since the proof-of-concept of CAR T cells has been validated, current developments include the control of cell expansion or avoidance of CD19 escape. Therefore, in order to fully benefit from preclinical and clinical studies, there is a need for noninvasive tracking of the transfused T cells in patients to determine their biodistribution, viability, and functionality[101]. Several strategies may be proposed: either *ex vivo* preloaded nanoparticles on CAR T cells or *in vivo* administration of nanoparticles after CAR T cell infusion-nanoparticles serving as imaging or contrast agents. For instance, in mouse model, CAR T biodistribution has been monitored through radiolabeled-nanoparticles or contrast-agent-nanoparticles loaded into CAR T cells prior to cell infusion [102,103].Intrinsically, hematological malignant cells disseminate throughout the body. Detecting locations of cancer cells in the body is of particular importance in the case of hematologic cancers. In addition, *in situ* imaging alternatives to the invasive sampling of bone marrow are desirable for diagnosis and for residual disease follow-up. By proposing efficient targeting contrast agents, nanomedicine can greatly improve the diagnostic and localization of tumor cells (Kobayashi et al., 2005).

5.2 TO IMPROVE TUMOR ACCESSIBILITY

As mentioned above, one of the major limitations of cellular or nano-therapeutics is their poor mobility in the tumor microenvironment. Non-conventional strategies start to emerge, adding always more complexities to the therapeutics. One strategy is to make use of endogenous tumor environment stimulus, such as pH, redox, or hypoxia to trigger the release of the payload at the site of destruction[104]. Alternatively, nanosystems were designed to undergo *in situ* enzyme degradation reducing them to smaller units which can diffuse into the interstitial space. For instance, Wong *et al.* took advantages of extracellular metalloproteases to digest 100 nm gelatin particles

containing 10nm quantum dots. Indeed, metalloproteases are highly expressed at the sites of angiogenesis and at the invasive edges of tumors, where they cleave away the extracellular matrix opening routes for the tumor cells to invade the tissue. They demonstrated that after their release, the 10nm quantum dots can deeply penetrate into the tumor tissues. A variety of multistage drug delivery systems were also designed to promote tumor activated carrier-cell interactions, using triggered PEG detachment, targeting ligand emergence or charge reversal [104]. An alternative strategy, inspired by motile and invasive cells, is the active enzymatic degradation of the hindering matrix generated by protease associated with the therapeutic system. Thus, iron oxide nanoparticles coated with collagenase were magnetically driven through *in vitro* extracellular matrix, at a rate similar to invasive cells [105]. Other proteolytic surfaces include bromelain, an enzymatic complex belonging to the papain family and extracted from pineapple which contains a mixture of 9 proteases with distinct pH and enzymatic activities [106]. Local heating triggered by external sources has also been used to alter the tumoral environment and enhance delivery of nanomedicine, based on plasmonic nanoparticles such as gold nanoparticles [107,108]. Deeper penetration into tumoral tissues was also obtained *via* intracellular translocation. Thus, undecylenic acid conjugated porous silicon nanoparticles were internalized into tumor cells and subsequently excreted *via* lysosomal exocytosis, inducing a domino-like infection of adjacent cells. This mechanism was used to spread encapsulated doxorubicin deeply into both 3D-spheroid and *in vivo* tumor [109]. According to cancer type, hematological malignant cells originate from the bone marrow (*e.g.* leukemia, myeloma) or lymph node (*e.g.* lymphoma), and infiltrate all tissues including blood stream and solid tissues. Although hematological malignancies differ from solid tumors, some limitations of the CAR T therapy due to limited access to specific accumulation sites may be observed as well. Therefore, association of CAR T with tumoral tissue disruption can be envisaged to enhance the efficiency of the treatment. For instance, in mouse studies, Kennedy *et al.* used T cell as chaperones for gold nanoparticle delivery to enhance the efficacy of nanoparticle-based photothermal therapies and imaging applications by increasing gold nanoparticle tumor accumulation [110].

5.3 TO MANAGE TOXICITIES OF CAR T CELLS

Major toxicity such as severe cytokine release syndrome is intrinsically related to CAR T efficiency, and current developments tend to control it. Strategies to allow preferential CAR T cells removal include genetic "safety switch" or drug sensitivity[111]. In this perspective, nanoparticules could be specifically designed to target CAR T cell, making possible a selective apoptosis of those cells. Our group has recently reported on a vesicular platform, with a shell of inorganic nanoparticles named Hybridosomes® [112]. The large number of nanoparticles forming the shell is a clear advantage for imaging applications, since an enhanced contrast is observed. Initially designed for MRI, these Hybridosomes® can not only be prepared from iron oxide superparamagnetic nanoparticles but also from any types and combinations of inorganic particles with imaging or therapeutic properties. Therefore, they are suitable tools for multimodal imaging as well as theranostics. They can be prepared with a tunable size, in the range 60-100nm, which will drive them to accumulate around the leaky regions of tumor vasculature and also encapsulate drugs. Interestingly, the Hybridosomes® exhibit outstanding mechanical and colloidal stability over long periods of time (~year). It is clear that Hybridosomes® and several other nanomedecines can address many of the challenging issues of hematological cancer diagnosis and therapy. They can be functionalized with anti-CD19 to target tumor cells, and encapsulate anticancer drugs. Particularly, Hybridosomes® can easily be tailored to effect multistage delivery by releasing the individual nanoparticles in the tumor microenvironment, or functionalized with extracellular-matrix eating proteases to find their way through the tumor cells. Most interesting will be probably to combine CAR T and nanomedicine. Thus, an innovative strategy related to hematological diseases is the magnetic sorting of sick cells, after attachment of a magnetic particle. In some cases such as malaria, the intrinsic magnetic properties of infected cells even allow magnetic sorting of unlabeled cells [113]. This could be used to lower the tumoral load (lymphodepletion) before treatment or alternatively remove CAR T after treatment.

LIST OF ABBREVIATIONS

ALL: acute lymphoblastic leukemia
Ag: Silver
Au: Gold
BCR: B cell receptor
B-ALL: B cell acute lymphoblastic leukemia
CAR: chimeric antigen receptor,
CL: Constant Light chain;
CH: Constant Heavy chain
Chol: Choline
CLL : chronic lymphocytic leukemia
DOTAP: 1,2-dioleoyl-3-trimethylammoniumpropane
DOPE: dioleoylphosphatidylethanolamine
DSPE: Distearoylphosphatidylethanolamine
EC: European Commission
EDC: (1-ethyl-3-(3- dimethyl-aminopropyl)carbodiimide hydrochloride
EggPC: Egg yolk phosphatidylcholine
EPR: Enhanced Permeation and Retention
Fab: variable region
Fc region: constant region
FDA: US Food-and-Drug-Administration
IFNγ: interferon gamma
IL6: interleukin 6
HD37-CCH: Hybridomas HD37-c-myc-Cys-His5 scFv
HSPC: hydrogenated soy phosphatidylcholine
LNP: liposomal nanoparticle
mAb: monoclonal antibody
MGITC: Malachite Green Isothiocyanate
MHC-Ig: Major Histocompatibility Complex-Immunoglobulin
MPS: mononuclear phagocyte system
MRI: Magnetic Resonance Imaging
MRI/CT: magnetic resonance imaging/ computerized tomography

MRI/PET: magnetic resonance imaging/ positron emission tomography
NHS: N-hydroxysuccinimide
NP:nanoparticle
PEG: Polyethylene glycol
PLGA: poly(lactic-co-glycolic acid)
PVP: polyvinylpyrrolidone
QD: quantum dots
RES: reticuloendothelial system
SERS: Surface Enhanced Raman Scattering
SiO2: Silicon dioxide
siRNA:small interference RNA
SMCC: N-succinimidyl 4-(N maleimidomethyl)cyclohexane-1-carboxylate
SPDP: N-succinimidyl 3-(2-pyridylthio)propionate
SPECT: single, photon emission computed tomography
scFv: single-chain variable fragment
SYK: Spleen Tyrosine Kinase
TCR: T cell receptor
TEM: transmission electron microscopy
UCNPs: up-converting nanoparticles
VL: Variable Light chain
VH: Variable Heavy chain

ACKNOWLEDGEMENTS/FUNDING

This work is supported by SFR Biosit UMS CNRS 3480 - INSERM 018 (Call Interdisciplinary Project, SC, FG, MBT), Université de Rennes 1 (Call Scientific Challenge, SC, FG, MBT), French Ministry of Research (HJ, FS), the Sociétés d'Accélération du Transfert de Technologies Ouest Valorisation (SC, FG, FS), Ligue régionale contre le cancer (comity 22, 35, 56, 79, 41) (MBT), the Société française de lutte contre les cancers et les leucémies de l'enfant et de l'adolescent and the Fédération Enfants et Santé (MBT), a private donator Mrs. M-Dominique Blanc (MBT), the CNRS (MBT, FG), and the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7/2007-2013) under REA grant agreement n°291851 (MBT).

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3. Les Hybridosomes comme plateforme en nanomedecine

La nanomedecine est devenue depuis une dizaine d'année un champ multidisciplinaire pour porter les nanotechnologies vers les domaines de la santé et la médecine. Elle implique un grand nombre de disciplines scientifiques incluant la physique, la chimie, la biologie, l'engineering et les sciences médicales. Elle fait partie des domaines prometteurs pour les thématiques de santé de notre société puisqu'elle englobe bon nombre de problématiques comme le diagnostic, le traitement ainsi que la question du suivi des patients. De nombreuses questions sont encore à soulever pour amener la nanomedecine au-delà du transfert clinique. Qu'il s'agisse d'agents thérapeutiques, de diagnostic en imagerie, diagnostic *in vitro* ou encore appareil médicaux, les challenges sont nombreux pour franchir ces longues et coûteuses étapes précliniques puis cliniques.

Nous allons discuter dans cette partie de quelques challenges dans lesquels les Hybridosomes pourraient s'inscrire et apporter des solutions en tant que plateforme multifonctionnelle en nanomedecine.

3.1 Imagerie ciblée

La fonctionnalisation des Hybridosomes a été envisagée avec l'anticorps anti CD-19, marqueur des cellules du lignage B afin de cibler. Comme nous l'avons vu précédemment dans l'article 3, de très nombreuses stratégies de bioconjuguaison existent et présentent toutes avantages et inconvénients. Ainsi deux stratégies ont été envisagées : (i) fonctionalisation du PAA par l'anticorps puis assemblage des Hybridosomes avec le PAA fonctionnalisé, (ii) greffage de l'anticorps sur les Hybridosomes. Ces études de couplages ont été poursuivies par P. Martin au cours de son stage deuxième année de Master au sein de notre groupe.

La première stratégie de la modification du PAA n'a pas permis de conclure à la fonctionnalisation par l'anticorps avec les réactifs de couplace classique NHS/EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide / N-Hydroxysuccinimide). En effet, après avoir purifié le milieu réactionnel par dialyse dans un premier temps, puis par colonne échangeuse d'ions, la détection UV des différentes fractions n'a pas permis de mettre en évidence une nette séparation des PAA couplé et PAA non couplé. De plus, ni l'analyse par électrophorèse, ni par chromatographie d'exclusion stérique (SEC) de ces deux fractions n'ont permis de mettre en évidence une espèce de taille attendue supérieur au poids moléculaire du PAA.

Les études de stabilité des Hybridosomes vis-à-vis du pH présentées dans le chapitre 2 semblaient être favorables, puisque les Hybridosomes sont stables dans les conditions de réalisation du couplage via NHH/EDC (pH 4,5). Nous avonc donc également testé la seconde stratégie de en utilisant ces réactifs (Figure 42).

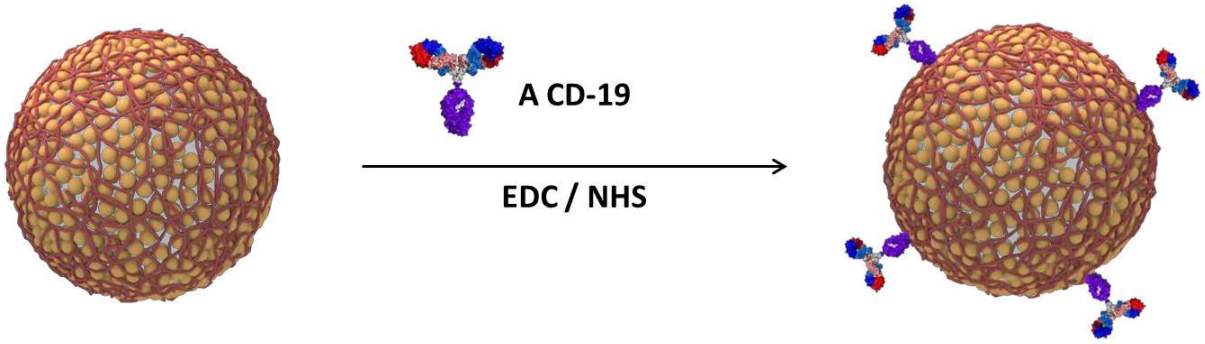

Figure 42: Stratégie de post-fonctionalisation des Hybridosomes par l'anti CD-19 par couplage peptidique classique EDC/NHS.

Les premiers résultats semblent montrer qu'un couplage à lieu. Des analyses en cytométrie de flux, à l'aide d'un anticorps secondaire fluorescent, semblent indiquer l'efficience du couplage sur les Hybridosomes. Ces résultats ont été obtenus par des analyses en cytométrie de flux, réalisées par D. Y. Danger (EFS Biosit), après marquage des Hybridosomes cnojugués par un anticorps secondaire fluorescent. Cependant, durant le couplage des problèmes d'agrégation et/ou de destructions des Hybridosomes ont été rencontrés, ce qui rend l'interprétation et la quantification du couplage encore compliqués.

D'autres stratégies de fonctionnalisation sont envisagées dans la suite de ce projet, dans le but d'avoir un meilleur contrôle des conditions de couplage, notamment des interactions acides / amines intra et inter espèces (Hybridosomes et anticorps). En ce sens, l'utilisation de bras espaceurs pourrait permettre d'accroître l'efficacité de la bioconjuguaison et de conserver la fonctionnalité de l'anticorps.

3.2 Vers d'autres applications

3.2.1. Potentialités en imagerie multimodale

Chaque technique d'imagerie préclinique ou clinique présente des résolutions différentes et apportent des informations complémentaire (fonctionnelles ou anatomique). De nombreux travaux visent à combiner des nanomatériaux pour former un agent de contraste actif dans plusieurs modes d'imagerie ce qui permettrait par exemple d'orienter un patient vers la technique d'imagerie la plus

facilement disponible ou encore d'imager les tissus mous (en IRM) en les positionnant par rapport au squelette (CT).

Les IONP font déjà partie de nombreuses solutions en imagerie multimodale, par exemple lorsqu'elles sont combinés à des oxydes de Gd (agents de contraste positifs avec des relaxivités longitudinales r_1) offrant des agents de contrastes polyvalents r_1-r_2 en IRM.[114] Par exemple, des oxydes de fer ont été combinés à des particules d'or pour la multimodalité IRM-CT,[115] à des radionucléides pour la multimodalité TEP-IRM (Tomographie par émission de positrons)[116] ou encore à des agents de contraste en imagerie à ultrasons.[117] Ces nanomatériaux hybrides offrent déjà des solutions très prometteuses dans l'imagerie multimodale.

Les particules à base de lanthanides apparaissent depuis quelques années comme très prometteuses également. En effet, les propriétés uniques des lanthanides offrent de nombreuses solutions en nanomédecine et les UCNP trouvent déjà de nombreuses applications en imagerie des rayons X[118], PET/SPECT[119], IRM[120] et en imagerie multimodale.[121]

Les Hybridosomes présentent la possibilité de combiner plusieurs types de nanocristaux et des Hybridosomes tri-fonctionnels ont été présentés dans le Chapitre 2. De manière simple, il est possible de combiner les propriétés d'un grand nombre de nanoparticules et les possibilités d'imagerie multimodale ne semblent pas limitées par le procédé. Certains développements proposent déjà des nanoparticules trimodales Ultrasons/CT/IRM,[122], PET/CT/Imagerie proche infrarouge,[123] SPECT/IRM/ Imagerie optique[124].

3.2.2. Promesses en thérapie

Les propriétés des nanomatériaux à base d'or, d'oxydes magnétiques, de lanthanides permettent aussi le développement de traitements en cancérologie. Les effets thérapeutiques se basent sur les réponses (en général élévation de la température) des différentes nanoparticules à différents rayonnements. Par exemple, sous l'application d'un champ magnétique, certaines nanoparticules magnétiques peuvent dégager de la chaleur et ainsi induire la mort cellulaire. Cette technique nommée hyperthermie a déjà montré des résultats très encourageants sur certains modèles de cancers.[125,126]

Les particules d'or présentent des propriétés radiosensibilisantes et peuvent elles aussi être utilisées en thérapie. Lorsqu'elles sont soumises à des rayonnements plus ou moins énergétiques, elles sont capables de produire des espèces oxygénées réactives (ROS) comme l'oxygène singulet $^1O_2$ ou triplet $^3O_2$.

Ces espèces sont responsables de dommages intracellulaires et peuvent induire l'apoptose cellulaire et la nécrose. On parle dans ce cas de thérapie photodynamique (PDT).[127] Grace à d'autres stimulis lumineux, des développements sont aussi prometteurs en thérapie photothermale (PTT)[128] comme pour les particules magnétiques. L'hyperthermie peut également être engendrée par des stimulis ultrasons ou micro-ondes.[129] Les UCNP présentent aussi ces caractéristiques et concentrent tout autant de développements depuis quelques années. [121,130]

Dans ce contexte, les Hybridosomes pourraient aussi apporter de nouvelles solutions et l'étude de leur réponse à différents stimuli mérite d'être explorée. Ainsi leur réponse à un champ magnétique, à une source lumineuse, à une source micro-onde ou à une source ultrasons pourrait produire des effets thermiques, générer des espèces réactives (ROS), ou encore génération de photons γ à partir des lanthanides. Encore une fois, l'universalité du procédé d'assemblage ne semble pas limiter les possibilités offertes par la multiplicité des nanoparticules utilisables.

3.2.3. Un long chemin vers des applications cliniques

Plusieurs « nanomédicaments » existent depuis des années et sont commercialement proposées. Ainsi, on retrouve les liposomes et les micelles de polymères comme principaux vecteurs de principes actifs contre le cancer et qui font leurs preuves depuis des dizaines d'années déjà.[131] Les liposomes sont aussi utilisés pour des applications antibiotiques et antifongiques grâce aux propriétés de l'amphotericine B par exemple.[132] Il existe également sur le marché des principes actifs couplés à des protéines comme le très connu placlitaxel[133] sont également utilisés dans le traitement des cancers du sein, du foie et du pancréas.

Concernant les nanoparticules inorganiques, aucune solution clinique en thérapie n'a encore vu le jour et obtenu l'approbation de la FDA synonyme de graal et de mise sur le marché. Seul deux cas se sont hissés aux stades de tests cliniques en phase II et III. Il s'agit d'un facteur de nécrose tumorale TNFα couplé à une particule d'or de 30 nm connue sous le nom de Aurimune (CYT-6091) et commercialisé par Cytimmune.[134] Le second, développé par Nanobiotix, repose sur un oxide d'Hafnium et poursuit actuellement des tests cliniques en phase III en radiothérapie. Plusieurs cas d'oxydes de fer ont cependant été approuvés au sein de l'union européenne en tant qu'agent de contraste et certains sont encore en cours d'évaluation clinique pour l'approbation de la part de la FDA. C'est le cas du Sienna+® commercialisé en Europe par Endomag et de Abdoscan approuvé seulement en Suède depuis 1993 mais qui n'est pas parvenu à s'étendre sur le globe.

Le principal bénéfice clinique attendu des nanomédicaments est la combinaison des atouts des particules en imagerie, en thérapie mais surtout en multithérapie. Cependant, les développements cliniques de nouveaux principes actifs sont toujours très coûteux et longs ce qui fait tendre vers l'utilisation de molécules déjà approuvées pour en améliorer les bénéfices cliniques. Leur nanoformulation peut améliorer leur délivrance, leur efficacité ou encore diminuer leur toxicité. Dans le but de maximiser ces bénéfices, les développements en théranostic pourraient tendre de plus en plus vers la multithérapie. Cet idéal peut être envisagé soit en combinant plusieurs agents de ciblages pour différents cancers, soit en utilisant plusieurs principes actifs (anticancéreux, antifongiques, etc) mais aussi en combinant plusieurs modalités d'imageries.

4. Conclusion

Nous avons discuté dans ce chapitre des potentialités de Hybridosomes en nanomédecine. Plusieurs points semblent encourageants dans le développement des Hybridosomes en tant que nanoplateforme pour le théranostic :

- Possibilité de combiner tout type de nanocristaux hydrophobes : imagerie, thérapie, diagnostique
- Capsules de taille ajustable 50 – 500 nm et sphères de taille de l'ordre du micron.
- Encapsulation de molécules hydrophobes
- Fonctionnalisation de surface par une ou plusieurs entités (biomolécules pour ciblage, radionucléides, etc)

Cependant nous avons évoqué les difficultés liées aux grand nombre d'études nécessaire en préalable à une application clinique et qui nécessitent donc des quantités importantes. A ce jour, les études présentées sur les Hybridosomes restent à l'échelle de quelques dizaines de µg et des efforts sont à mettre en œuvre dans la compréhension du procédé afin d'envisager une montée en l'échelle du procédé d'assemblage. De plus, la question du rendement est primordiale et n'a pas été abordée dans cette thèse. La synthèse des nanoparticules, les étapes d'assemblage et d'encapsulation, les étapes de couplage de biomolécules ou radiosensibilisants, ainsi que toutes les étapes de lavages et de stérilisation des objets finaux sont autant de points à adresser promettant encore de nombreuses perspectives de recherche et développement.

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Chapitre V

Vers l'élaboration de nouveaux nanomatériaux à base de clusters octaédriques de molybdène.

1. Contexte initial

Nous avons vu dans le chapitre 1 que plusieurs stratégies « template » permettent l'obtention de nanocapsules et nanospheres, dont la paroi est composée de particules. Nous avons aussi vu des cas d'assemblages ou les particules sont de taille nanométrique, micrométrique mais qu'il est aussi possible dans ces stratégies d'assembler des entités de taille moléculaire. Nous allons nous intéresser ici à la famille des clusters atomiques. Il s'agit d'un ensemble d'atomes ou de molécules qui ont une taille intermédiaire entre la molécule et le solide bulk. Introduit dans les années 1960 par F.A Cotton pour référencer les composés comportant des liaisons métal-métal (M-M), on parle également de clusters dans le cas des carboranes par exemple, pour les édifices composés de bore, carbone et hydrogène. Ces clusters métalliques peuvent présenter des propriétés magnétiques, optiques et red/ox.

Nous avons exploré le comportement de clusters métalliques au sein de l'interface liquide/liquide mise en évidence précédemment. Ce projet est issu d'une collaboration avec l'équipe Chimie du Solide Matériaux de l'ISCR, dont un des axes de recherche porte sur les clusters de métaux et intermétalliques. Les clusters de métaux de transitions forment des édifices de géométrie linéaire, triangulaire, tétraédrique et octaédriques lorsqu'ils comportent 2, 3, 4, ou 6 atomes respectivement. Dans cette étude nous nous sommes plus particulièrement intéressés aux clusters octaédriques de molybdène de type $\{Mo_6L_{14}\}^{2-}$. La nomenclature particulière permet de prendre en compte la structure octaédrique et la position des 14 ligands dans ce cas Figure 43.

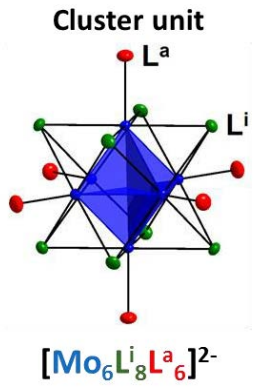

Figure 43: Unité de cluster octaedrique demolybdène de type $[Mo_6L^i_8L^a_6]^{2-}$.

Nous présentons ici un article en cours d'élaboration à propos des assemblages formés ainsi que des premières caractérisations obtenues.

2. Article 5 : Self-Assembly of A2[Mo6Li8La6] cluster into new hollow nanocapsules

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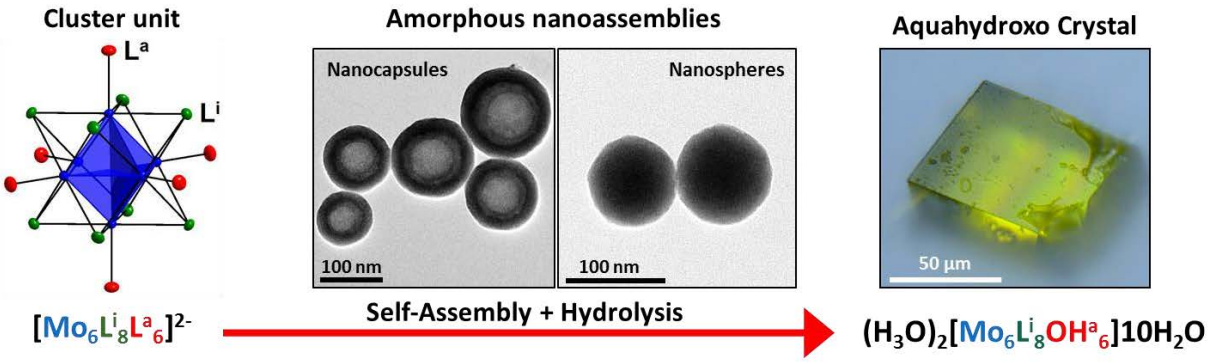

Self-Assembly of $A_2[Mo_6L^i_8L^a_6]$ cluster into new hollow nanocapsules

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INTRODUCTION

Octahedral Mo_6 nanoclusters are known as robust species with various properties: magnetic, electronic and luminescent which confer them attractivity in the development of light energy conversion), optoelectronic, lighting or theranostic applications. The $[Mo_6L^i_8L^a_6]^{2-}$ clusters units (where L^i is the inner ligand and L^a is the apical ligand) constitutes the basic building block in Mo_6 octahedral cluster chemistry. Thanks to their versatility, Mo_6 clusters offer plenty of possibilities as building block entities in order to reach nanoarchitectonics materials for several applications.[1] They have been used as clustomesogen entities in the field of liquid crystals.[2] The $\{Mo_6X_8\}^{m+}$ core has been covalently functionalized either with a variety of ligands such as carboxylate and phosphine in order to modify and study their behavior as liquid crystals.[3] Such new developments take the advantage of the iono-covalent behavior of the M-L^a bond that enable the functionalization with organic moieties by ligand exchange without any modification of the $\{Mo_6L_8\}^{m+}$ core.[4,5] Clusters have also been encapsulated into silica shell leading to multifunctional core@shell materials when combined to luminescent zinc oxide nanocrystals, plasmonic gold or magnetic iron oxide nanoparticles.[6–8] However, their use in the development of nanoarchitectonics materials often requires several steps either to functionalize the building block or to prepare its encapsulation into silica or polymer matrix.[9]

Molecular molybdenum clusters are of great interest for their catalytic performances regarding water splitting and hydrogen production. Special attention has been paid so far to Mo sulfide MoS_x because of its high stability and excellent electrocatalytic properties.[10, 11] More recently, new catalytic species such as Mo oxides MoO_x[12], and Mo nitrides MoN_x, gained significant attention, as it has been shown that they can exhibit strong catalytic properties regarding HER. More recently, $[Mo_6L^i_8L^a_6]^{2-}$ clusters units have been grafted on graphene oxide surface and showed interesting stability and efficiency toward Hydrogen Evolution Reaction (HER).[13]

In a previous work we have introduced a new process to assemble several inorganic nanocrystals.[14] We evidenced

that solvent droplets in miscible mixtures can template the self-assembly of iron oxide, quantum dots or gold nanoparticle. Along this process, nanoparticles with sizes from 5 to 10 nm, shown properties to self-assemble into soft hybrids nanocapsules.[15] In the present work, we investigate the behavior of Mo_6 clusters in miscible mixture in order to reach new architectures.

Proof of concept

We hypothesized that molybdenum octahedral clusters units could also self-assemble in THF/Water mixtures to form vesicular structures using the same process. Clusters dispersed in THF were simply diluted with water. The system is let to mature for 24h before eating at 40°C for 15h allowing solvent evaporation. TEM analyses were performed after this step. Following this procedure the system goes to the self-assembly of metal clusters. We observe either nanocapsules with $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ (Figure 1a) or nanospheres with $Cs_2[Mo_6Br^i_8Br^a_6]$ (Figure 1b). Other $A_2[Mo_6L^i_8L^a_6]$ compounds were engaged in the process and similar spherical structures were observed for $(NH_4)_2[Mo_6Br^i_8F^a_6]$ (Figure S1a1 and a2) and $(NH_4)_2[Mo_6Br^i_8Br^a_6]$ (Figure S1c1 and c2). However, such observations were made at an early stage of the process, during the maturation time, precisely after 2h and 10h respectively. Figure S1b and d shows that crystals are also present in the samples. It also advocates different kinetics reactions either in the self-assembling and the eventual crystallization phenomenon and confirms that different phenomenon occurs which render the monitoring complicated. We also performed others experiments changing several conditions of the process such as maturation and heating times, temperature of heating and conditions of pressure by opening or closing the reactor during the heating step. The TEM observations are presented Figure S5 and Figure S6. We will try to propose a growth mechanism based on these studies later on.

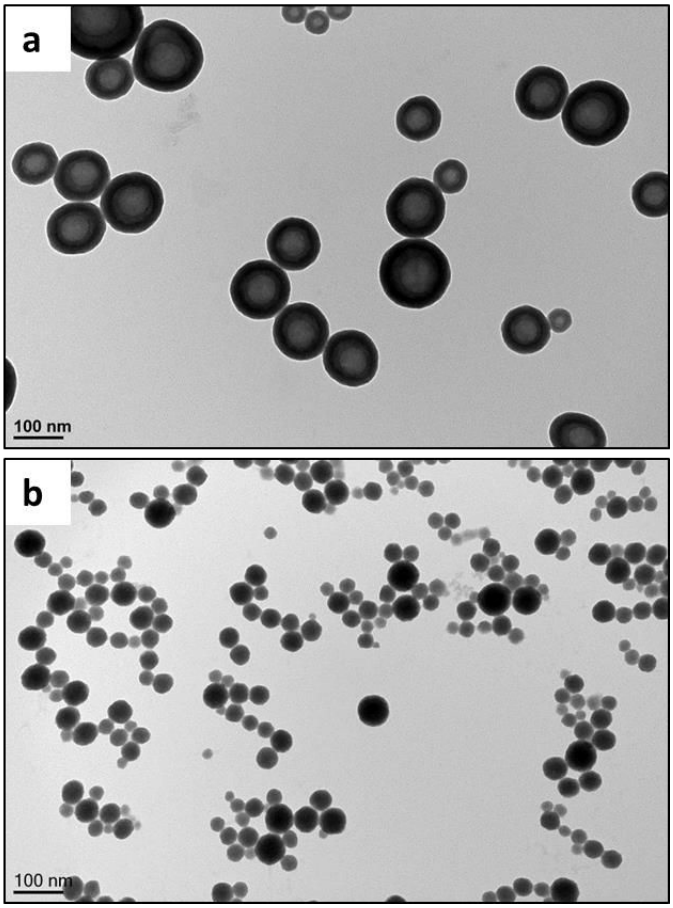

Figure 1: TEM micrograph of nanoassemblies obtained after 24 h maturation at RT and 15h at 40 °C from (a) $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ and (b) $Cs_2[Mo_6Br^i_8Br^a_6]$.

In this context, in order to simplify the monitoring of self-assembling, we decided to heat up the suspensions at 40°C maintaining the reactor closed, right after the addition of water. From this reaction time, noted T_0, we performed DLS measurements (Figure 2) for up to 50h (Figure 3a).

DLS measurements at T_0 (Figure 2) evidence both the $[Mo_6Br^i_8(NCS)^a_6]^{2-}$ cluster unit and droplets in the suspension. However, important scattering contrast within the suspension between clusters and droplets makes DLS observations difficult to process along the self-assembly. DLS measurements evidence an increase of the $Z_{average}$ from 150 nm at T_0 up to a plateau at 275 nm reached after T_{0+18h}. Based on our previous work and DLS measurements in Figure 2, $Z_{average}$ value of 150 nm could correspond to droplets structures in THF/Water mixture.

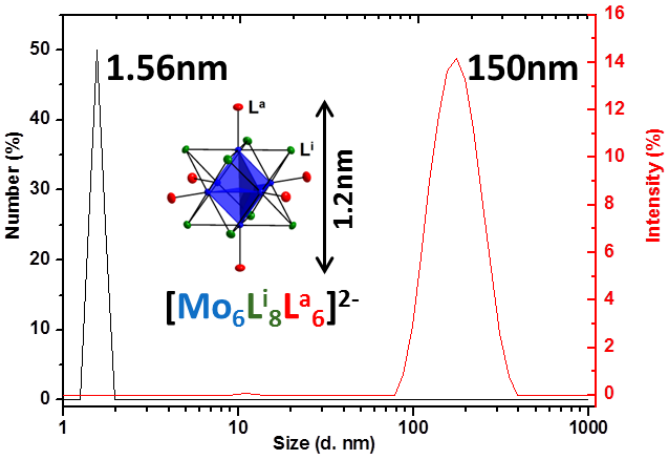

Figure 2: DLS measurements of $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ in THF/Water mixture at T_0.

During the process we performed TEM analysis of the suspensions at T_0 (Figure 3b), T_{0+10h} (Figure 3c) and T_{0+30h} (Figure 3d). At the early stage of the process T_0, TEM micrographs show numerous vesicular structures of around 100 nm (Figure 3b). Observations also shows aggregates with a size of around 25 nm, agglomerated on TEM grids (Figure S2a).

At a later stage T_{0+10h}, we continue to observe both kinds of structures (Figure 3c center): the Figure 3c left zoom shows dark rough aggregates with sizes between 60-100nm and Figure 4c right zoom shows vesicular structures Vesicular structures appeared deflated thin nanocapsules as shown in the right inset. After 30 h, we clearly evidence nanocapsules with a diameter of around 250 nm and a thickness of 40 nm (Figure 3d). Altogether these results suggest that two populations of structures are growing simultaneously which is supported by observations at T_{0+10h}, of intermediates nanocapsules between aggregates and capsules (Figure S2b).

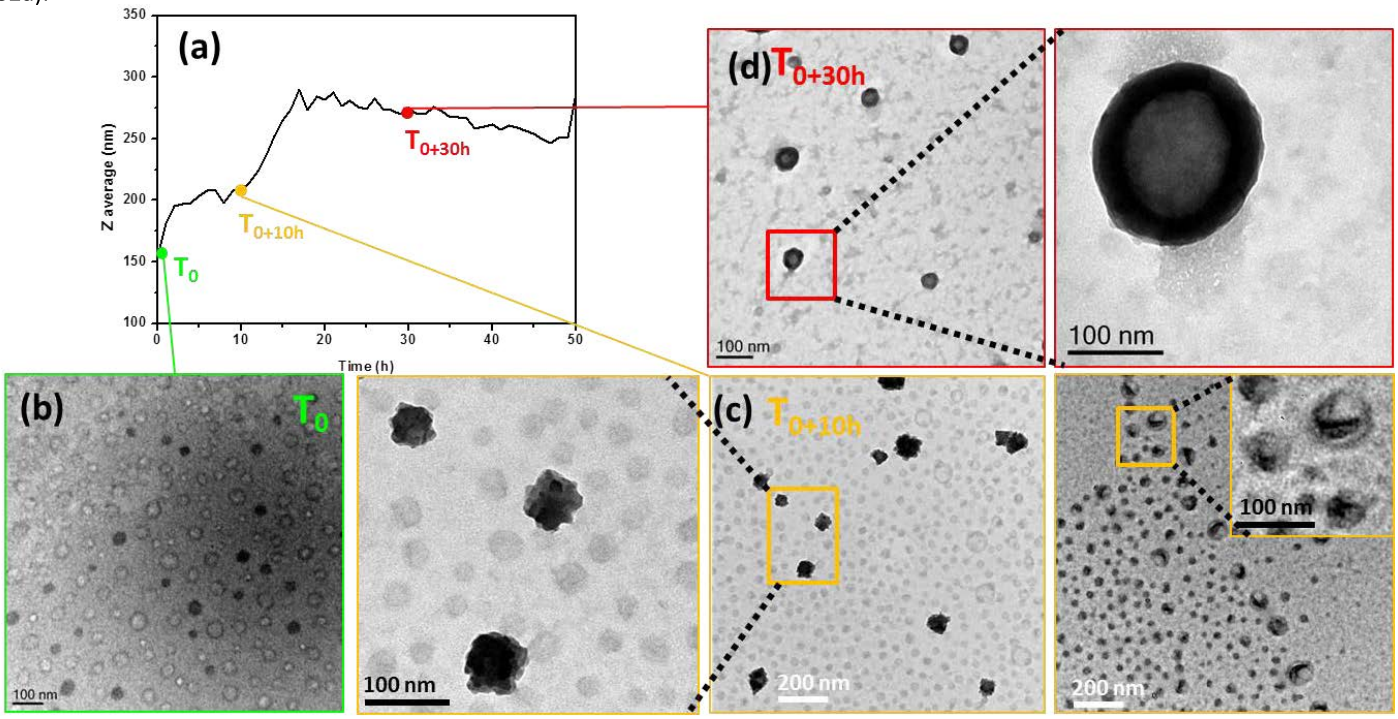

Figure 3: Growth monitoring of nanocapsules obtained from $(NH_4)_2Mo_6Br^i_8NCS^a_6$ at 40°C. (a) Monitoring of $Z_{average}$ of the suspension obtained by DLS in function of time and TEM micrographs of the suspension at (b) T_0, (c) T_{0+10h} and (d) T_{0+30h}.

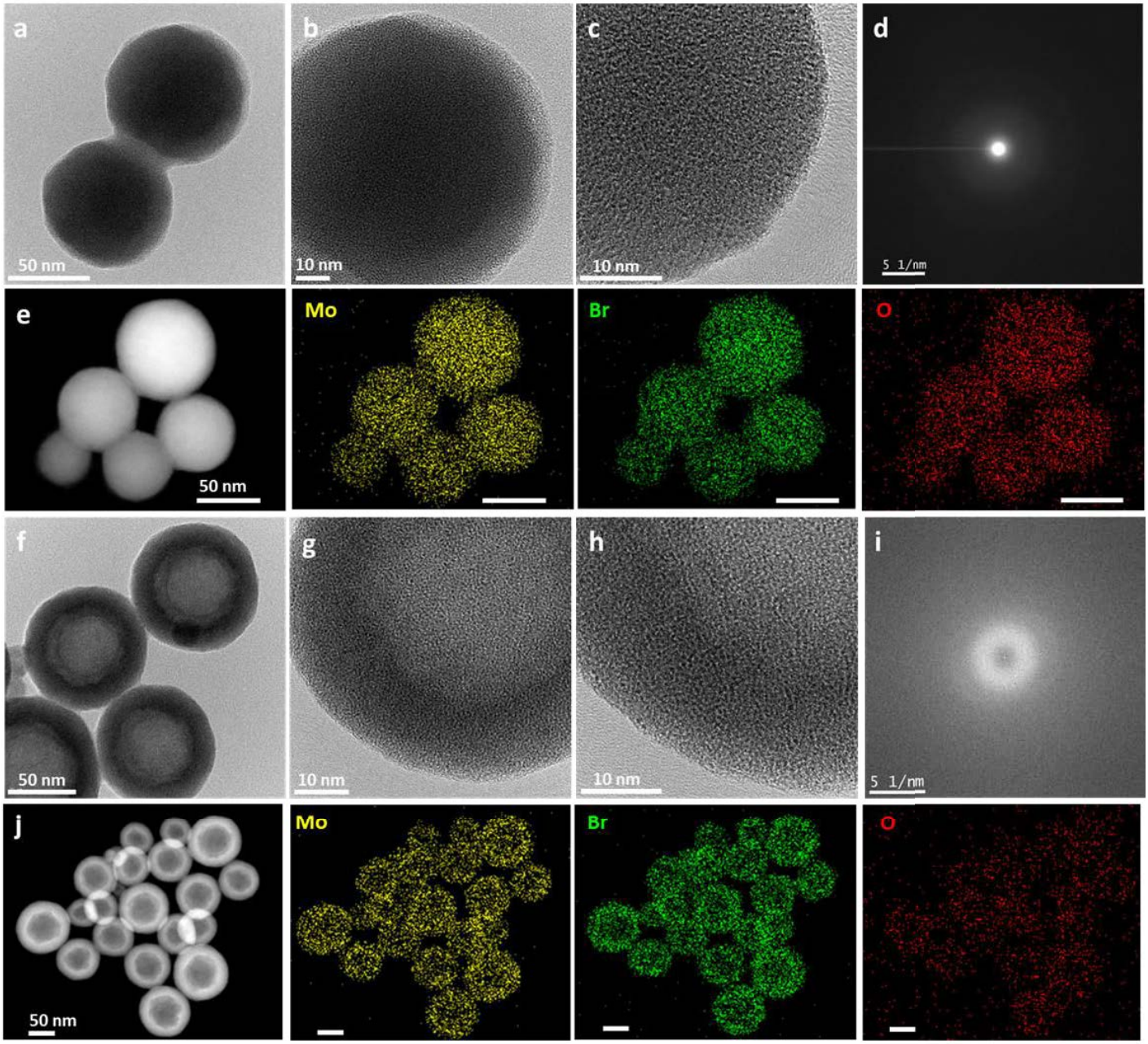

Figure 4: High resolution TEM and chemical mapping of Nanospheres and Nanocapsules. (a, b, c) High resolution TEM micrographs of nanospheres, (d) Diffraction pattern of nanospheres, (e) STEM-EDS elemental mapping of nanospheres, (f, g, h) High resolution TEM micrographs of nanocapsules, (i) Diffraction pattern of nanocapsules, (j) STEM-EDS elemental mapping of nanocapsules

The observation of nanocapsules seems to confirm the interfacial self-assembly of $[Mo_6Br^i_8(NCS)^a_6]^{2-}$ cluster unit as suggested in our previous work with nanoparticles.[14] From these observations we suggested the following mechanism in the case of $[Mo_6Br^i_8(NCS)^a_6]^{2-}$ unit: At the early stage (T_0), cluster unit self-assemble into spherical structures, probably at an interface, as suggested by numerous nanocapsules observed Figure 3c. At the same time (T_0), the clusters units also aggregate into small aggregates that agglomerate on the TEM grid upon drying as shows Figure S2a. Over time, both structures mature. On the one hand, interfacial assembling continues leading to the thickering of the nanocapsules (Figure 3c right zoom) (T_{0+10h}). On the other hand, the cluster units aggregates which are observed at the early stage T_0 (Figure S2a) continues to feed the growth of raspberries aggregates (Figure 3c and Figure S2a) (T_{0+10h}).

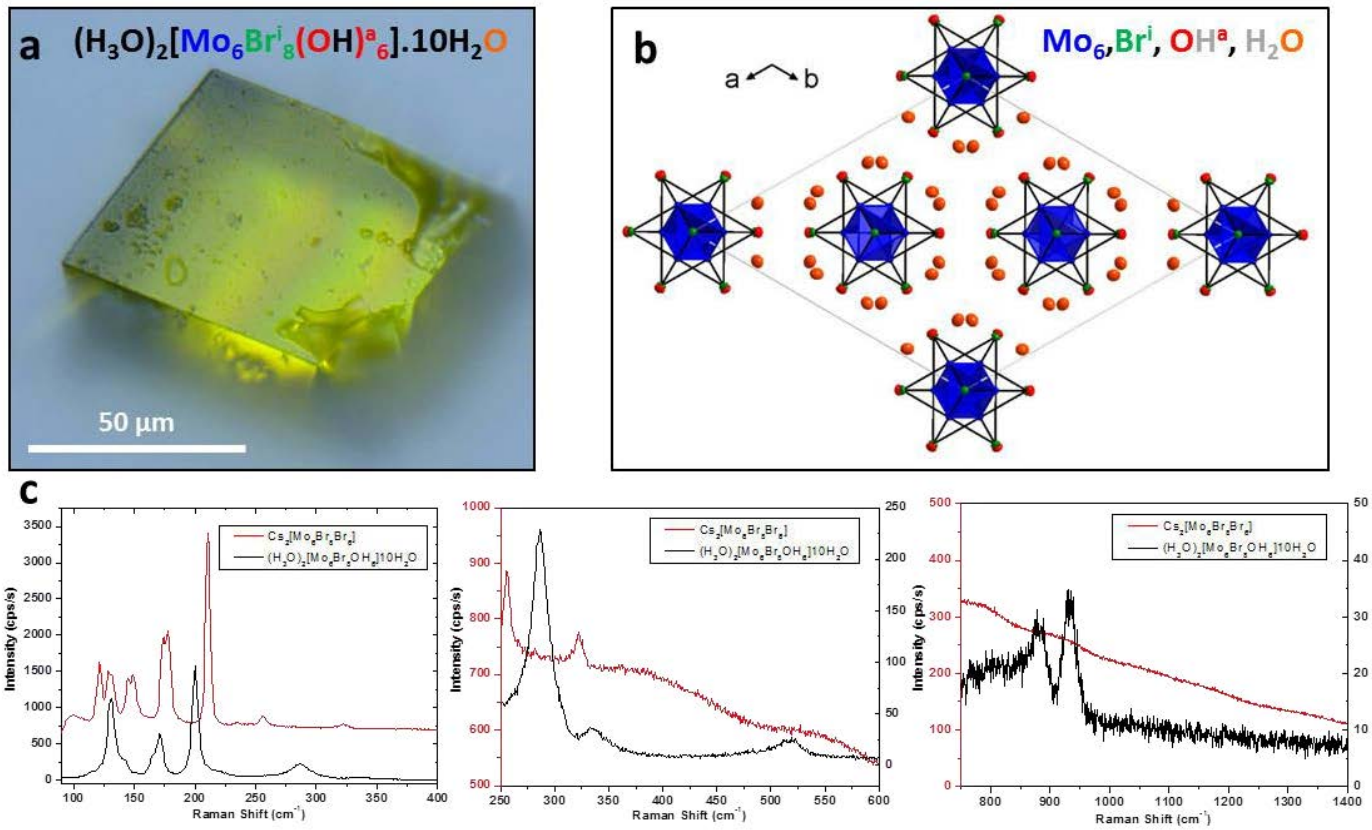

Figure 5: Aqua/Hydroxo Crystal Characterization. (a) Optical Microscopy of $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$, (b) Projection obtained from RX structure, (c) Raman Spectra of $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$.

Finally, observations made after T_{0+10h} only show some larger nanocapsules Figure 3d (T_{0+30h}). Cluster raspberries aggregates observed at the early stages disappear, probably at the advantage of nanocapsules. This might be due to Ostwald ripening. This is supported by several other observations at T_{0+24h}, T_{0+34h}, T_{0+47h} (Figure S2c-e). DLS measurements in function of time confirm the growth phenomenon (Figure 3a).

High resolution TEM (HRTEM) analysis were specifically performed on the typical nanospheres and nanocapsules presented obtained from $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ and $Cs_2[Mo_6Br^i_8Br^a_6]$ respectively. Figure 4a-c and f-g indicates the amorphous constitution of the nanospheres (Figure 4b an c) and nanocapsules (Figure 4g and h). These observations are supported by the complete absence of diffraction pattern in SAED images Figure 4d and i. From STEM-EDS element mapping Figure 4e1-e4, we clearly evidence that Mo, Br and O are uniformly distributed within the nanosphere. The colocalization of Mo, Br and O is also shown in the case of nanocapsules Figure 4j1-j4. Therefore, STEM-EDS element mapping confirm the presence of oxygen within nanoassemblies and strongly suggests the hydrolysis of molybdenum cluster. Nevertheless, the EDS chemical mapping does not succeed to quantify the oxygen amount. Hence, the precise chemical composition of the compounds forming the spheres/capsules cannot be determined at this stage.

It is important to mention that the stability of the clusters in aqueous solutions is still not completely understood. However, the substitution of apical ligands (L^a) by hydroxyl groups, or water molecules has been confirmed.[16] Thus, many hydrates,[17] hydrolysed molybdenum[18] clusters and aquahydroxo crystals have been reported.[13] Interestingly, the crystallization phenomenon suggested from Figure S1b and d led to the growth of microscopic crystals, during the self-assembly of $(NH_4)_2[Mo_6Br^i_8F^a_6]$, $(NH_4)_2[Mo_6Br^i_8Br^a_6]$ and $Cs_2[Mo_6Br^i_8Br^a_6]$ (Figure 5a).

The crystal structure was determined by single crystal X-ray measurements and confirms the crystallization of the well define aquahydroxo complex $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ in R-3m space (Table S1) as previously reported.[13]

Thus, in the crystal all apical Br^a ligands are substituted by OH ligands forming the $[Mo_6Br^i_8(OH)^a_6]^{2-}$ units. The new Mo-O bond distances are in adequation with previous work (Table S2).[13] The crystalline structure is maintained by water of crystallization, thus creating a network with hydroxyl ligands through H-bonding as shown in Figure 5b. The crystalline compound $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ may exhibit interesting proton conductivity properties which are under investigation

.

To better understand the chemical transformation during the process, Raman spectroscopy studies were performed. Only a few studies have been devoted to the vibrational spectroscopy investigation of $A_2[Mo_6L^i_8L^a_6]$ compounds. $[Mo_6L^i_8L^a_6]$ clusters units belong to the O_h point group, and the selection rules allow ten vibration modes in Raman.[19] It is important to note that the symmetry may be degraded by the environment of the complexes in crystalline or amorphous structures and more than the predicted number of vibrations may be observed. In our case, the expected modes correspond to breathing motions of the Mo_6 cage, L^i_8 face-bridging cube and apical L^a_6 ligands. The complete attribution for the cluster compounds $Cs_2[Mo_6Br^i_8Br^a_6]$ and $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ is presented Figure S3.

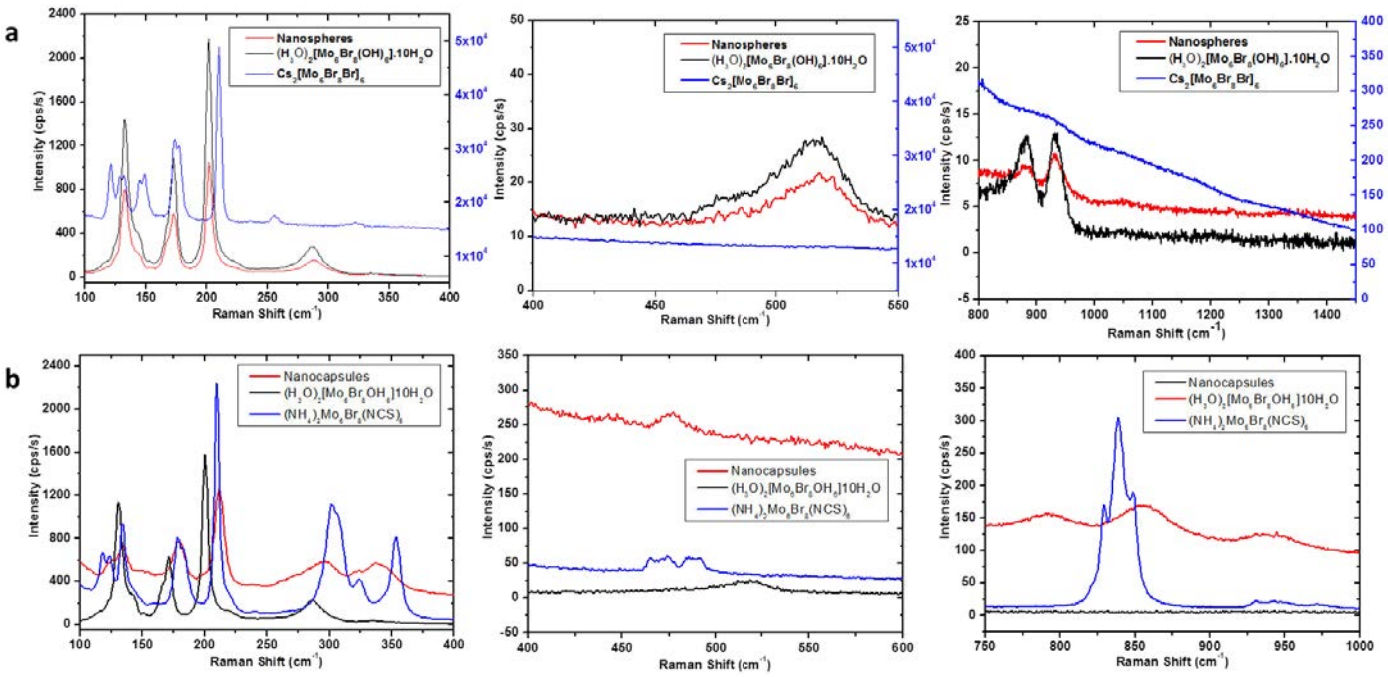

Figure 6: Raman characterization of the precursors, nanocapsules, nanospheres and aquahydroxo crystal. (a) Raman spectra of $Cs_2[Mo_6Br^i_8Br^a_6]$ (blue line), $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ (black line) and nanospheres (red line), (b) Raman spectra of $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$ (blue line), $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ (black line) and nanocapsules (red line).

The main vibration modes are observed for $Cs_2[Mo_6Br^i_8Br^a_6]$ precursor with characteristic bands at 121, 129, 132, 145/149, 174/178, 210, 256 and 322cm^{-1}. Bands at 121 cm^{-1}, 129 cm^{-1} and 322 cm^{-1} are attributed to Mo_6 cage, bands at 132 cm^{-1}, 174/178 cm^{-1} and 210 cm^{-1} are attributed to Mo-Br^i_8 vibrations, and finally bands at 145/149 to the Mo-Br^a_6 bonds in line with previous results.[19] In the case of $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$ three vibration bands corresponding to the Mo_6 cage (118 cm^{-1}, 124 cm^{-1} and, 323 cm^{-1}) and the three vibrations bands for Mo-Br^i_8 bonds (134 cm^{-1}, 178/183 cm^{-1} and 210 cm^{-1}) are observed. However, the bands at 301 cm^{-1} and 353 cm^{-1} are attributed to typical vibrations of Mo-N bonds.[20] The deformation vibration of the C≡N bond in isothiocyanate groups is responsible for the broad band in the range of 460-490 cm^{-1} and its second order at 950 cm^{-1} (Figure S3b). A third area shows the typical stretching vibrations of C-S with strong multiple bands at 829 cm^{-1}, 839 cm^{-1}, 849 cm^{-1}. Finally, the two bands at 2055 cm^{-1} and 2071 cm^{-1} are typically correlated to the stretching vibrations of C≡N bond in isothiocyanate groups. All these attributions are in good agreement with previously reported characterization of $(n\text{-}Bu_4)_2[Mo_6Br^i_8NCS^a_6]$.[20]

In order to further investigate the hydrolysis of apical ligands, we attempted the Raman study of the crystal compound $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$, presented Figure 5c. In comparison to the precursor, we observe the disappearance of the band at 149 cm^{-1}, corresponding to the Mo-Br^a_6 vibration which is not expected anymore. This trend is correlated with the emergence of new bands related to the new Mo-O bond. New bands rise at 220, 287, 517, 881 and 931 cm^{-1}. These bands could be attributed to the Mo-O vibration by comparison with previous studies of molybdenum oxides,[21] polyoxomolybdates.[22] In such compounds, the Mo-O bond valence and oxidation state of Mo is manifold and up to 15 bands were observed in some case.[21] Symmetry modes could also multiply the possible Raman bands observed, thus complicating assignments of the Mo-O vibrations frequencies. However, the Mo-O vibrations has been correlated to the bands at 229 cm^{-1} and 292 cm^{-1} in $Na_2[Mo_6Cl^i_8(OMe)^a_6]$ and $Na_2[Mo_6(OMe)^i_8(OMe)^a_6]$ clusters compounds respectively.[19] Moreover, all the bands at 287 cm^{-1}, 517 cm^{-1}, 881 cm^{-1} and 931 cm^{-1} have been assigned to Mo-O vibration in many molybdenum oxides[21–24] and polyoxomolybdates[25]. In addition, the band at 287 cm^{-1} is very close to the one at 282 cm^{-1} assigned to Mo-F vibration in $(TBA)_2[Mo_6Br^i_8F^a_6]$ compound.[26] In this context, observations made from Figure 5 confirm the assignment of new bands at 220, 287, 517, 881, 931 cm^{-1} to the Mo-O vibrations in $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ aquahydroxo crystal. To further confirm and quantify the hydrolysis of apical ligands, X-ray photoelectron spectrometry measurements (XPS) and calculations are still on going.

The Figure 6a shows Raman spectra of nanospheres together with those of $Cs_2[Mo_6Br^i_8Br^a_6]$ precursor and $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ aquahydroxo crystal. All the vibrations bands (132, 143, 171, 201, 220, 287, 335, 517, 881, 931 cm^{-1}) previously assigned for $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ are present in the spectrum of nanospheres. This confirms that the $\{Mo_6Br^i_8\}^{4+}$ core is preserved during the self-assembly. It also confirms that the nanospheres are constituted of the $[Mo_6Br^i_8OH^a_6]^{2-}$ units similarly to $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ aquahydroxo crystal. Raman studies were also performed in the case of nanocapsules and compared to their precursor $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$. The Figure 6b presents the Raman spectra of nanocapsules and of $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$ and $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$. The presence of bands at 126 cm^{-1}, 134 cm^{-1}, 178 cm^{-1} and 210 cm^{-1} confirms that the octahedral cluster is maintained in nanocapsules as well. The bands related to the vibration of Mo-N bonds (301 cm^{-1} and 353 cm^{-1}) are also observable in nanocapsules spectrum but are broadened and less intense than in the case of the precursor. This is most probably related to the amorphous structure and a different environment of the cluster. In addition the band at 287 cm^{-1}, attributed to Mo-O as previously mentioned, seems to be present but overlapped by the larger signal of Mo-N vibration. This information strongly suggests the presence of Mo-O bond, thus attesting of a partial substitution of NCS^a apical ligands. Moreover, the same observations are made for the deformation vibrations of C≡N bonds (470 and 490 cm^{-1}), for stretching vibrations of C-S bonds (829 cm^{-1}, 839 cm^{-1}, 849 cm^{-1}) and for stretching vibrations of C≡N bonds. Indeed, all bands appeared

broadened compared to $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$, which is typically attributed to a phenomenon of amorphization of the structure.

When compared to the NH_4NCS salt, the narrowed bands of the $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$ in both 750-850 cm^{-1} (Figure S4a) and 2075cm^{-1} zones (Figure S4c), suggest a similar environment of the NCS group. However, regarding nanocapsules sample, bands in both areas appears broadened and or shifted. This confirms that isothiocyanate groups undergoes significant chnges during the sefl-assembly, and suggests that isothiocyanate group in nanocapsules sample is in several environments. It may be explain by a trapped thiocyanate ion.

XPS analysis should confirm this observation with an atomic quantification of all elements.

Lots of efforts are made in the development of solar energy and electrochemical water splitting is one of the promising ways to store sustainable energy resources in the form of hydrogen. In water splitting cell, the Hydrogen Evolution Reaction (HER) takes place at the cathode and oxygen evolution reaction (OER) takes place at the anode. (ref) These two reactions are favoured in acidic conditions, which can be a problem in the stability of catalysts. Amongst many molybdenum compounds presenting electrocatalytic activities, Feliz *et al.* has been proved that $[Mo_6Br^i_8(OH)^a_6]^{2-}$ units generated in situ shows catalytic activity toward HER.[13] Most of the studies report activities in acidic or alkaline media and achieving HER in neutral media is still a challenge to overcome instability of catalysts and limit destructive corrosion.

In order to evaluate the catalytic performance of the molybdenum octahedral clusters and new nanoassemblies, we engaged nanospheres and nanocapsules for hydrogen evolution reaction (HER). We established here a qualitative and comparative electrochemical study between $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$, $Cs_2[Mo_6Br^i_8Br^a_6]$ and their respective nanocapsules and nanospheres assemblies. We compared activities to Pt, the best catalyst known for HER. The four species were deposited at the surface of a glassy carbon (GC) disk electrode. For comparison, the electrocatalytic activities of bar GC electrode and of the Pt electrode were also measured. Figure 7a reports current densities obtained for the first linear sweep voltammetry experiment (LSV) of $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ (red line), nanocapsules obtained from $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ (orange line), $Cs_2[Mo_6Br^i_8Br^a_6]$ (blue line), nanospheres obtained from $Cs_2[Mo_6Br^i_8Br^a_6]$ (cyan line), compared to control electrode of GC (red dashed line) and Pt (black dashed line) in 1 M KNO_3 pH 7. Formation of gas bubbles, which certainly corresponds to the production of H_2, was observed at the vicinity of the electrode surface. Current densities of 10 mA/cm² are reached between 1.55 V and 1.70 V for all cluster compounds, whereas GC only affords this current density when 2 V is applied. LSV curves evidence that all species display a catalytic effect toward HER. Moreover, higher current densities are measured when nanocapsules and nanospheres are used, compared to their respective precursors $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ and $(NH_4)_2[Mo_6Br^i_8Br^a_6]$ at a given potential.

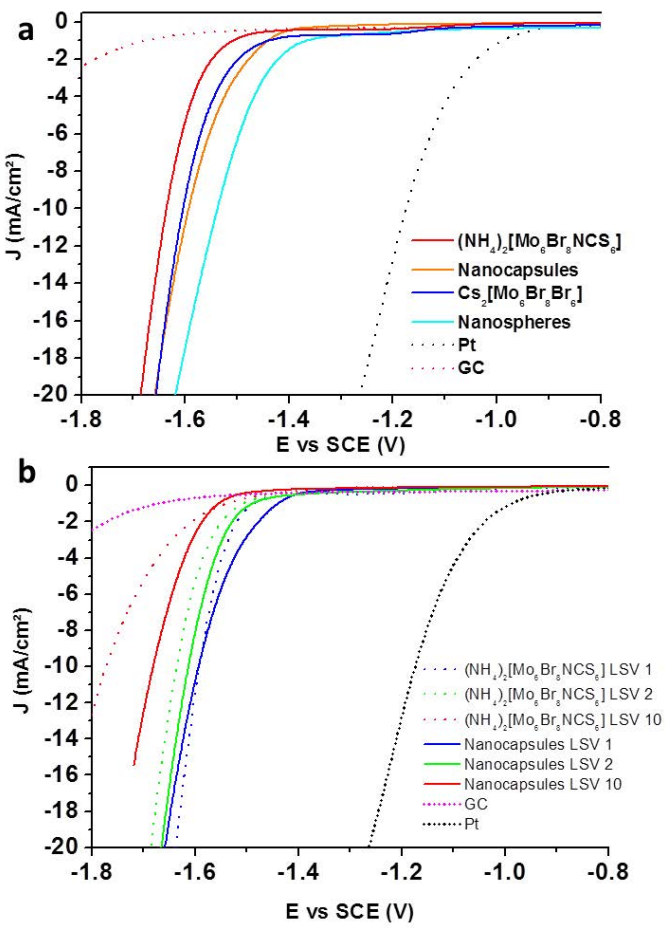

Figure 7: LSV curves of $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$, nanocapsules, $Cs_2[Mo_6Br^i_8Br^a_6]$, nanospheres, Pt and GC in Ar-purged 1M KNO_3 pH 7 solution. (a) First LSV curve, (b) Successive LSV curves (1st, 2nd, 10th curves) for $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$ and nanocapsules. Scan rate: 100 mV.s^{-1}

To investigate the stability of the coating of nanocapsules or of $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ during the course of HER, we have compared the first, the second and the tenth LSV curves recorded in the same conditions. Figure 7b clearly evidences an evolution of the different LSV for both species, which could either suggest a degradation of species at the electrode or a release from the GC surface. However, this evolution is delayed in the case of nanocapsules.

In this preliminary electrochemical study, we only focused on current densities. First made observations based on plotted LSV curves, does not give all information about the reactivity. Further considerations are needed on specific catalytic species in $A_2[Mo_6L^i_8L^a_6]$ cluster compounds of our study, for example Tafel's plot.To further examine stability and reactivity, XPS analyses are ongoing and will give a deeper understanding about oxidation state and stability of present elements during the course of HER.

CONCLUSION

In summary, new nanoassemblies exclusively based on octahedral molybdenum clusters have been prepared through an innovative self-assembling approach in mixtures of miscible solvents. We showed that several spherical structures can be obtained by changing the precursor. Well defined nanosphere and nanocapsules were obtained. To the best of our knowledge this is the first self-assembly into nanospheres and hollow nanocapsules exclusively composed of octahedral molybdenum cluster. Chemical analysis of these new nanomaterials indicates a partial hydrolysis of the cluster units in the case of nanocapsules and a complete hydrolysis in the case of nanosphere. These nanoassemblies could exhibits interesting catalytic properties as they contain $[Mo_6Br^i_8(OH)^a_6]^{2-}$ units. Such units have already shown electrocatalytic efficiency towards HER when grafted on graphene oxide. More generally, this work open perspectives in the growth of unique nanospheres and nanocapsules based on cluster. Additional characterizations, such as magnetic and fluorescence properties may be addressed later on this study.

Method

Materials.

THF was purchased by VWR. All aqueous solutions were prepared with MilliQ water (18,2 MΩ.cm^{-1}) purified using a Purelab Prima system.

Synthesis of octahedral molybdenum clusters.

Molybdenum octahedral cluster compounds $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$, $Cs_2[Mo_6Br^i_8Br^a_6]$, $(NH_4)_2[Mo_6Br^i_8Br^a_6]$, $(NH_4)_2[Mo_6Br^i_8F^a_6]$, were synthesized according to previous methods. (Refs)

Self-Assembly of nanobeads and nanocapsules.

A solution of octahedral cluster precursor (100 µL, 156 µg) was diluted in THF. Then 800 µL of water was added and the mixture vigouring vortexed. The suspension is let to rest for 24 h before heat up in a dry bath at 40°C during 15 h. The obtain suspension was then centrifuged at 10 000 rpm for 10 min and suspended in 1mL of water.

Raman spectroscopy

Raman spectra were recorder by a LabRam HR800 Raman microscope (Horiba Scientific). To avoid fluorescence noise and to preserve the sample, the excitation wavelength was 785 nm from a XTRA (TOPTICA) laser diode. Measurements have been performed in back scattering configuration with a 100x objective. Power laser was adjusted to avoid sample heating.

Electron Microscopy

Transmission electron microscopy was performed using a JEOL JEM-1400 operating at 120 kV and equipped with a GATAN Orius 1000 camera. High resolution TEM micrographs, negative staining STEM and Energy dispersive X-ray spectroscopy analysis, were performed on a JEOL JEM-2100F at 200 kV. Scanning electron microscopy was performed using a JEOL 7100f ttls at 10 kV voltage, equipped with a tungsten tip and a Everhart-Thornley detector.

Light Scattering Measurments.

Diffusion Light scattering (DLS) monitoring was perfomed with a Nanosizer (Malvern Instruments).

Electrocatalytic behaviour towards HER.

The electrocatalytic activities were recorded using an Autolab electrochemical analyzer (PGSTAT 30 potentiostat/galvanostat from EcoChemie B.V.) in a three-electrode setup. A saturated calomel electrode (SCE) and platinum wire were used as the reference electrode and as the counter electrode, respectively. The working electrode is a glassy carbon disk electrode of 3 mm diameter. Nanocapsules and nanospheres were simply dropcasted onto the GC surface. The amount of deposited of nanocapsules and nanospheres were quantified through QCM measurements, prior to electrochemical measurements. Suspensions were first dropcasted on a platinized quartz surface and then the variation of the nominative frequency was converted to a mass according to Sauerbrey equation: $\Delta m_p = -k\Delta f$ with k=1.1ngHz^{-1}. Typically, a 20 µL drop of nanocapsules solution gives 4.907 µg of nanocapsules. The dropcast of 100 µl of these suspensions on the GC electrode (diameter 3mm) thus correspond to a catalyst loading of 2.8mg/cm² on the working electrode. Electrochemical measurements were performed with a scan rate of 100 mV.s^{-1} in KNO_3 1 M (pH 7) as electrolyte. The electrocatalytic activities of $(NH_4)_2[Mo_6Br^i_8Br^a_6]$ and $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$ were measured in the same conditions to enable comparison.

Single-Crystal X-ray Diffraction.

Single crystal of $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ were obtained from different precursors: (1) $(NH_4)_2[Mo_6Br^i_8F^a_6]$, (2) $(NH_4)_2[Mo_6Br^i_8Br^a_6]$, and (3) $Cs_2[Mo_6Br^i_8Br^a_6]$. The crystal structure measurements were carried out on APEX II Bruker AXS spectrometer using Mo-K_α X-ray wavelength (CDIFX, ISCR, Rennes). Measurements were performed at room temperature (T = 293(2)K) for (1) and (2), and at low temperature (T = 150(2)K) for (3). The structures were solved by direct methods using SIR97 program[27] and then refined with full-matrix least-square methods based on F^2 (SHELXL-2014)[28] using the WinGX software package.[29] Multi-scan absorption correction was applied using SADABS. All non-hydrogen atoms were refined with anisotropic atomic displacement parameters. Hydrogen atoms were not considered in the structural model. Publishing data were computed using CRYSCALC program (T. Roisnel, local program, 2017). Crystal data for (1): sample size = 0.09 × 0.08 × 0.03 mm^3, space group *R*-3*m*, T = 293(2)K, a = 15.2780(6) Å, c = 11.2035(6) Å, V = 2264.7(24) Å^3, Z = 3, M_f = 1535.17 g.mol^{-1}, ρ_{calc} = 3.377 g.cm^{-3}, θ = 2.382 to 33.152°, 5773 reflections collected, 1055 unique reflections, R_{int} = 0.0934, completeness to theta max = 0.993, 32 refined parameters, final R_1 [I > 2σ] = 0.0388, final wR_2 [I > 2σ] = 0.0844, largest difference peak and hole were +1.895 and -1.207 e.Å^{-3}. Crystal data for (2): sample size = 0.17 × 0.12 × 0.07 mm^3, space group *R*-3*m*, T = 293(2)K, a = 15.2966(18) Å, c = 11.2188(15) Å, V = 2273.4(8) Å^3, Z = 3, M_f = 1535.17 g.mol^{-1}, ρ_{calc} = 3.364 g.cm^{-3}, θ = 3.572 to 33.200°, 19971 reflections collected, 1065 unique reflections, R_{int} = 0.0611, completeness to theta max = 0.998, 32 refined parameters, final R_1 [I > 2σ] = 0.0188, final wR_2 [I > 2σ] = 0.0521, largest difference peak and hole were +0.666 and -0.490 e.Å^{-3}. Crystal data for (3): sample size = 0.16 × 0.08 × 0.07 mm^3, space group *R*-3*m*, T = 150(2)K, a = 15.2675(4) Å, c = 11.1594(3) Å, V = 2252.7(2) Å^3, Z = 3, M_f = 1535.17 g.mol^{-1}, ρ_{calc} = 3.395 g.cm^{-3}, θ = 3.582 to 45.260°, 18107 reflections collected, 2244 unique reflections, R_{int} = 0.0484, completeness to theta max = 0.997, 32 refined parameters, final R_1 [I > 2σ] = 0.0285, final wR_2 [I > 2σ] = 0.0550, largest difference peak and hole were +1.207 and -1.056 e.Å^{-3}.

Acknowledgments

The authors want to thanks ScanMat platforms, in particular THEMIS for TEM microscopy, SIR for Raman studies.

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Supporting Information

Figure S1: TEM microscopy of nanoassemblies obtained from various octahedral clusters.

Figure S2: TEM micrographs of structures obtained during the self-assembly of $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$.

Table S1: Atomic coordinates, site occupancy and equivalent isotropic displacement parameters of $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$.

Table S2: Average bond distances in $[Mo_6Br^i_8(OH)^a_6]^{2-}$ cluster units.

Figure S3: Raman characterization and assignments of Raman virations of octahedral molybdenum clusters precursors.

Figure S3: Single crystal data collection and structure refinement of $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$.

Figure S4: Raman study of isothiocyanate group in $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$, nanocapsules compared to NH_4NCS salt.

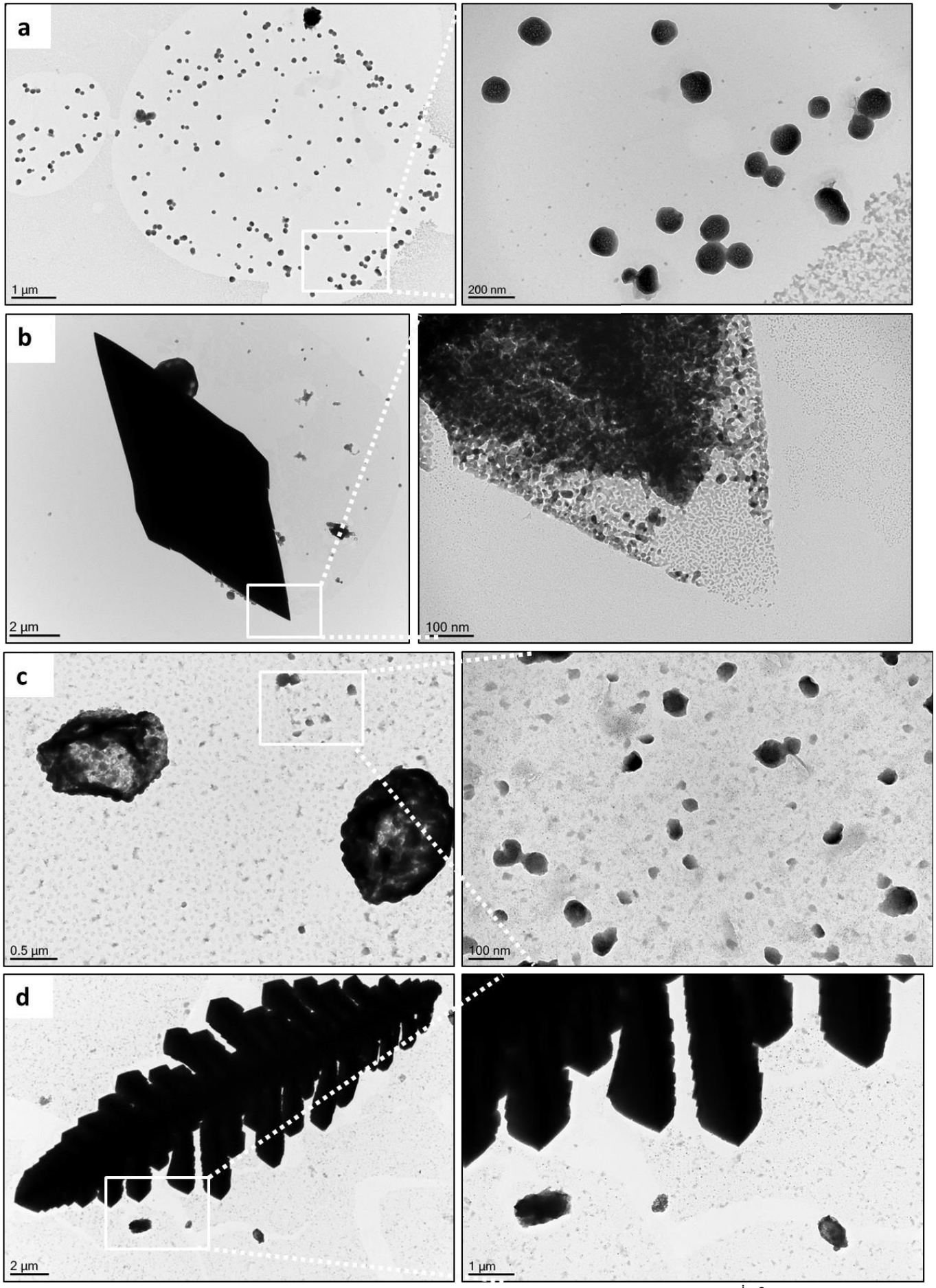


Figure S1: TEM micrographs of assemblies obtained from octahedral clusters (a,b) $(NH_4)_2[Mo_6Br^i_8F^a_6]$ after 2h of maturation and (c,d) $(NH_4)_2[Mo_6Br^i_8Br^a_6]$ after 10h of maturation.

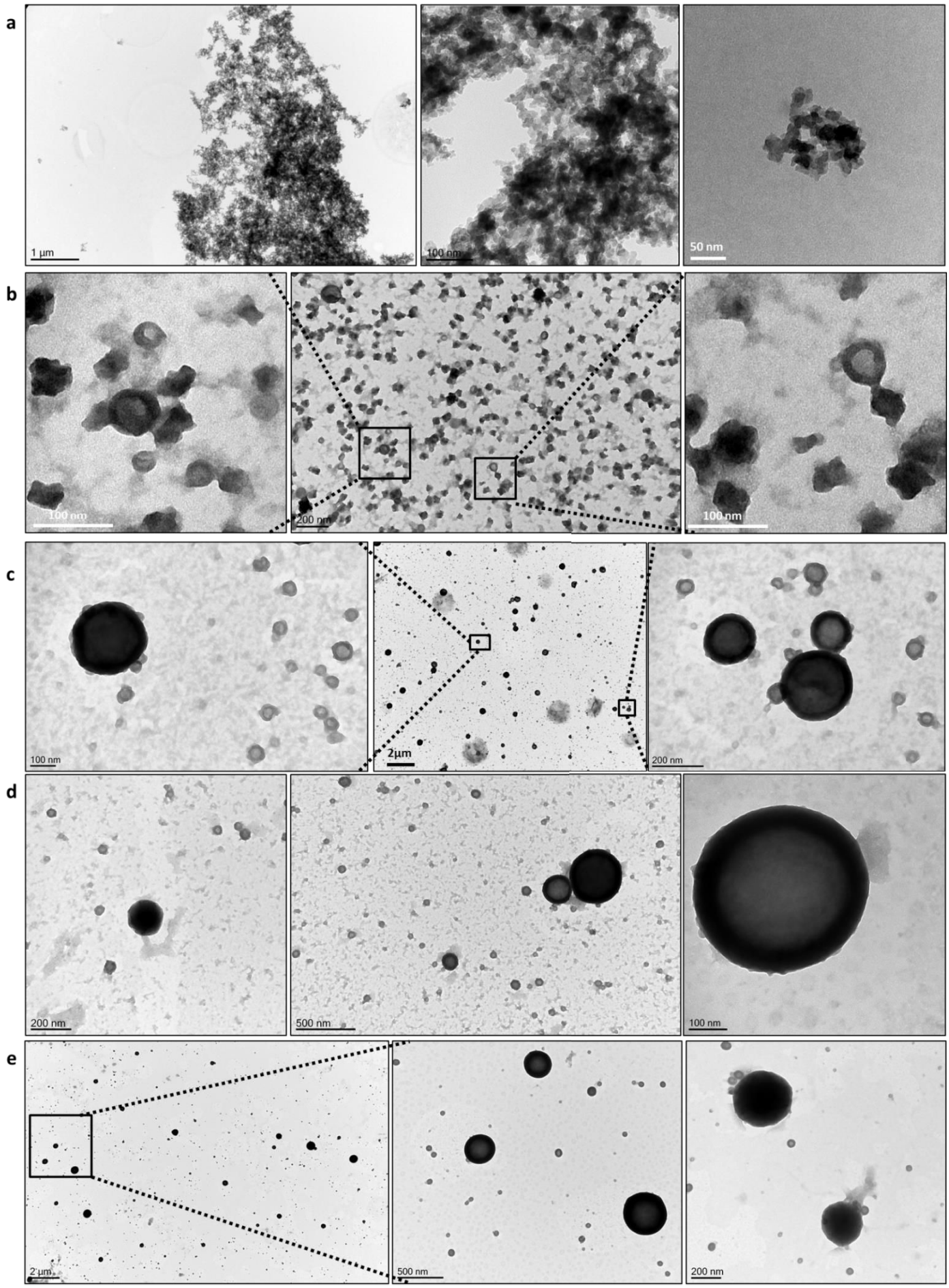


Figure S2: TEM micrographs of structures obtained during the self-assembly of $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ at 40°C in closed reactor at (a) T_0, (b) T_{0+10h}, (c) T_{0+24h} , (d) T_{0+34h} and (e) T_{0+47h}.

(1)						
Atom	Site	x	y	z	Occ.	U(eq)
Mo	18h	0.1144(1)	x/2	0.5958(1)	1.00	0.0116(2)
Br^i(1)	6c	0	0	0.7858(2)	1.00	0.0168(3)
Br^i(2)	18h	0.2284(1)	x/2	0.4069(1)	1.00	0.0189(2)
O^a	18h	0.2416(4)	x/2	0.7081(5)	1.00	0.0241(11)
O (water)	36i	0.2108(4)	-0.0259(3)	0.8802(4)	1.00	0.0409(12)

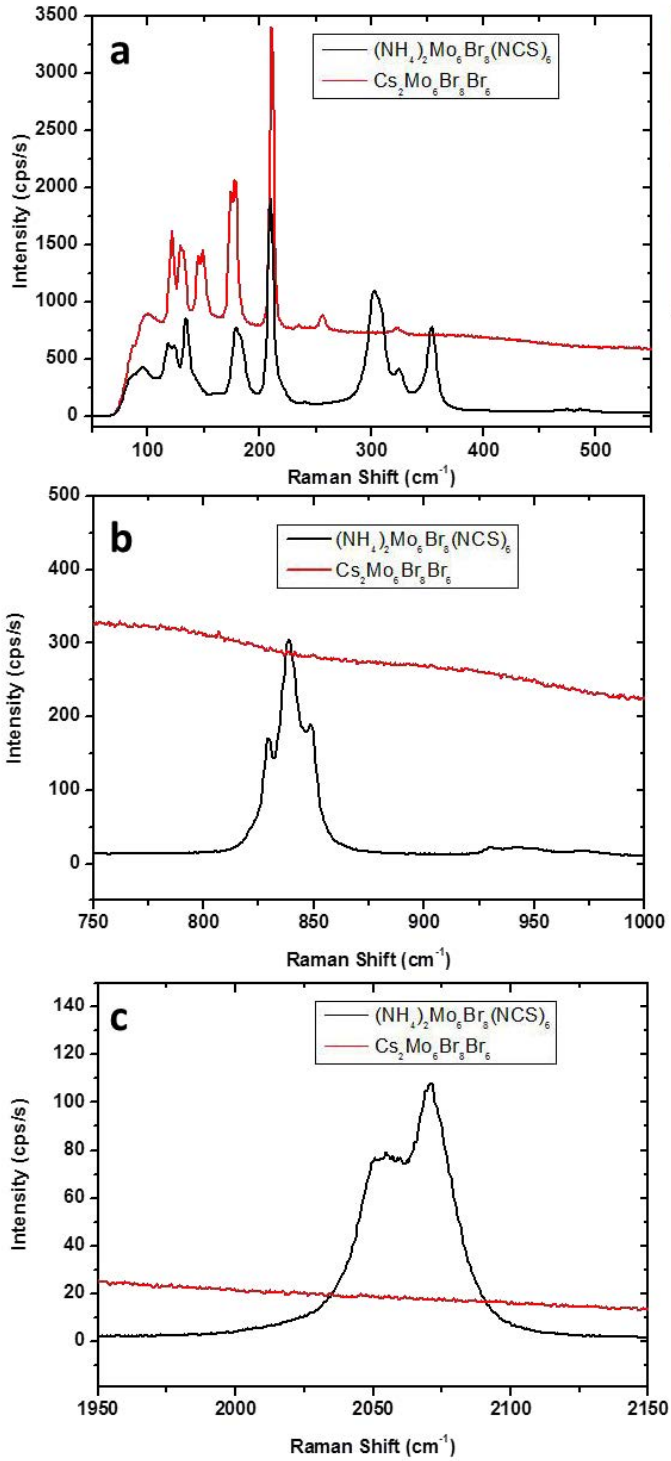
(2)						
Atom	Site	x	y	z	Occ.	U(eq)
Mo	18h	0.1145(1)	x/2	0.5959(1)	1.00	0.0112(1)
Br^i(1)	6c	0	0	0.7860(1)	1.00	0.0165(2)
Br^i(2)	18h	0.2285(1)	x/2	0.4068(1)	1.00	0.0184(2)
O^a	18h	0.2418(2)	x/2	0.7087(2)	1.00	0.0243(5)
O (water)	36i	0.2111(2)	-0.0257(2)	0.8799(2)	1.00	0.0401(5)

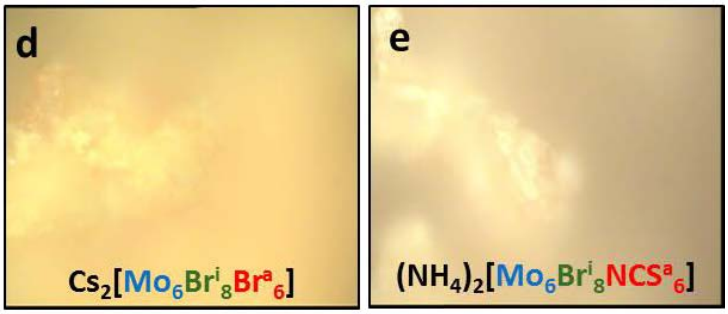
(3)						
Atom	Site	x	y	z	Occ.	U(eq)
Mo	18h	0.1147(1)	x/2	0.5965(1)	1.00	0.0059(1)
Br^i(1)	6c	0	0	0.7877(1)	1.00	0.0090(1)
Br^i(2)	18h	0.2292(1)	x/2	0.4064(1)	1.00	0.0100(1)
O^a	18h	0.2423(2)	x/2	0.7103(2)	1.00	0.0147(4)
O (water)	36i	0.2106(2)	-0.0264(2)	0.8799(2)	1.00	0.0231(3)

Table S1: Atomic coordinates, site occupancy and equivalent isotropic displacement parameters of $(H_3O)_2[Mo_6Br^i_8(OH)^a_6].10H_2O$ obtained from different precursors: (1) $(NH_4)_2[Mo_6Br^i_8F^a_6]$, (2) $(NH_4)_2[Mo_6Br^i_8Br^a_6]$, and (3) $Cs_2[Mo_6Br^i_8Br^a_6]$.

Precursor	Temperature (K)	Mo-Mo (Å)	Mo-Br^i (Å)	Mo-O^a (Å)
(1)	293(2)	2.625(2)	2.611(2)	2.101(5)
(2)	293(2)	2.629(1)	2.616(1)	2.109(2)
(3)	150(2)	2.631(1)	2.618(1)	2.111(2)

Table S2: Average bond distances in $[Mo_6Br^i_8(OH)^a_6]^{2-}$ cluster units





f

$Cs_2Mo_6Br^i_8Br^a_6$		$(NH_4)_2Mo_6Br^i_8(NCS)^a_6$	
ν (cm^{-1})	Assigments		ν (cm^{-1})
121	Mo-Mo	Mo-Mo	118
129	Mo-Mo	Mo-Mo	124
132	Mo-Br^i_8 (E_g)	Mo-Br^i_8 (E_g)	134
145/149	Mo-Br^a_6 (A_{1g})		
174/178	Mo-Br^i_8 (T_{2g})	Mo-Br^i_8 (T_{2g})	178/183
210	Mo-Br^i_8 (A_{1g})	Mo-Br^i_8 (A_{1g})	210
256	Mo-Br_8		
		Mo-NCS^a_6 (E_g)	301
322	Mo-Mo (A_{1g})	Mo-Mo (A_{1g})	323
		Mo-NCS^a_6 (A_{1g})	353
		δ(NCS) (A_g, B_g)	470/490
		ν(CS) (A_g, B_g)	829/839/849
		2δ(NCS)	950
		ν(CN)	2055/2071

Figure S3: Raman Characterization and assignments of octahedral molybdenum clusters precursors. (a, b, c) Raman Spectra of $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ (black line) and $Cs_2[Mo_6Br^i_8Br^a_6]$ (red line), (d,e) Optical micrograph of the acquisition zone for $Cs_2[Mo_6Br^i_8Br^a_6]$ and $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ respectively. (f) Raman shifts and assignments for precursors.

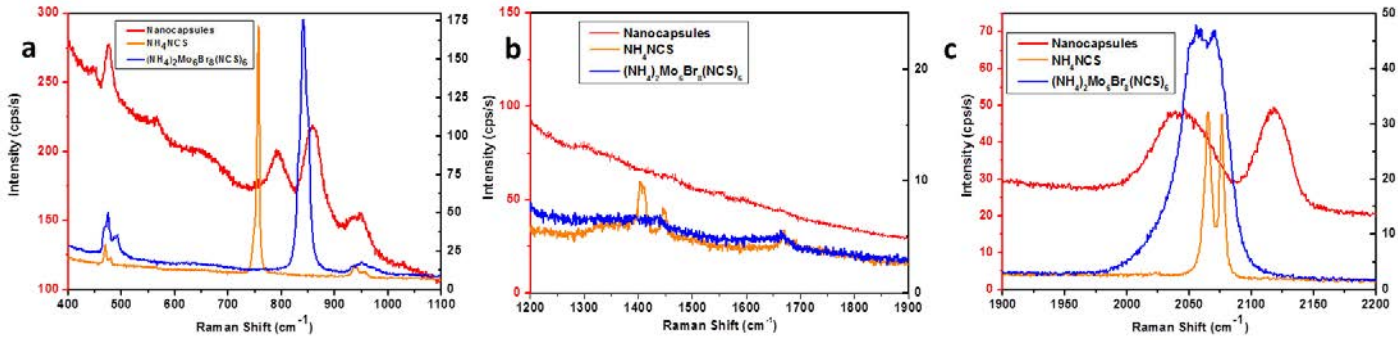


Figure S4: Raman spectra of isothiocyanate group in $(NH_4)_2[Mo_6Br^i_8NCS^a_6]$ (blue line), and nanocapsules (red line) compared to NH_4NCS salt (orange line).

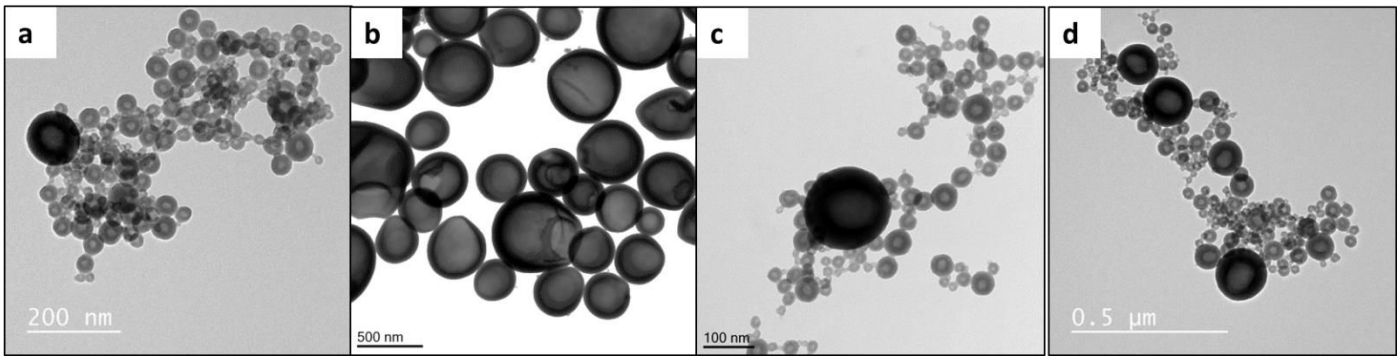


Figure S5: TEM micrographs of structures obtained during the self-assembly of $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ at 40°C in open reactor at (a) $T_{0+13h30}$, (c) T_{0+24h} , (d) T_{0+72h} and (e) T_{0+96h}.

We observe from Figure S5b blown up nanocapsules of over 500 nm. Some Nanocapsules appeared exploded which suggest a difference of pressure between the inside and outside of the capsule.

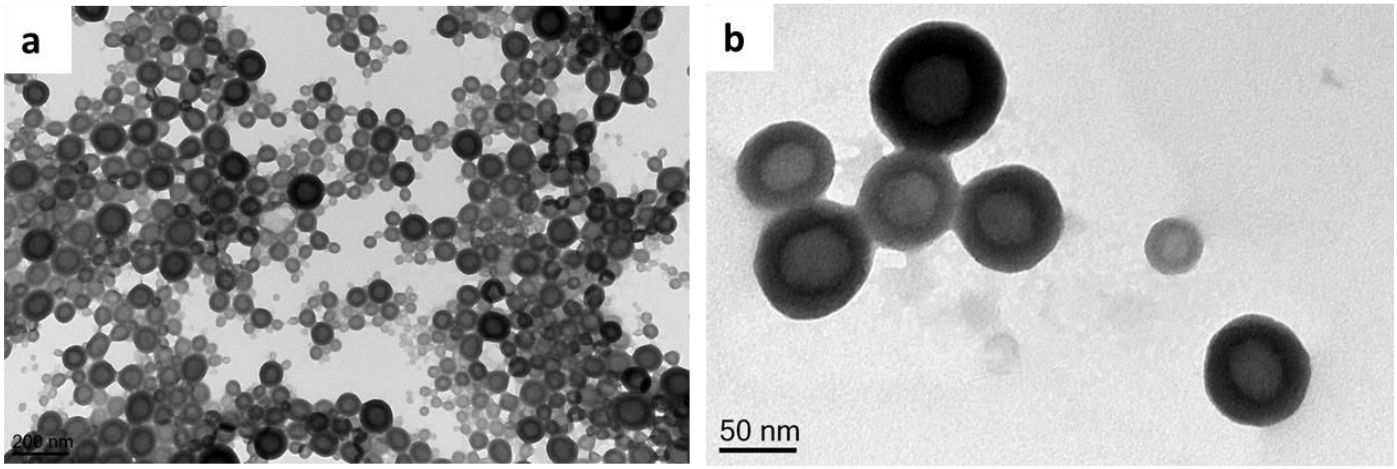


Figure S6: TEM micrographs of structures obtained during the self-assembly of $(NH_4)_2[Mo_6Br^i_8(NCS)^a_6]$ in close reactor without any heating step at $T_{0+100\ days}$.

Conclusion Générale

Les nanotechnologies sont devenues en quelques dizaines d'années un sujet d'intérêt dans de nombreux domaines. Elles suscitent l'intérêt de nombreuses communautés aussi bien académiques qu'industrielles dû à leur haut potentiel en recherche et développement. Les propriétés mécaniques, chimiques, optiques, électroniques, magnétiques des nanoparticules très différentes des propriétés à l'état massif, offrent d'innombrables domaines d'exploration dans les branches de la physique, la chimie, la biologie, l'ingénierie etc. Dans ce contexte interdisciplinaire, l'objectif initial de cette thèse était double:

- étudier les interactions entre nanoparticules et protéines dans le but d'appréhender l'influence des nanomatériaux sur la structure et l'activité des biomolécules.
- étudier les interactions entre nanoparticules et polymères afin de stabiliser des agrégats d'oxydes magnétiques

Rapidement, l'exploration des mélanges ternaires solvant/eau/nanoparticules a permis de mettre en évidence à la fois le comportement particulier du mélange miscible eau/THF, ainsi que le comportement interfacial de nanoparticules d'oxydes de fer au sein de ce mélange.

Il a été confirmé par diffusion de la lumière que le mélange d'eau de de THF conduit à l'apparition de structures dont la taille caractéristique est de l'ordre d'une centaine de nanomètres. Ces structures que nous avons d'abord appelés gouttes restent encore de nature indéterminée. L'existence et la particularité de ces « gouttes » semblent les rapprocher du phénomène Ouzo. En effet, les conditions semblent réunies puisque le THF et l'eau sont miscibles. Ces observations suggèrent la présence d'un soluté qui, dans un mélange riche en eau (70-90%), serait contraint à précipiter. Cependant, ce soluté n'a pas été clairement identifié jusqu'à présent bien que le stabilisant du THF soit plus que suspecté et que son rôle dans l'apparition de ces structures a déjà été rapporté. Des études portant sur la qualité du THF, ou d'autres solvants miscibles à l'eau pour lesquels des structures ont été rapportés (alcools etc) devraient permettre une étude plus précise du système ternaire dans la zone Ouzo.

Dans le cas ou des nanoparticules sont en suspension dans les conditions propices à la nanoprécipitation, nous avons observé l'auto-assemblage de ces dernières à l'interface liquide/liquide alors créée. Ce phénomène n'avait jusque-là peu ou pas été observé pour des nanoparticules inorganiques seules. De plus, l'explication de l'assemblage de nanocristaux sous la forme de nanocapsules par l'effet Ouzo n'a pas été rapportée. Ces assemblages ont été stabilisés par différents polymères et ont donné naissance aux Hybridosomes.

Par la suite, la composition des Hybridosomes a été modulée grâce à l'utilisation de particules d'or, de quantum dots, de particules up-converting, apportant ainsi un caractère multifonctionnel aux nanocapsules de par leur constituants inorganiques. Des Hybridosomes magnétiques, luminescents, plasmoniques et up-converting ont été obtenus. La grande diversité, pour ne pas dire les quasi infinies possibilités, de synthèse de nanoparticules de taille entre 2 et 10 nm et fonctionnalisées par des ligands hydrophobes, permet d'envisager presque toute les compositions chimique pour les Hybridosomes.

Le procédé d'assemblage à permis de piéger un fluorophore au sein de l'Hybridosome, démontrant ainsi que la fluorescence des capsules peut aussi être de nature moléculaire. Cette preuve de concept, permet aussi de valider l'emprisonnement d'une molécule hydrophobe et d'envisager les applications de relargage.

De plus, le caractère hybride de la capsule, apporté par un poly(acide acrylique) qui maintient la paroi de nanoparticules, confère des comportements réactifs. Dans notre cas, les propriétés acides du PAA ont permis d'induire la destruction des capsules. Le stimulus peut aussi être varié grâce aux nombreux polymères réactifs à la température, à un courant électrique, à un champ magnétique par exemple. La diversité des polymères, autant par leur nature que par leur structure, permet là encore un degré de liberté conséquent dans le choix de la propriété et le design d'Hybridosomes à façon. Par exemple, l'utilisation de polymères naturels comme les polysaccharides, ou à l'inverse des polymères synthétiques de dérivés de polyvinyle, permet d'envisager la construction de matériaux aux applications dans la science du vivant ou plus proche des matériaux pour l'énergie.

L'aspect hybride apporté par le polymère, en plus d'être structurant, permet aussi une fonctionnalisation supplémentaire. Dans le chapitre 3, nous avons montré la fonctionnalisation du PAA par un groupement alcyne, pour ensuite assembler les Hybridosomes avec le PAA-alcyne. Cette étape à rendu possible l'utilisation des Hybridosomes-alcyne comme partenaire dans la réaction de cycloaddition de Huisgen (chimie click). Par un procédé de chimie électroclick, nous avons montré qu'il était possible de réticuler des Hybridosomes sur une surface électro-active et ainsi créer des films tout en conservant les spécificités des capsules. La pluralité des Hybridosomes a permis de construire de nouveaux films à la fois nanostructurés et multifonctionnels. Des coatings à la fois pH et électro-réactifs, ont aussi apportés la preuve du relargage du Bodipy emprisonné dans le film. D'importantes étapes ont été franchies lors cette étude et les résultats obtenus sont encourageants pour le développement de nouveaux matériaux qui pourraient trouver des applications en catalyse, avec le choix de matériaux electroactifs, mais également en optique grâce aux particules plasmoniques.

La première application des Hybridosomes se situe du côté de la nanomedecine. Les Hybridosomes magnétiques ont déjà permis de confirmer que ces propriétés magnétiques rendent possible le développement d'agents de contrastes en IRM. Nous avons montré que leur efficacité est comparable à un agent commercial préclinique (Feraspin, Miltenyi). Dans le domaine biomédical, les applications apparaissent très nombreuses également notamment en imagerie multimodale, imagerie ciblée et relargage. Des études concernant le comportement des Hybridosomes vis-à-vis de différents rayonnements devraient rapidement confirmer ou infirmer si la thérapie photothermique ou encore la thérapie photodynamique font partie du champ d'applications possibles. Des études sur l'encapsulation de principes actifs devraient aussi conforter le potentiel des Hybridosomes en nanomedecine. Malgré les coûts financier et temporel élevés de ces différentes études, elles viendront accroitre les chances de valorisation des Hybridosomes dans les années futures. Les Hybridosomes apporteront certainement des éléments réponses dans le développement de certaines solutions en nanomédecine, bien que le challenge soit considérable.

Pour finir, nous avons montré dans le chapitre 5, que le phénomène mis en évidence lors du procédé permet l'assemblage de clusters métalliques. Ainsi, de nouveaux matériaux à base de clusters ont aussi été synthétisés sans l'ajout d'une quelconque autre matrice. Ces édifices adressent également de nouvelles questions à la science des matériaux et ouvrent de nouvelles possibilités dans le domaine des « nanoarchitectonics » c'est-à-dire dans l'assemblage et la construction d'édifices fonctionels, structurés de l'échelle nanométrique à l'échelle macroscopique.