Université du Québec
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EFFETS DES NANOPARTICULES D'ARGENT SUR LES COMPOSÉS ORGANIQUES SOUFRÉS D'ORIGINE BIOLOGIQUE

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[Le lâche meurt à petit feu, tous les jours. Chaque fois qu'il s'enfuit devant le danger et laisse les autres souffrir à sa place. Chaque fois qu'il est témoin d'une injustice et ce dit : "ça n'a rien à voir avec moi." **Chaque fois qu'un homme risque sa vie pour les autres, et survit, il devient plus que ce qu'il était avant**.

– David Gemmell : Loup blanc]

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RÉSUMÉ

Il est connu que l'argent ionique ainsi que les nanoparticules d'argent (AgNPs) ont une forte affinité pour les composés soufrés aussi bien inorganiques qu'organiques. De nombreuses études ont en effet démontré des interactions entre les AgNPs et des acides aminés soufrés tels que la cystéine via le groupement thiol de la molécule. De plus, certaines recherches ont proposé que les AgNPs puissent se lier aux protéines via leurs groupements thiols.

L'objectif principal de ce mémoire était d'analyser les interactions des AgNPs avec les composés soufrés organiques tels que la cystéine et l'albumine et de comparer les résultats obtenus avec les effets des ions Ag^+ sur ces mêmes molécules. Par ailleurs il s'est présenté une occasion de rédiger une revue de littérature afin d'expliquer le comportement, les transformations et le devenir des AgNPs une fois introduites dans l'environnement aquatique ; cette revue fait partie de ce mémoire.

Suite à des expériences de spectroscopie RAMAN et de matrices d'excitation-émission de fluorescence, des interactions entre l'argent ionique avec le soufre du groupement thiol de la cystéine ont été démontrées. De plus, les effets des ions Ag^+ et des AgNPs sur la protéine d'albumine ont été mesurés. Les résultats obtenus ont montré des effets plus importants en présence des ions Ag^+ que des AgNPs. Il a été proposé que les ions argent, étant plus petits que les AgNPs, aient un accès facilité à certains sites de liaisons sur la protéine d'albumine en comparaison des nanoparticules. Finalement, il a été indirectement observé que bien que les AgNPs aient des effets plus faibles que l'argent ionique sur l'albumine, elles sont tout de même capables d'affecter directement la protéine. À notre connaissance, aucune étude antérieure n'avait comparé les effets des ions argent à ceux des nanoparticules d'argent sur la protéine d'albumine.

Le mémoire suivant est constitué d'une introduction générale, d'un travail de recherche concernant les interactions entre les AgNPs et les composés soufrés organiques, d'une revue

de littérature concernant les transformations des AgNPs dans les milieux aqueux ainsi que leurs effets sur les organismes aquatiques, et d'une conclusion générale.

Mots clés : [AgNPs, ROS, soufre, albumine, fluorescence, RAMAN, environnement]

ABSTRACT

It is well known that silver ions as well as silver nanoparticles (AgNPs) have a high affinity for organic and inorganic sulfur compounds. Indeed, many studies have demonstrated the interactions between AgNPs and small amino acids such as cysteine through the thiol group of the molecules. Moreover, some studies suggested that AgNPs could bind to proteins through their thiol groups.

The main objectives of this study were to analyse the interactions of AgNPs with sulfur organic compounds such as cysteine and albumin as well as to compare the obtained results with the effect of silver ions on these same organic compounds. Furthermore, there was an opportunity to produce a literature review on the behavior, transformations and future of AgNPs once in the aquatic environment. This review takes part in this document.

Experiments using RAMAN spectroscopy and excitation-emission fluorescence matrix have demonstrated interactions between ionic silver and sulfur of the thiol group of cysteine. Also, effects of Ag^+ ions and AgNPs on the protein of albumin have been measured. Results showed that the effects were enhanced in presence of silver ions compared with AgNPs. It was suggested that as silver ions are smaller than AgNPs, they may have promoted access for specific binding sites of albumin compared to the nanoparticles. Finally, it has been indirectly observed that although AgNPs had less impact on albumin than the silver ions, they are still able to directly affect the protein. To our knowledge, no previous study had compared the effects of silver ions and AgNPs on the protein of albumin.

This study includes a general introduction, a study on the interactions of AgNPs and sulfur organic compounds, a literature review on transformations of AgNPs in aquatic medium as well as on aquatic organisms, and a general conclusion.

Keywords: [AgNPs, ROS, sulfur, albumin, fluorescence, RAMAN, environment]

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INTRODUCTION GÉNÉRALE

Argent et ses propriétés antibactériennes

Depuis les dernières décennies, l'augmentation de l'apparition de bactéries de plus en plus multi-résistantes aux antibiotiques a mené à un regain d'intérêt envers l'étude de l'argent et de ses propriétés antibactériennes. En effet, il est connu, depuis l'antiquité, que l'argent est utilisé afin de lutter contre les maladies et les infections. Plusieurs mécanismes d'action antibactériens de l'argent sont observés, tels que la perturbation de l'ADN de ces microorganismes et l'inhibition de la synthèse d'ARN, la destruction des membranes, la perturbation du transport d'électrons, l'altération du métabolisme et la liaison de l'argent aux protéines modifiant ainsi leur comportement (Prabhu and Poulose 2012, Yang et al. 2013, Marambio-Jones and Hoek 2010a, Morones et al. 2005, Zhang et al. 2016a, Zhou et al. 2015). Il est largement admis que l'une des causes des liaisons de l'argent aux protéines est sa capacité à se lier à leurs groupements sulfure, ce qui affecte les protéines, les processus cellulaires et le métabolisme des bactéries (Bragg and Rainnie 1974, Liau et al. 1997).

Argent ionique et les composés soufrés

L'argent est, avec l'or, le mercure, le cuivre, et le plomb, un métal mou de classe B et se comporte comme un acide de Lewis capable d'accepter un doublet d'électrons. C'est pourquoi il peut se lier avec des ligands mous via la formation d'une liaison covalente sigma (Alshammari 2017, Bell and Kramer 1999, Smith et al. 2002). En effet, les métaux mous ont des nuages électroniques qui peuvent être déformés et sont fortement polarisables (Smith et al. 2002). L'argent a donc la capacité de se lier à des ligands tels que le soufre réduit (S^{2-}) pour lequel il possède une affinité de plusieurs ordres de grandeur supérieure à celle de ligands comme l'azote ou l'oxygène (Alshammari 2017, Smith et al. 2002).

Argent ionique et composés soufrés inorganiques

L'argent est capable de se lier au soufre inorganique présent dans des molécules telles que H_2S, HS^- et $(H)S^{2-}$, dont les concentrations des diverses espèces dans le milieu dépendent principalement du pH et de la concentration en oxygène dissous (Bell and Kramer 1999). Le

produit de la liaison de l'argent ionique et du soufre dépend principalement des concentrations des réactifs. Habituellement, dans l'environnement naturel les concentrations d'argent sont relativement faibles (0.001-0.01μgL^{-1}) (Alshammari 2017, VI and VI).

En solution, l'argent (Ag(I)) et le soufre (S(-II)) vont former des complexes pouvant être mononucléaires (AgL_n) ou polynucléaires (Ag_mL_n), où L représente les ligands soufrés HS^-, $S^{-2}{}_p$, et HS_p et où S_p représentent les espèces polynucléaires du soufre (Bell and Kramer 1999). À faible concentration en soufre et en argent, le complexe prédominant sera $AgHS^0$. En augmentant les concentrations en soufre, de nouvelles formes apparaissent en suivant l'ordre croissant ci-dessous (Bell and Kramer 1999).

$$Ag+ \rightarrow AgHS^0 \rightarrow Ag(HS)_2^- \rightarrow (AgS)_nS^{2-} \rightarrow Ag_mL_n$$

$AgHS^0$ est décrit comme étant probablement un doublet covalent lié, alors que dans le cas de $Ag(HS)_2^-$ il s'agit plus probablement d'une chaine linéaire HS-Ag-HS. L'augmentation des concentrations de soufre jusqu'à des concentrations de l'ordre du micromolaire va mener à la formation de chaines polynucléaires en zigzag tels que $(HS\text{-}Ag\text{-}S\text{-}Ag\text{-}HS)^{2-}$ (Bell and Kramer 1999). La taille de ces chaines dépendra notamment des concentrations d'argent et de soufre, mais également du pH du milieu. En effet, l'augmentation du pH tend à faire augmenter la prédominance des espèces polynucléaires (Bell and Kramer 1999).

La liaison de l'argent avec le soufre est également capable de former un solide, Ag_2S, particulièrement stable et très faiblement soluble dans l'eau (Bell and Kramer 1999, Hillman et al. 2007, Sharma and Chang 1986). Ce complexe est vraisemblablement le devenir ultime de toutes les formes de complexes Ag-S, que le soufre soit d'origine inorganique ou organique (Bell and Kramer 1999). Ces transformations pouvant se produire soit via la réaction des thiolates d'argent avec des ions hydrosulfures (H_2S/HS^-) menant à la libération du mercaptan ou via la dégradation oxydative d'un composé organique menant ainsi à la formation de l'Ag_2S insoluble. En revanche, dû à sa forte stabilité et sa quasi-insolubilité, la réaction inverse de Ag_2S vers des composés Ag-soufre organique/inorganique est chimiquement peu probable.

Argent ionique et composés soufrés organiques

De nombreuses études ont montré que l'argent ionique peut se lier avec le soufre présent dans les molécules organiques. Les forces de liaisons des complexes soufre-Ag sont près de six ordres de grandeurs supérieurs à celles du soufre avec les carboxylates bien que la stabilité des complexes soufre-argent formés dépende des espèces avec lesquelles l'argent se lie (Bell and Kramer 1999). En effet, l'argent peut se lier avec le soufre des groupements thiols possédant des liaisons covalentes simples avec le carbone, notamment avec les mercaptans, thiophenols, thioacides, et thioethers. Il peut également se lier avec le soufre possédant une double liaison pour le carbone comme dans les groupements thioketones, thioaldheydes, thionoacides, thioamides, thiocarbamates, et dithiocarbamates (Bell and Kramer 1999). La liaison de l'argent aux groupements mercaptans forme des liaisons sigma particulièrement fortes avec des constantes de formation log K_f proche de 13. En revanche les liaisons de l'argent avec des soufres possédant une double liaison ont des valeurs log K_f proche de celles des amines (K_f de methylamine = 3.06) (Bell and Kramer 1999) (voir Tableau 1). De plus, la présence de plusieurs groupements amines peut augmenter la valeur K_f (Bell and Kramer 1999).

Tableau 1. Valeurs des log $K_{formation}$ (K_f) pour quelques exemples de composés argent-soufre

Composés	pK_f
2-Mercaptoethanol $HOCH_2CH_2SH$	13.2
Cystéine $^-OOCCH(NH3^+)CH_2SH$	11.9
Thiourée H_2NCSNH_2	7.11
Diméthyle sulfure CH_3SCH_3	3.7

Tableau modifié de Bell et Kramer 1999

Les interactions de l'argent avec des molécules soufrées organiques peuvent mener à la formation de structures complexes et stables qui peuvent également former des agrégats. En effet, il a été

montré que les liaisons entre l'argent ionique et les groupements thiols de composés organiques forment des chaines en zigzag -S(R)-Ag-S(R)-. La taille et la complexité de ces chaines dépendent majoritairement des concentrations de soufre et d'argent présentes en solution (Bell and Kramer 1999, Leung et al. 2013, Pakhomov et al. 2010). Ces chaines peuvent aussi s'associer entre elles afin de former des structures en feuillets (Bell and Kramer 1999). Deux mécanismes de formation des feuillets sont proposés. Dans un premier cas, il est suggéré que les groupements terminaux des molécules organiques, liées à l'argent via leurs groupements thiols, se lient entre eux par liaison hydrogène et forment ainsi des feuillets (Bell et al. 1997, Bell and Kramer 1999). Dans le second cas, il a été proposé qu'une chaine –S(R)-Ag-S(R)- ait ses groupements organiques orientés dans une direction, alors qu'une autre chaine les aura orientés dans la direction opposée. Les atomes d'Ag de chaque chaine se retrouvant à proximité (~ 3.20 Å), ils peuvent interagir entre eux et former des structures en feuillets suffisamment grandes et complexes pour mener à leur agrégation (Dance et al. 1991 cité dans Bell and Kramer 1999). De plus, Dance et al 1983 (cité dans Bell et al. 1997, Bell and Kramer 1999) ont observé que les chaines argent-thiols issues des complexes argent avec du 3-methylpentan-thiolate peuvent subir une torsion interne et ainsi former des structures en doubles hélices. Ces doubles hélices sont dues à la présence d'un angle à 90° entre les unités Ag-S-Ag. De ce fait, un twist est imposé sur le quatrième argent à cause des fragments de la molécule organique (Figure 1) (Bell et al. 1997, Dance et al. 1983).

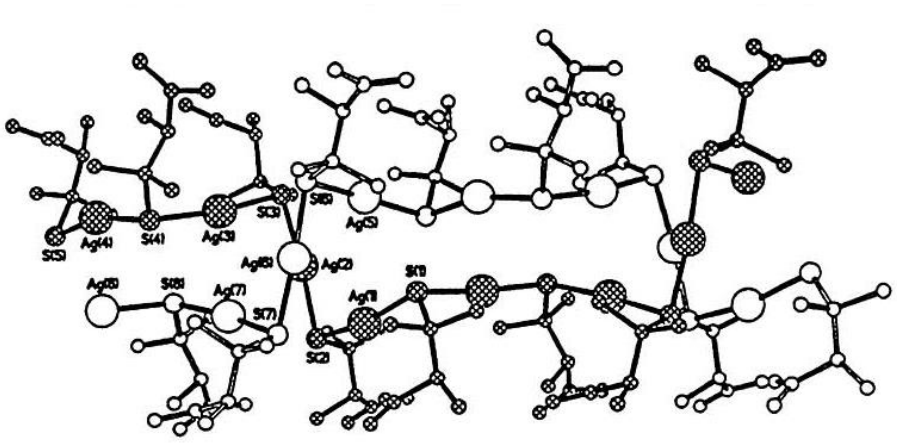

Figure 1 : Structure en double hélice entre deux penicillamines liées à l'argent (de Bell et al 1997)

Finalement, les travaux de Tunaboylu et Schwarzenbach (1971) (cités dans Adams and Kramer 1999) ont montré qu'à des concentrations en soufre supérieures à 10^{-4} mol*L^{-1} l'argent a une forte tendance à former des complexes polynucléaires avec les ligands thiols lorsque l'argent est présent à de fortes concentrations. En revanche, lorsque les concentrations en argent sont faibles, comme celles présentes dans l'environnement, alors les complexes mononucléaires seront prédominants.

Argent ionique et cystéine

L'une des molécules la plus simple et très répandue contenant des groupements thiols est la cystéine. Il s'agit d'un petit acide aminé soufré ($C_3H_7NO_2S$) présent dans de nombreux peptides et protéines. De plus, la dimérisation de la cystéine via des ponts disulfures forme la cystine qui est en partie responsable de la structure tertiaire des protéines. De nombreuses études ont observé la liaison de l'argent avec la cystéine (Alekseev et al. 2012, Bell et al. 1997, Komarov et al. 2008, Leung et al. 2013). Il est reconnu que la cystéine possède des propriétés réductrices. De ce fait, elle est capable de se lier aux ions Ag^+ via la formation d'une liaison covalente avec le soufre de son groupement thiol (Alekseev et al. 2012, Bell and Kramer 1999, Komarov et al. 2008). Ces liaisons forment des complexes dont trois formes sont majoritaires; AgHcys, Ag_2cys, et Ag_2Hcys^+ (Alekseev et al. 2012). Les études de Pakhomov et al. (2004) ont montré que le mélange de solutions de cystéine avec une solution d'$AgNO_3$ menait à la formation d'un gel, dont la force dépend des ratios argent : cystéine. En effet, à des concentrations en Ag^+ 1.4 à 1.5 fois supérieures aux concentrations de cystéine, la force des gels était maximale, alors qu'a des ratios de 1 : 1, ils ont observé la formation de précipités insolubles AgS-$CH_2CH(NH_2)$COOH, réaction déjà décrite par Cheronis (1954) (cité dans Pakhomov et al. 2004). L'induction de gels en présence d'Ag^+ et cystéine a été attribuée non seulement à la présence de thiolates d'argent via la formation d'une liaison soufre-argent, mais également leurs interactions entre eux afin de créer deux sortes d'oligomères (Komarov et al. 2008, Pakhomov et al. 2010). Dans le premier cas, les thiolates d'argent interagissent directement entre eux menant aux complexes $[Ag\text{-}S\text{-}Cys]_n$, alors que dans le second cas les thiolates d'argent se lient aux ions Ag^+ en excès [Ag-S-cys]…Ag+…[Ag-S-cys]. Ces oligomères peuvent se retrouver sous la forme de grappes de 1-100 nm qui mènent à la gélification du milieu. Ces grappes sont formées principalement via les interactions faibles entre l'argent (lié au thiol d'une cystéine par une liaison covalente) et le soufre d'une autre cystéine ; ainsi que grâce aux ponts hydrogène des groupements carboxyliques et amides des différents

thiolates d'argent. Bell et al. (1997) suggèrent que ces structures sont le résultat de la formation de longues colonnes composées de chaines en zigzag –Ag-S(R)-Ag-S(R)-.

Nanoparticules d'argent

Durant les deux dernières décennies, et avec l'apparition des nanotechnologies, un immense intérêt a été porté aux nanoparticules d'argent (AgNPs). Le terme nanoparticule est associé à toute particule dont au moins une dimension mesure entre 1 et 100 nm (ISO 2008). De manière générale, elles peuvent être constituées d'un seul élément et sont alors dites homogènes ; ou de différents éléments, on parle alors de nanoparticules hétérogènes. Dans les deux cas, elles peuvent être présentes dans l'air, les sols, les sédiments et l'eau.

De par leur petite taille, les AgNPs possèdent un grand rapport surface/volume, ce qui leur confère une grande réactivité ainsi que des propriétés physiques et chimiques exceptionnelles. Grâce à leur grande réactivité, les AgNPs ont une forte propension à interagir entre elles, mais également avec leur environnement et les molécules présentes dans le milieu (Carlson et al. 2008b, Maillard et al. 2004, SCENIHR 2014).

Les AgNPs font partie des nanoparticules d'origine anthropique les plus abondamment produites et utilisées (elles sont présentes dans plus de 400 produits) (Vance et al. 2015). Elles sont principalement employées pour leurs propriétés antibactériennes (Abdel Rahim and Ali Mohamed 2015b, Lara et al. 2009) et se retrouvent dans les produits d'utilisation courante tels les cosmétiques, les emballages alimentaires, les textiles, et certains produits médicaux afin de limiter la propagation de bactéries (SCENIHR 2014). La plupart des AgNPs sont constituées d'un noyau Ag^0 (Levard et al. 2012) ainsi que d'un recouvrement de molécules organiques, il s'agit le plus souvent de recouvrements citrate, polyvinylpyrrolidone (PVP), acide arabique, ou albumine. De plus, les AgNPs existent sous plusieurs formes et tailles ainsi que la présence ou non de différents recouvrements (Figure 2) (Levard et al. 2012).

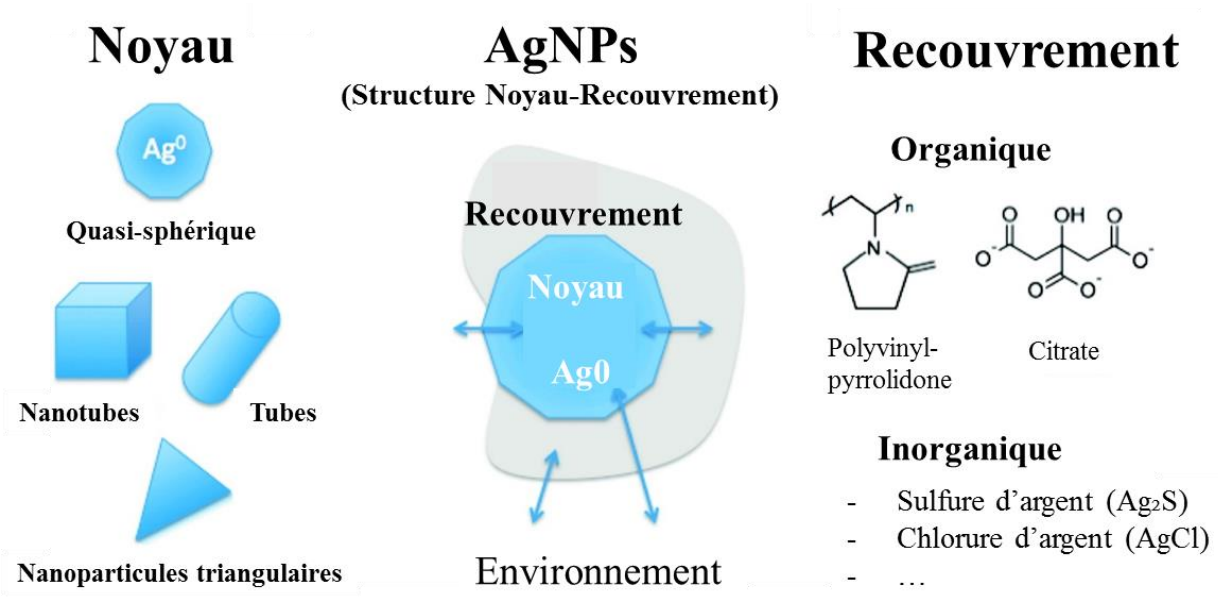

Figure 2 : Formes et recouvrements des AgNPs (modifié de Levard et al 2012)

À cause de leur importante utilisation pour leurs propriétés antibactériennes (notamment dans les textiles), les AgNPs sont libérées dans l'environnement, principalement via l'évacuation des eaux usées (SCENIHR 2014, USEPA 2009). Une fois dans l'environnement, elles pourront interagir avec les composantes du milieu et subir diverses transformations (Osterheld et al 2018, dans Kumar et al. 2018).

Transformations des AgNPs dans l'environnement : agrégation, dissolution, et oxydation/réduction

Les premiers types de transformation que peuvent subir les AgNPs sont les phénomènes d'agrégation. Ces phénomènes peuvent prendre deux formes, l'homoagrégation ou l'hétéroagrégation. L'homoagrégation s'observe lorsque des particules de même nature s'associent en une plus grosse particule, une sorte de granulat homogène. En revanche, l'hétéroagrégation se produit lorsque plusieurs nanoparticules de nature différente s'associent pour former un petit solide réunissant des constituants hétérogènes. Ces derniers sont alors constitués de NPs et d'un ou plusieurs autres types de particules de l'environnement. Dans les milieux naturels,

l'hétéroagrégation est largement dominante (Lowry et al. 2012b). L'agrégation des AgNPs peut modifier leur fixation à des surfaces tel le verre, des roches, des métaux mais aussi sur la matière organique en suspension et sur des organismes vivants (Handy et al. 2008, Smith et al. 2007). Le pH et la salinité de l'eau peuvent jouer un rôle important dans l'agrégation des AgNPs, qui s'assemblent plus dans l'eau salée que dans l'eau douce (Afshinnia and Baalousha 2017, Zhou et al. 2016). Les phénomènes d'agrégation peuvent également influencer le comportement des AgNPs en augmentant leur taille ce qui peut avoir des effets sur leur transport, sédimentation, réactivité, interactions et toxicité (Osterheld et al 2018, dans Kumar et al. 2018). Les phénomènes d'agrégation dans l'environnement sont donc des phénomènes particulièrement complexes qui dépendent de nombreuses forces ainsi que des variations de l'environnement et influencent le devenir des AgNPs.

Le deuxième groupe de transformations regroupe les phénomènes de dissolution des AgNPs. Il s'agit de processus qui dépendent du type et de la présence ou non d'un recouvrement, de l'état d'agrégation des NPs, et des conditions de l'environnement (pH, salinité, présence de macromolécules) (Dobias and Bernier-Latmani 2013, Liu and Hurt 2010b, Zhang et al. 2016a). En effet, la présence d'un recouvrement aura tendance à stabiliser les AgNPs et donc réduire leur dissolution. De plus, les recouvrements sont également capables de capter les ions relâchés par les AgNPs et ainsi limiter la présence d'argent ionique dans le milieu (Ostermeyer et al. 2013). La dissolution des AgNPs, aboutira à une diminution de la taille des particules ainsi qu'à la libération d'ions issus des NPs, modifiant leurs propriétés chimiques et toxicologiques (Dobias and Bernier-Latmani 2013, Li et al. 2010a).

L'une des causes de la dissolution des AgNPs sont les phénomènes d'oxydation/réduction (Peretyazhko et al. 2014, Zhang et al. 2016b). Ce sont des processus chimiques durant lesquelles un électron est transféré de la forme réduite du matériau, vers sa forme oxydée. De manière générale, les zones oxygénées favorisent le plus souvent les réactions d'oxydation, alors que les zones plus anaérobiques favorisent la réduction des éléments (Lowry et al. 2012b). Dans les milieux dynamiques tels les zones tidales, les réactions redox peuvent être cycliques et les AgNPs peuvent subir plusieurs états de réduction et d'oxydation.

Nanoparticules d'argent avec le soufre inorganique

Tout comme pour les ions argent, il a été démontré que les AgNPs sont capables d'interagir et se lier avec le soufre inorganique présent dans leur environnement. Kim et al (2010) ont observé la présence de nanoparticules d'argent soufrées (Ag_2S) dans les boues de stations d'épuration. Ils ont alors suggéré que ces nanoparticules soufrées étaient soit le résultat des interactions d'argent ionique avec le soufre ou des AgNPs qui ont subi des phénomènes de réduction de l'argent présent à leur surface par le soufre présent dans les eaux des égouts. Les nanoparticules sont ainsi recouvertes d'une couche d'Ag_2S fortement insoluble et vont par la suite sédimenter pour se retrouver dans les boues d'égouts. Il a été démontré que la sulfuration des AgNPs dans les stations d'épuration se produit principalement dans les zones anaérobiques où les concentrations de soufre y sont plus importantes (Kent et al. 2014). Néanmoins, la sulfuration des AgNPs peut également se produire dans les zones aérobies bien que le soufre y soit présent sous des formes différentes. Des études de laboratoire ont démontré que la sulfuration des AgNPs n'est pas un processus uniforme; c'est pourquoi des AgNPs partiellement recouvertes de soufre sont toujours capables de libérer des ions Ag^+ (Kent et al. 2014, Levard et al. 2011b, Liu et al. 2011). Il a été observé que la sulfuration des AgNPs peut mener à la formation de nano-ponts de Ag_2Sentre les nanoparticules (Levard et al. 2011b, Reinsch et al. 2012). La présence et la taille de ces ponts dépendent de la concentration de soufre présente et mènent à la formation de structures fractales. Ces modifications altèrent les propriétés de surface des nanoparticules telles que leur charge ainsi que leur vitesse de dissolution. Levard et al (2011b) ont suggéré que la formation des ponts Ag_2S est le résultat de l'oxydation des AgNPs menant à la libération d'ions Ag^+ qui vont par la suite réagir avec le soufre présent dans le milieu et ainsi re-précipiter afin de former des ponts Ag_2S entre les nanoparticules. En effet, il a été démontré que pour être sulfuré, les AgNPs peuvent au préalable subir un phénomène d'oxydation (Levard et al. 2012, Levard et al. 2011b, Liu et al. 2011). La sulfuration des AgNPs requière l'oxydation de deux Ag afin qu'ils réagissent avec S^{2-} et forment Ag_2S. L'oxydant majoritaire en solution étant l'oxygène dissout les phénomènes d'oxydosulfuration peuvent être décrits comme suit (Liu et al. 2011).

$$4Ag + O_2 + 2H_2S \rightarrow 2Ag_2S + 2H_2O$$

ou

$$4Ag + O_2 + 2HS^- \rightarrow 2Ag_2S + 2HO^-$$

De plus, il a été observé que la sulfuration des AgNPs dépendait des concentrations de soufre présent et que deux voies de formation différentes existent dépendamment des concentrations en soufre (Figure 3).

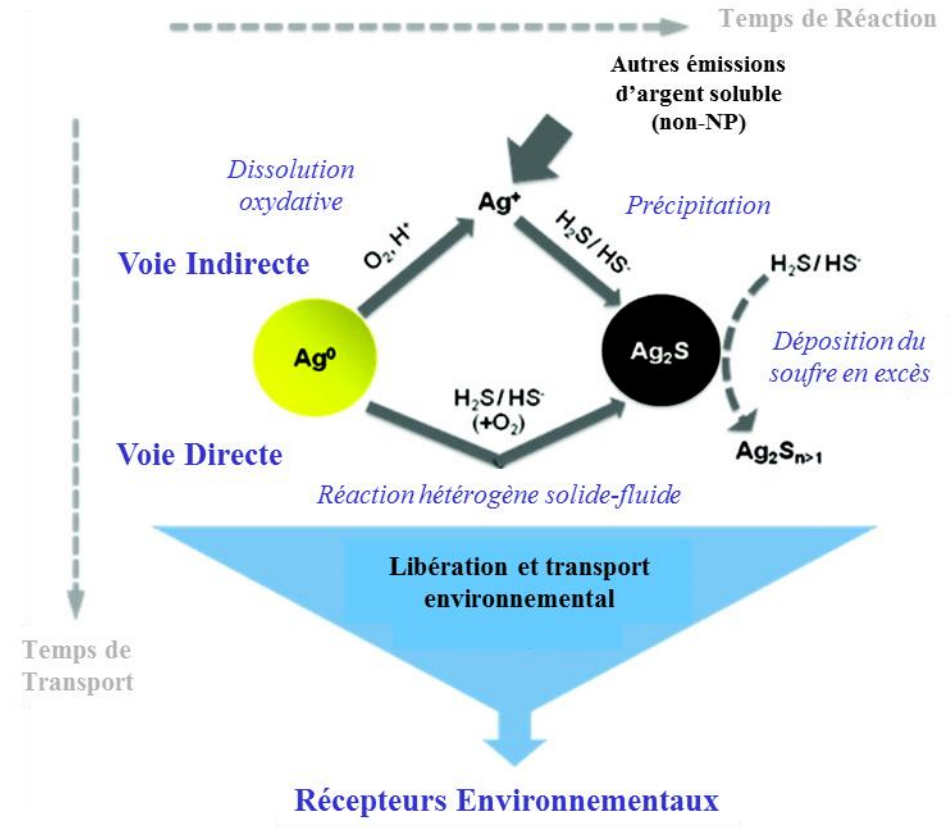

Figure 3 : Voies de sulfatation des AgNPs (modifié de Liu et al 2011)

La première voie est la voie indirecte qui se produit lorsque les concentrations en soufre sont faibles. Dans ce cas-là, les AgNPs subissent un premier phénomène d'oxydation/dissolution en ions intermédiaires Ag^+ suivi par la liaison entre les ions argent et le soufre présents en solution ce qui aboutit ultimement à la formation de nanoparticules d'argent Ag_2S-NPs. La seconde voie est directe et a lieu lorsque les concentrations en soufre sont élevées. L'oxydosulfuration se passe alors directement via une réaction particule-fluide et le soufre réduit se fixe directement à la surface des AgNPs (Liu et al. 2011). Lowry et al 2012 ont observé que dans des conditions

environnementales, l'oxydosulfuration des AgNPs est plus lente que dans des conditions de laboratoire. Ils ont attribué cela au plus faible ratio soufre/Ag ainsi qu'à la présence d'autres métaux dans l'environnement pouvant entrer en compétition avec les AgNPs pour le soufre. De plus, le manque d'oxygène présent dans certaines zones de l'environnement peut également jouer un rôle dans cette différence, en limitant l'oxydation des AgNPs. Leurs résultats suggèrent donc que la voie majoritaire de sulfuration des AgNPs dans l'environnement serait la voie indirecte.

Il est généralement admis que des AgNPs possédant à leur surface une couche d'Ag_2S sont fortement insolubles et stables de par la forte insolubilité de l'acanthite, un sulfure d'argent (Djoković et al. 2009, Kent et al. 2014, Kim et al. 2010, Levard et al. 2012). Cependant, certaines études récentes ont montré que des Ag_2S-NPs pouvaient malgré tout subir des phénomènes d'oxydation et ainsi libérer des ions Ag^+. Le traitement de Ag_2S-NPs avec de l'ozone peut notamment induire l'oxydation et la dissolution des nanoparticules et ainsi libérer des ions Ag^+ dans le milieu (Thalmann et al. 2015). De plus, Li et al. (2016b) ont observé que les Ag_2S-NPs subissent de l'oxydation lorsqu'elles sont en présence de Fe(III). Cette oxydation a été attribuée à la présence de radicaux hydroxyles formés lors de la réduction de Fe(III) en Fe(II) en présence de lumière, les ions Ag^+ sont par la suite réduits en Ag grâce au fort pouvoir réducteur de Fe(II). De façon plus générale, il est possible de dissoudre Ag_2S en présence d'un couple oxydo-réducteur très puissant.

Nanoparticules d'argent et soufre organique : cystéine

Les AgNPs sont capables de se lier au soufre organique, notamment le soufre présent sous forme de groupements thiols tels que celui de la cystéine. De ce fait, la cystéine peut servir de molécule de recouvrement pour les AgNPs et permettre leur stabilisation (Mandal et al. 2001, Varghese et al. 2009). En outre, les AgNPs possédant déjà un recouvrement pouvant se lier à la cystéine libre en solution (Csapó et al. 2012, Gondikas et al. 2012). La liaison de la cystéine aux AgNPs peut mener au remplacement de leur recouvrement initial par l'acide aminé et ainsi modifier leur comportement et propriétés de surface (Gondikas et al. 2012, Toh et al. 2014). De plus, le pH de la solution de AgNPs-cystéine peut influencer le comportement des AgNPs (Baranova et al. 2016, Csapó et al. 2012). Baranova et al. (2016) ont observé qu'à pH < 4 une solution contenant des AgNPs-cystéine devient visqueuse. Ils ont attribué ce phénomène à la formation de polymères de supramolécules. Avec l'augmentation du pH, les polymères de supramolécules commencent à

former des agrégats et la viscosité diminue. Ces agrégats sont issus de la liaison covalente des AgNPs à la cystéine via son groupement thiol puis à la formation de ponts hydrogène entre le groupement carboxyle d'une cystéine liée à une AgNP et le groupement amine d'une autre molécule de cystéine liée à une autre AgNP (Figure 4) (Choi et al. 2003, Csapó et al. 2012, Mandal et al. 2001). Ces résultats sont en accord avec les observations de Csapo et al. (2012) qui ont démontré qu'à 3< pH < 7, les AgNPs recouvertes de cystéine subissent des phénomènes d'agrégation. En effet, lorsque le pH se situe entre ces valeurs, le groupement carboxyle de la cystéine est négativement chargé (COO^-) et le groupement amine est positif (NH_3^+), induisant l'attraction et la liaison de molécules de cystéine entre elles et l'agrégation des nanoparticules. En revanche, lorsque le pH est inférieur à 3, le groupement carboxyle est protoné (COOH) et si le pH est supérieur à 7 le groupement amine perd une charge (NH_2). Dans les deux cas, il y a répulsion électrostatique entre les molécules de cystéine et inhibition de l'agrégation de AgNPs

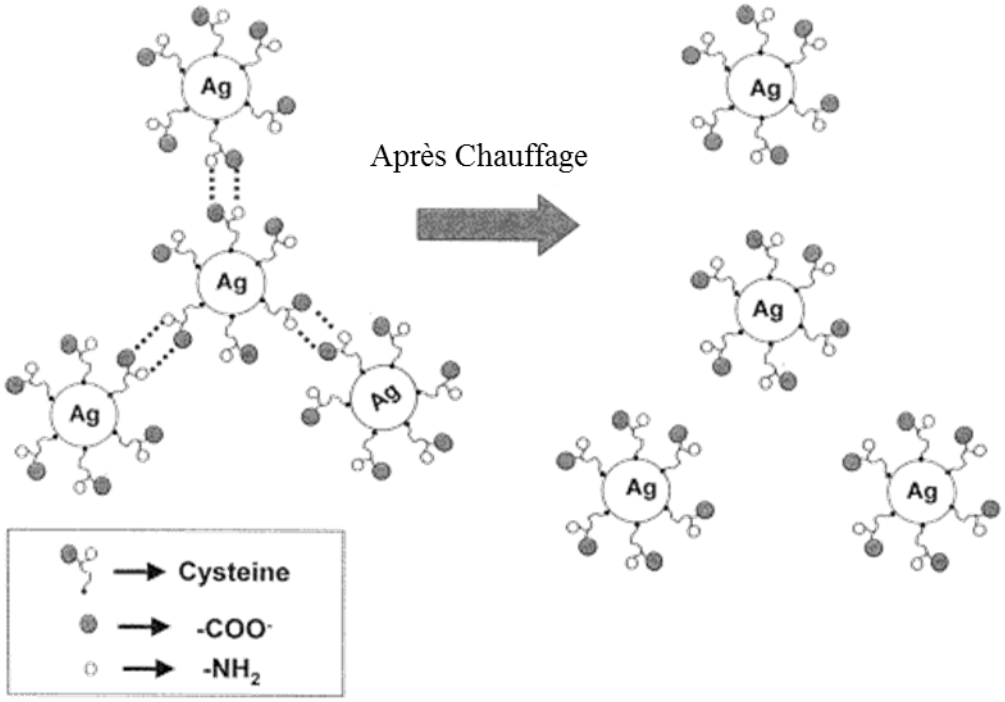

Figure 4 : Agrégats d'AgNPs via la formation de ponts hydrogène entre des groupements carboxyles et amines de molécules de cystéine (modifié de Mandal et al. 2001)

En outre, Gondikas et al. (2012) ont démontré que la présence de cystéine peut augmenter la dissolution des AgNPs recouvertes de citrate ou PVP. Dans leur étude, en l'absence de cystéine

10% de l'argent total est présent sous forme ionique alors que suite à l'ajout de l'acide aminé, jusqu'à 47% de l'argent s'est dissout. De plus, la composition du recouvrement de surface modifie le taux ainsi que la vitesse de dissolution des AgNPs. De fait, leurs résultats montrent que la vitesse de dissolution des AgNPs-citrate est trois fois plus rapide que celle des AgNPs-PVP.

Nanoparticules d'argent et protéines : formation d'une couronne

La sorption de protéines à la surface des AgNPs via leurs groupements sulfures peut mener à la formation d'une couronne de protéines à la surface des AgNPs. La présence d'une telle couronne sur les NPs modifie leur taille ainsi que leur composition/recouvrement de surface, conférant ainsi de nouvelles propriétés physiques et biologiques aux AgNPs. Ceci affectera alors les réponses physiologiques, l'absorption cellulaire, l'activation de voies métaboliques, le transport, l'accumulation et la toxicité des nanoparticules au sein des organismes (Nel et al. 2009b, Rahman et al. 2013). De nombreux paramètres affectent la composition, l'épaisseur et la conformation des protéines de la couronne, tels que la taille, la forme, la courbure, la solubilité des AgNPs, ainsi que leurs voies d'administration et la nature de l'environnement dans lequel se trouvent les NPs.

Il est admis que lors de l'entrée de NPs dans un milieu physiologique contenant des protéines, les NPs vont immédiatement être recouvertes de protéines. Selon l'effet Vroman, les protéines les plus abondantes dans le milieu vont dans un premier temps recouvrir les NPs, puis elles seront par la suite remplacées par des protéines moins abondantes mais possédant une meilleur affinité pour les AgNPs (Devineau et al. 2013, Vroman et al. 1980). Ces phénomènes sont dynamiques, et à tout moment une protéine peut se désorber des NPs pour être remplacer par une autre.

De manière générale, deux types de couronne sont décrits : (1) les couronnes "dures" (hard coronna), constituées de protéines possédant une forte affinité pour les NPs et qui restent fixées aux particules; (2) les couronnes "molles" (soft coronna) qui sont habituellement rapidement formées, mais avec des protéines de plus faible affinité et qui se désorbent facilement (Devineau et al. 2013, Rahman et al. 2013).

En outre, l'interaction d'une protéine sur une NP et la formation d'une couronne mène très souvent à des modifications ou à des pertes plus ou moins importantes de la structure des protéines (Devineau et al. 2013, Rahman et al. 2013).

L’albumine

Dans les tissus biologiques des vertébrés, l’une des protéines la plus abondantes est l’albumine qui représente près de 60% des protéines du plasma sanguin avec des concentrations avoisinant 40 mgL^{-1}, un diamètre de 8.5 nm et un poids moléculaire de 66.5 kDa (Dockal et al. 1999, Kiselev et al. 2001, Kratz 2008, Mariam et al. 2014). L’albumine est synthétisée par le foie et sa demi-vie est de 19 jours (Kratz 2008). Cette protéine a de nombreuses fonctions et joue un rôle essentiel dans le métabolisme de lipides, la liaison et le transport de nombreuses molécules, notamment celles possédant des groupements thiols. L’albumine peut également se lier à des métaux comme le zinc, le cuivre, le calcium et le nickel présents dans le sang. Elle est importante pour la régulation de la pression osmotique et la régulation du pH sanguin (Dockal et al. 1999).

Le sérum d’albumine humain (HSA) est structurellement très proche du sérum d’albumine bovin (Figure 5). Il est composé de trois domaines homologues en hélice (I, II, III). Chaque domaine est constitué de deux sous-domaines appelés IA, IB, IIA, IIB, IIIA, IIIB (Dockal et al. 1999, Ghuman et al. 2005). Ce sont ces sous-domaines qui sont responsables des interactions de HSA avec d’autres molécules. Il est admis qu’il y a dans les sous-domaines IA et IIIA des sites de liaison de fortes affinités pour les petits acides aminés hétérocycliques ou aromatiques (Dockal et al. 1999, Sudlow et al. 1975). Les sous-domaines IB et IIIB possèdent des sites de liaison pour les acides gras à longues chaines (Carter and Ho 1994).

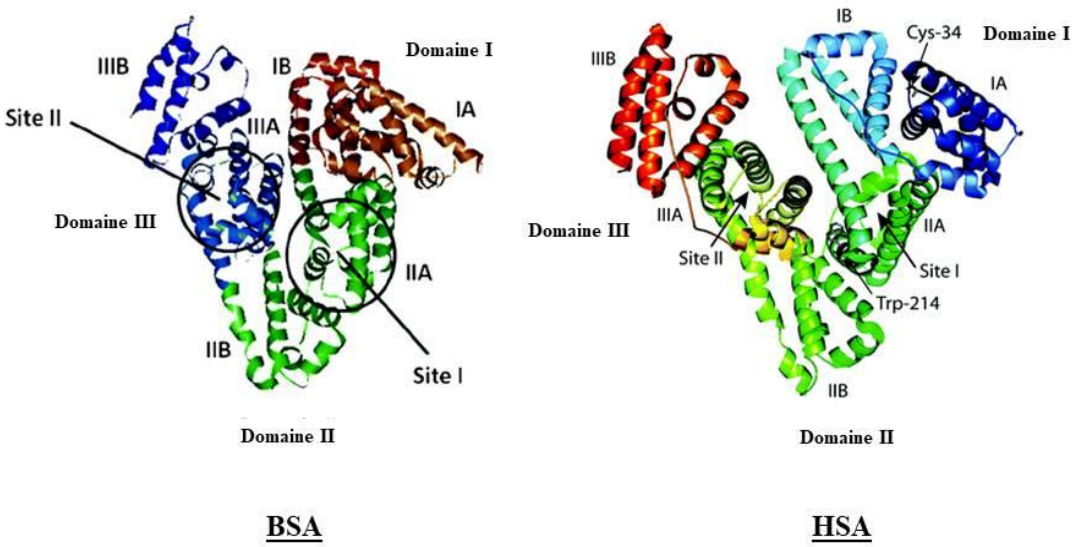

Figure 5 : Représentation de la structure tertiaire des protéines d'albumine bovine (BSA) et humaine (HSA) (modifié de Patra et al. 2012 et Park et al. 2009)

En outre, quatre sites de liaison des métaux ont été décrits chez l'albumine. 1) Le site N-terminal (NTS) qui est composé de la chaîne Asp-Ala-His. Son principal rôle est de lier le cuivre et le nickel (Bal et al. 2013, Kozłowski et al. 1999). 2) Le site A est un site capable de lier plusieurs métaux et est situé à l'interface des domaines I et II et il est le site de liaison préférentiel pour le Zn(II) ainsi qu'un excellent site de liaison pour le Cd et peut également lier le cuivre bien que moins efficacement que le NTS (Bal et al. 1998, Bal et al. 2013, Martins and Drakenberg 1982, Sadler and Viles 1996). 3) La localisation de ce site n'est pas encore clairement définie, mais il est capable de lier le cadmium avec autant d'affinité que le site A bien que son affinité pour le zinc soit nettement inférieur. Il semble être le site préférentielle pour la liaison de Mn(II) (Bal et al. 1998, Bal et al. 2013, Sadler and Viles 1996). 4) Le dernier site de liaison des métaux est constitué de la cystéine 34 (cys34) et son environnement. Le groupement cys34 représente la seule cystéine dont le groupement thiol est libre dans l'HSA; les autres cystéines servant à former la structure de la protéine via des ponts disulfure entre les thiols des cystéines (Bal et al. 2013). Le site cys34 est le seul site de liaison de l'or sur HSA. Cette liaison se produit via une réduction du métal (Bal et al.

2013, Messori et al. 2011, Shaw III 1989). De plus, grâce à son thiol libre le groupement cys34 joue un rôle essentiel dans la liaison de molécules contenant des thiols.

Albumine et argent ionique

De par sa forte abondance et ses nombreux sites de liaison des métaux, l'albumine peut interagir avec l'argent présent dans les tissus sanguins. Shen et al. (2003) ont décrit deux types de sites de liaison des ions Ag^+ sur l'albumine. Il y a quatorze sites de liaison à forte affinité pour l'argent et de nombreux sites de faible affinité. Le mode de liaison de l'argent à l'albumine dépend des concentrations du métal en solution (Zhao and Wang 2011). À faibles concentrations en argent, des liaisons hydrogène et forces de van der Waals sont impliquées, alors qu'à fortes concentrations il s'agit d'associations électrostatiques. En outre, la liaison des ions Ag^+ induit des changements structuraux sur la protéine d'albumine. Une diminution des ratios d'hélices alpha et une augmentation des feuillets beta ainsi que des modifications du squelette ont été observées (Shen et al. 2003, Zhao and Wang 2011). Il a été suggéré que ces modifications lors de la liaison avec l'argent pouvaient présenter de nouveaux sites et ainsi faciliter la liaison de plus d'argent sur la protéine (Shen et al. 2003). Ces phénomènes se produisent lorsque la protéine se "desserre" et présente ses régions hydrophobes dans un environnement polaire (Zhao and Wang 2011). La liaison avec l'argent pourra également perturber le microenvironnement proche des résidus d'acides aminés aromatiques (tryptophane, tyrosine) ainsi que les ponts disulfures de la protéine (Shen et al. 2003). De leurs résultats, il est conclu que l'argent ionique peut se lier au soufre issu de la cys34, mais également aux acides aminés méthionines présents dans la protéine et des ponts disulfures. L'argent peut aussi se lier à l'azote issu de peptides déprotonés, d'imines et d'indoles. De plus, les modifications résultant de la liaison de l'argent à l'albumine peuvent être dues à la destruction de liaisons qui maintiennent la structure de la protéine, tels que les ponts disulfures et d'autres liaisons plus faibles (Shen et al. 2003, Zhao and Wang 2011).

Albumine et nanoparticules d'argent

L'albumine est capable de chélater les ions argent, mais également de se lier directement à la surface des AgNPs (Ostermeyer et al. 2013). Lorsqu'elle est présente à forte concentration, elle peut remplacer les recouvrements présents à la surface des AgNPs et ainsi modifier leur comportement (MacCuspie 2011, Ostermeyer et al. 2013). L'adsorption de l'albumine à la surface

des AgNPs peut mener à la formation d'une couronne. Deux modèles de liaison de l'albumine aux AgNPs ont été proposés. Le modèle "side-on", c'est-à-dire sur le côté de la protéine, fait en sorte qu'il y a formation de nombreux ponts de basse énergie entre la surface des AgNPs et des molécules d'albumine (Ostermeyer et al. 2013). La liaison "end-on" a également été proposée, qui peut mener à une plus grande couverture d'albumine à la surface des AgNPs (Dasgupta et al. 2016, Ravindran et al. 2010).

L'adsorption des protéines à la surface des AgNPs se passe en deux temps. Elle est d'abord rapide puis ralentit progressivement, ce qui se traduit par des constantes de vitesse de réaction différentes (Dasgupta et al. 2016, Ravindran et al. 2010). Dasgupta et al. (2016) suggèrent que cela peut être dû à un nombre limité de sites libres pendant la phase initiale de la réaction et que les sites restant sont plus difficiles à atteindre. De ce fait, la réaction prendrait plus de temps. En opposition à cette hypothèse, les résultats de Zhang et al. (2015) n'ont montré qu'un seul site de liaison des AgNPs sur l'albumine.

Les liaisons entre les AgNPs et l'albumine résultent de processus spontanés issus d'interactions hydrophobes et électrostatiques (Ali et al. 2015, Mariam et al. 2011, Roy and Das 2014, Wang et al. 2017). Les résultats de Guo et al. (2015) montrent que les interactions entre les AgNPs et l'albumine sont régies par des forces de van der Waals et la formation de ponts hydrogène. De plus, ces réactions suivent une cinétique de réaction de pseudo-second ordre et une isotherme de Freundlich différente de 1, indiquant la présence de plusieurs sites d'absorption (Dasgupta et al. 2016, Ostermeyer et al. 2013, Ravindran et al. 2010, Wang et al. 2017). Ceci peut indiquer que l'adsorption de l'albumine se produit en plusieurs couches (Ravindran et al. 2010, Wang et al. 2017).

Des mesures de fluorescence de solutions contenant de l'albumine et des AgNPs ont montré que les AgNPs peuvent réduire la fluorescence de l'albumine de deux manières (Dasgupta et al. 2016, Guo et al. 2015, Roy and Das 2014, Zhang et al. 2015). Par une réduction dynamique, il y a collision entre les AgNPs et l'albumine, ce qui va diminuer la fluorescence de la protéine. Via une réduction statique, les AgNPs et l'albumine se lient et forment un complexe à un état fondamental non fluorescent. En outre, tout comme avec l'argent ionique, les interactions des AgNPs sur l'albumine modifient le microenvironnement de la protéine. Ceci engendre une réduction des hélices alpha et une augmentation des feuillets beta de la protéine (Ali et al. 2015, Iranfar et al.

2012, Mariam et al. 2014, Zhang et al. 2015) ce qui pourra altérer le squelette de la protéine (Guo et al. 2015). Cette déformation de la protéine expose les acides aminés hydrophobes et change ainsi le comportement et les liaisons qu'elle peut faire, notamment en exposant certains groupements situés dans des poches hydrophobes (Liu et al. 2009).

Mariam et al. (2014) ont observé une diminution significative du nombre de thiols libres lors de la liaison de l'albumine humaine avec les nanoparticules d'argent. Ceci suggère que le groupement cys34 de l'albumine est affecté par la liaison de la protéine aux AgNPs. Cependant, cette diminution des thiols libres est moins importante avec de l'albumine bovine (Guo et al. 2015). De plus, Gebregeoris et al. (2013) ont observé une diminution de la disponibilité des ponts disulfures lors de la liaison des AgNPs avec l'albumine. Cette perte de disponibilité n'est pas observée avec les ions Ag^+. De fait, ils suggèrent que les ponts disulfures sont potentiellement impliqués dans les interactions AgNPs-albumine. La liaison des AgNPs à l'albumine peut modifier le comportement et les propriétés physico-chimiques de la protéine. Ces liaisons vont notamment réduire l'efficacité catalytique de la protéine (Mariam et al. 2014) comme suggéré pour la liaison des AgNPs aux résidus lysine de la protéine modifiant ainsi sa structure secondaire. La réduction de l'activité catalytique de l'albumine n'est pas observée en présence d'ions Ag^+ de sorte que ce phénomène a été attribué à un effet direct des AgNPs. En opposition à cette perte d'activité catalytique, Iranfar et al. (2012) ont démontré que la présence d'AgNPs pouvait augmenter les capacités de liaison de l'albumine pour certaines molécules. En effet, ils ont observé que l'ajout d'AgNPs permettait une meilleure exposition du groupement tryptophane permettant ainsi à la molécule de ciprofloxacin de former des complexes plus stables avec l'albumine.

Il est à noter que le pH physiologique correspond à un pH supérieur au point isoélectrique de l'albumine (4.5 et 4.9). Ainsi, de nombreux groupes fonctionnels de la protéine sont négativement chargés, notamment les soixante groupements lysines, qui peuvent former des interactions attractives de Coulomb avec les AgNPs (Dasgupta et al. 2016, Ravindran et al. 2010).

La taille des AgNPs joue également un rôle important dans les interactions AgNPs-albumine ainsi que les modifications physico-chimiques de la protéine qui en résultent. Les petites AgNPs ont de plus fortes interactions avec l'albumine que les grosses nanoparticules et peuvent ainsi plus altérer la structure et le comportement de la protéine (Zhang et al. 2015). Iranfar et al. (2012) ont montré que des AgNPs de différentes tailles ont des comportements différents lors de l'induction de

complexes HSA-ciprofloxacin, indiquant que le cirpofloxacin a de plus fortes affinités pour HSA lorsque les AgNPs sont grandes.

Des phénomènes d'agrégation des particules AgNPs-albumine ont été observés et attribués à des changements structuraux de la protéine menant à une diminution des répulsions stériques (Mariam et al. 2011). Cependant, à cause de la forte affinité de l'albumine pour l'argent, la liaison de cette dernière à la surface des AgNPs peut également augmenter leur vitesse de dissolution afin de lier les ions Ag^+ (Ostermeyer et al. 2013). Les ions libérés vont se fixer sur les groupements thiols de l'albumine. Il a été suggéré que la chemisoption de ces ions sur l'albumine peut mener à de l'instabilité des AgNPs et augmenter leur dissolution (Ostermeyer et al. 2013).

Des méthodes spectroscopiques ont été développées afin d'analyser et mesurer les interactions de composés entre eux. Ces méthodes comprennent de la spectroscopie Raman, ainsi que la spectroscopie de fluorescence. Ces deux techniques peuvent être utilisées pour analyser les interactions de l'argent ionique ou nanoparticulaire avec des composés organiques (Diaz Fleming et al. 2009, Iranfar et al. 2012, Mariam et al. 2011, Martina et al. 2012, Miškovský et al. 1998).

Techniques d'étude des interactions argent soufre

Spectroscopies Raman et fluorescence

Les techniques de spectroscopie sont d'excellents outils permettant de mieux comprendre le comportement de liaison entre une molécule organique et un autre composé. Ces techniques reposent sur le principe que les molécules possèdent un état fondamental stable qui peut être excité via l'absorption de photons menant à différents états d'excitation. La spectroscopie permet de mesurer l'émission de photons lors du retour d'un état excité à l'état fondamental. La spectroscopie Raman est basée sur la diffusion inélastique des photons et mesure l'excitation ou la relaxation vibrationnelle des molécules (Notingher 2007, Smekal 1923). Cette diffusion fait suite à l'excitation d'une molécule cible. Les changements d'énergie des photons diffusés correspondent aux niveaux d'énergie vibrationnels de la molécule. De plus, chaque groupe fonctionnel d'une molécule possède des énergies de vibration qui leur sont propres de sorte que chaque molécule possède son propre spectre Raman. La spectroscopie Raman est utile afin d'analyser quels groupes fonctionnels sont affectés lors de la liaison de deux molécules entre elle. En effet, lors de la liaison les groupes fonctionnels sont modifiés et leur énergie vibrationnelle change. Ces phénomènes sont

comparables à des ressorts sous tension où les ressorts représentent les liaisons chimiques qui forment les molécules. Lorsqu'une nouvelle liaison est formée, la tension sur les ressorts change de même que la diffusion vibrationnelle est modifiée. Ainsi, en comparant le spectre Raman d'une molécule seule avec le spectre de la molécule lié avec un autre composé, il est possible de déterminer quels sont les groupements fonctionnels affectés.

La spectroscopie de fluorescence permet d'étudier les interactions entre des sondes et des protéines cibles. Le principe de fluorescence consiste à exciter l'échantillon à l'aide de photons pour le faire passer de son état fondamental à un état excité stable de plus faible énergie. Cet échantillon va ensuite passer par différentes étapes de relaxation afin d'atteindre un état excité minimal. Une fois ce niveau d'excitation minimal atteint, l'échantillon va retourner à son état fondamental via l'émission de photons sous forme de fluorescence (Figure 6). Lors de l'analyse d'interactions avec des protéines, cette technique repose sur l'émission de fluorescence de certains acides aminés aromatiques suite à l'absorption de lumière ultra-violette. Les acides aminés en question sont le tryptophane (Trp), la tyrosine (Tyr) et la phénylalanine (Phe). Le Trp étant l'acide aminé émettant le plus fort signal de fluorescence (Albrecht 2008, Masters 2008). Ces acides aminés sont très sensibles aux changements de leur microenvironnement ainsi qu'aux modifications de structures de la protéine. Ainsi, le signal de fluorescence va varier avec les modifications de la conformation des protéines lors de leur liaison. La comparaison des signaux de fluorescence de l'échantillon en présence ou en absence de la sonde permet de donner des informations concernant le niveau de liaison moléculaire entre la sonde et la protéine, les constantes de liaison, et les mécanismes de liaison (Albrecht 2008, Masters 2008).

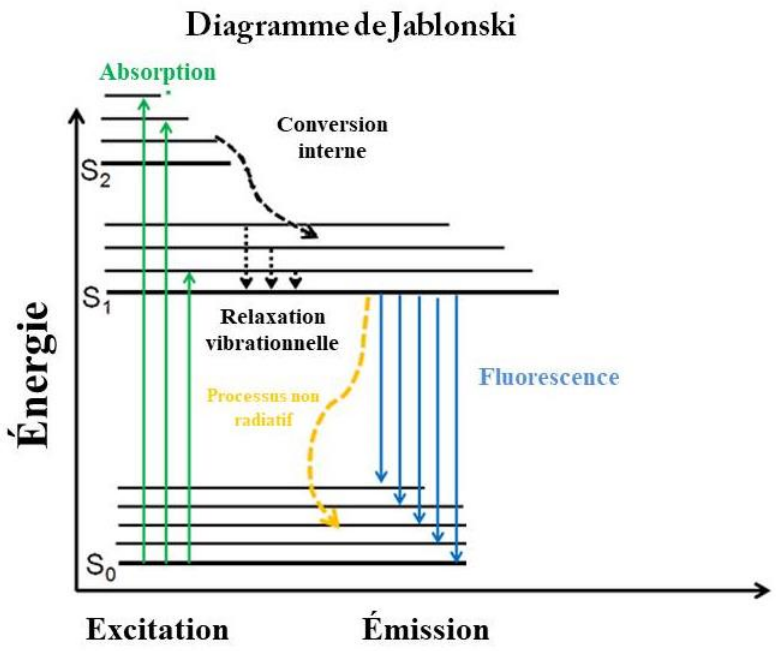

Figure 6 : Principe de la fluorescence et diagramme de Jablonski modifié de http://www.ibs.fr/recherche/groupes-de-recherche/groupe-dynamique-et-cinetique-des-processus-moleculaires-m-weik/pixel/photophysique-des-proteines (consulté le 05.09.2017)

Les objectifs des travaux

Le but principal de cette étude fut d'analyser les interactions des AgNPs et des ions Ag^+ avec des composés organiques soufrés ainsi que déterminer l'importance du soufre dans ces interactions. Le premier objectif était donc d'observer les interactions entre l'argent ionique ou les AgNPs avec un petit acide aminé soufré (la cystéine) et de déterminer l'existence de potentiels sites de liaison entre les formes d'argent et la cystéine. Ceci a été effectué à l'aide d'expériences de spectroscopie Raman Le second objectif fut d'analyser, par mesure de fluorescence, les interactions des AgNPs avec des protéines complexes en utilisant l'albumine comme protéine modèle. Finalement, le dernier objectif fut de comparer les effets des ions Ag^+ sur la cystéine et l'albumine, aux effets des AgNPs sur ces mêmes molécules soufrées. À ce jour et à notre connaissance, cette étude est la première à comparer directement l'importance des effets des AgNPs et des ions Ag^+ sur la cystéine et l'albumine, et à clairement démontrer un effet conjointdes ions Ag^+ et des AgNPs.

Ce mémoire est constitué d'une introduction générale en français, suivie d'un chapitre intitulé "Effects of silver nanoparticles on biological sulfated organic compounds" et un second chapitre intitulé "Nanotoxicity of silver particles : from environmental spill to effects on organisms", et une conclusion générale. Le chapitre 1couvre les résultats obtenus lors du projet de recherche, avec une interprétation des résultats ainsi qu'une discussion. Quant au chapitre 2, il s'agit d'une revue de littérature concernant les transformations et interactions des AgNPs dans le milieu marin et avec les organismes qui s'y trouvent (bactéries, algues, zooplancton, macro-invertébré, et poissons).

CHAPITRE 1

EFFETS DES NANOPARTICULES D'ARGENT SUR LES COMPOSÉS ORGANIQUES SOUFRÉS D'ORIGINE BIOLOGIQUE

1.1. RESUMÉ EN FRANÇAIS

Les AgNPs sont connues pour avoir une forte affinité envers les composés soufrés qu'ils soient organiques ou inorganiques. De plus, les AgNPs peuvent intervenir dans divers processus métaboliques et physiologiques chez les organismes vivants. L'absorption de nanoparticules d'argent par les organismes vivants peut mener à leur présence dans le sang où elles peuvent réagir avec les composés présents, et particulièrement avec les molécules contenant du soufre. Dans le sérum sanguin, l'albumine est la protéine la plus abondante, de même qu'un transporteur important des métaux. Cette protéine possède notamment un groupement cys34 avec un groupement thiol libre fortement réactif et reconnu pour se lier aux métaux. Ce groupement cys34 libre représente d'ailleurs la majorité des groupements thiols dans le système sanguin. C'est pourquoi on suppose que les AgNPs présentes dans le biofluide interagissent avec la protéine via le groupement cys34 et peuvent induire des modifications de la protéine.

Des mesures de spectroscopie RAMAN ont mis en évidence l'importance du soufre pour les interactions entre argent et cystéine, supportant ainsi l'hypothèse d'un rôle majeur du groupement cystéine libre (cys34) de l'albumine dans les interactions de la protéine avec les nanoparticules d'argent. Les résultats obtenus par l'utilisation de la fluorescence EEMF (Excitation Emission Matrix Fluorescence) de l'albumine en présence de concentrations croissantes d'AgNPs ou d'ions argent ont montré un effet plus important des ions argent sur l'albumine que ceux des AgNPs. Cependant, cette étude a pour la première fois, à notre connaissance, démontré des impacts directs des nanoparticules d'argent sur l'albumine et également suggéré des effets additifs des ions Ag^+ et des nanoparticules sur la fluorescence de la protéine. La présence de plusieurs sites probables d'interaction entre les AgNPs et/ou ions argent sur l'albumine ont également été mis en évidence.

Cet articles intitulé "*Effects of silver nanoparticles on sulfur-containing organic compounds from biological origin*" est le fruit de mon travail dans lequel j'ai réalisé l'essentiel de la recherche, du développement de la méthode à l'interprétation des résultats jusqu'à la rédaction de la première version de l'article. Les professeurs Jean-Pierre Gagné et Émilien Pelletier ont fourni l'idée originale du projet ainsi que leur aide et conseils tout au long du projet. Ils ont également pris part à la relecture et la correction de l'article.

1.2. EFFECTS OF SILVER NANOPARTICLES ON SULFUR-CONTAINING ORGANIC COMPOUNDS FROM BIOLOGICAL ORIGIN

1.3. INTRODUCTION

During the last decade, the increasing use of AgNPs for their antibacterial properties has led to their release in the environment. Once in the environment, AgNPs could interact with surrounding inorganic and organic compounds, thus altering their physical and chemical behavior. One of the major transformations of AgNPs with environmental compounds is their interactions with sulfur molecules.

Silver can interact with organic sulfur present in thiol groups (Alekseev et al. 2012, Cecil 1950, Dance et al. 1983, Hillman et al. 2007, Pakhomov et al. 2004). Indeed, it has been observed that silver ions strongly bind to the sulfur of thiols present on cysteine through the formation of a covalent bound (Bell and Kramer 1999). These bindings may result in different complexes such as AgHcys, Ag_2cys and Ag_2Hcys^+ (Alekseev et al. 2012). Moreover, these complexes can induce the formation of chain like structures by two different pathways. In one case silver thiolates directly bind together forming $[Ag\text{-}S\text{-}Cys]_n$. However, when silver ions are in excess, the silver thiolates can bind with the excess of silver ions thus resulting in [Ag-S-cys]…Ag+…[Ag-S-cys] chains (Komarov et al. 2008, Pakhomov et al. 2010). Further, it has been postulated that such chain-like structure could have a zigzag conformation composed of –Ag-S(R)-Ag-S(R)-(Andersson 1972) (cited in Bell et al. 1997) as the C–S–C angle is approaching 90° (Cremlyn 1996) and could be relatively similar with Ag–S–Ag.

Silver nanoparticles (AgNPs) are also able to covalently bind organic sulfur. Indeed, cysteine has been used as a coating material for AgNP stabilisation (Mandal et al. 2001, Varghese et al. 2009). Furthermore, AgNPs coated with other organic compounds such as citrate or polyvinylpyrrolidone can still bind cysteine to their surface (Csapó et al. 2012, Gondikas et al. 2012). In this regard, it has been observed that cysteine can replace the coating of AgNPs, thus affecting their behavior (Gondikas et al. 2012, Toh et al. 2014). However, depending on the pH of the solution of cysteine-

AgNPs, their behavior and stability might be altered. Indeed, although cysteine can be used to stabilise the AgNPs, an increase of the pH will induce aggregation of the cysteine-AgNPs through the formation of hydrogen bounds between the carboxyl and amine groups of cysteine molecules (Choi et al. 2003, Csapó et al. 2012, Mandal et al. 2001). With pH between 3 and 7, cysteine-AgNPs aggregate as their carboxyl group gets negatively charged (COO^-) while the amine group is positively charged (NH^+). This is not the case at pH below 3 or over 7 where the carboxyl group is protonated (COOH) (pH<3) or the amine group is deprotonated (NH_2^+) (pH>7). In both cases, the cysteine-AgNPs are electrostatically repulsed (Csapó et al. 2012). Finally, it has been proved that AgNPs are also able to bind to large peptides and proteins containing thiol groups such as the glutathione and penicillin (Leung et al. 2013).

In vertebrates, albumin is the major protein of the blood plasma as it represents around 60% of the total of proteins. It has a molecular weight of 66.5 kDa and is constituted of three domains I, II, and III, each of them being subdivided into two sub-domains A and B which result in many binding sites (Dockal et al. 1999). Albumin is synthetized by the liver and has multiple functions as it acts as a transporter of hormones and drugs; it also plays a major role in the metabolism of lipids, the regulation of the osmotic pressure, and control of the pH (Carter and Ho 1994, Dockal et al. 1999, Kratz 2008). Albumin also acts as a metal ligand as it can bind zinc, copper, calcium, and silver. In human, the albumin also called human serum albumin (HSA) contents 34 cysteines, but only one is a free thiol, the other ones being involved in the formation of disulfide bounds. The free cysteine is called cys34 and is involved in the binding of thiols containing compounds as well as certain metals.

Indeed, it has been observed that two types of binding could be involved during the binding of silver ions to albumin with a few high affinity sites and numerous low affinity sites (Shen et al. 2003). At low concentrations of silver, hydrogen bound and van de Waals forces were involved, while at high silver contents electrostatic associations were the major forces (Zhao et al. 2011). It has been shown that the binding of silver ions to albumin induces changes in the tri-dimensional structure of the protein with alterations of the ratio of alpha helix and beta sheets (Shen et al. 2003, Zhao et al. 2011). It has been suggested that this loosening of the protein structure could present hydrophobic regions as well as new potential binding sites (Zhao et al. 2011). It has been suggested that silver ions could bind to cys34, but also to other amino acids like methionine as well as to

disulfide bridges (Shen et al. 2003). Furthermore, by binding to albumin, silver ions could induce the destruction of structural bindings of the protein such as the disulfide bounds which could explain the loosening of the protein (Shen et al. 2003, Zhao et al. 2011).

While albumin is able to bind to silver ions, it has been shown that it also binds to the surface of AgNPs (Ostermeyer et al. 2013). Indeed, albumin can be used as a coating material or even replace the coating already present on the surface of the AgNPs (MacCuspie 2011, Ostermeyer et al. 2013). Two binding models have been suggested for albumin on AgNPs, both resulting on the formation of a corona around the nanoparticle. The first model is the side-on model in which the protein binds to the AgNPs by its side so that many bounds of low energy are created between the protein and the nanoparticle (Ostermeyer et al. 2013). The second model is the end-on binding in which the bottom of the protein binds to the AgNPs, therefore, more proteins can bind to each nanoparticle (Dasgupta et al. 2016, Ravindran et al. 2010). It has been postulated that the binding of albumin is spontaneous and driven by electrostatic and hydrophobic interactions (Ali et al. 2015, Mariam et al. 2011, Roy and Das 2014, Wang et al. 2017, Zhang et al. 2015), while Guo et al. (2015) showed that such binding were due to van der Waal forces and hydrogen bounds.

Furthermore, Mariam et al. (2014) have observed a diminution of free thiols of albumin when the protein was in presence of AgNPs. As the only free thiol of albumin is the cys34, such diminution tends to indicate that the presence of AgNPs could alter this binding site. This could happen either directly through a direct interaction of the AgNPs or silver ions released by the nanoparticle, with the cys34; or indirectly by a disturbance of the protein structure. Indeed, as already observed with silver ions, the binding of AgNPs with albumin induces disturbances of its structure. This alteration results in a loosening of the protein, a loss of fluorescence, and modifications of the ratio alpha helix versus beta sheets (Ali et al. 2015, Iranfar et al. 2012, Zhang et al. 2015). Also, reduction of the availability of disulfide bridges has been shown during the binding of AgNPs to albumin which has not been observed with silver ions (Gebregeorgis et al. 2013). Such observations tend to indicate that disulfide bridges might be involved in the binding of albumin with AgNPs and could also partially explain the structural modifications of the protein.

Although studies have shown that the presence of silver ions or AgNPs could alter the structure of albumin and reduce its fluorescence, to our knowledge, no studies have yet determined which fraction of the interactions between AgNPs and albumin is due to direct interaction with the

nanoparticle and which is due to the effect of the released ions by the nanoparticles. Indeed, it is known that solutions of silver nanoparticles are never fully exempt of silver ions, thus it is hard to precisely determine if the observed effects were due to the nanoparticles themselves or to the silver ions.

The main objective of this study was to compare the effects of silver ions and AgNPs on the fluorescence of human serum albumin (HSA). First, a characterisation of the interactions between silver ions, AgNPs, and HSA was performed by Raman spectroscopy. Then, the fluorescence of HSA was compared in presence of silver ions or AgNPs. To our knowledge, this is the first experiment to directly compare the effects of silver ions and AgNPs on HSA.

1.4. MATERIAL AND METHODS

1.4.1. Material

Human serum albumin >96% purity (HSA) (CAS: 70024-90-7), cysteine 97% purity ($HSCH_2CH(NH_2)CO_2H$) (CAS: 52-90-4), Sodium citrate dihydrate 99% purity ($C_6H_5Na_3O_7$ * $2H_2O$) (CAS: 6132-04-3), and silver nitrate 99% purity ($AgNO_3$) (CAS: 7761-88-8) were all purchased from Sigma Aldrich. Potassium phosphate monobasic 99.7% purity (KH_2PO_4) (CAS: 7778-77-0) as well as dibasic potassium phosphate 99.7% purity (K_2HPO_4) (CAS: 7758-11-4) were purchased at Fisher. Supracil quartz spectroscopy cells were purchased at PerkinElmer. All compounds were used as received without further purification.

1.4.2. Methods

1.4.2.1. Synthesis and characterization of the AgNPs

The silver nanoparticles (AgNPs) were prepared by reduction of silver nitrate with sodium citrate following a modified procedure of Jiang et al. (2007). Ten milliliters (10 mL) of a solution of $AgNO_3$, at 58.9 mM, were added to 185 mL of nanopure water under reflux. At the recovery of boiling, 5 mL of a solution of sodium citrate at 0.25% (w/v) was added dropwise. After 3 h of reaction under reflux, the system was cooled down at room temperature. The solution was filtered on 0.2 µm polycarbonate filter. The filtrate was centrifuged at 6000*g* for 15 min. The pellet was resuspended with 8 mL of nanopure water and centrifuged at 6000*g* during 15 min. This procedure was repeated three times to remove the excess of silver ions and citrate. After the purification process, the concentration of AgNPs and silver ions were determined by Inductively Coupled

Plasma-Mass Spectrometry (ICP-MS, Agilent 7500C^{TM}). Silver ion concentration was below the detection limit (0.015 µgAg.L^{-1}). The size of the AgNPs was measured by dynamic light scattering (DLS) and results showed that the nanoparticles had a hydrodynamic diameter (D_h) of 22 ± 5 nm. A stock solution of 5.42 mM of AgNPs was prepared. This solution was kept aside from light and under stirring. This solution was stable for months in dark.

1.4.2.2. Raman Spectroscopy

The characterization of the interactions between AgNPs, $AgNO_3$, cysteine, and HSA has been performed by Raman Spectroscopy and by Excitation Emission Matrix Fluorescence (EEMF) as described later. Raman spectra were obtained by using the ThermoScientific DXR Raman microscope. Analyses were performed between 3500 and 0 cm^{-1}. The laser wavelength was set at 633 nm with a power of 4 mW and the slit for the laser was 50 µm wide. 200 scans of 0.5 second each were performed per analysis with an automatic correction of fluorescence of 5. The spectra were normalized based on the intensity of the C-H peak at 2955 cm^{-1}. This peak was chosen for its stability in intensity and in frequency regardless its chemical environment (Tremblay and Gagne 2002). In order to avoid the effects of the buffer (especially for HSA with phosphate buffer), the solvent for all samples was nanopure water. Solutions were mixed together such as the obtained samples were cysteine alone, cysteine with $AgNO_3$, cysteine with AgNPs, cysteine with HSA, HSA with $AgNO_3$, HSA with AgNPs, and HSA with cysteine and $AgNO_3$. The concentrations of each reactant in all solutions when present were 500 mM of cysteine, 250 mM of $AgNO_3$ and HSA 250 µM. As total amount of AgNPs was limited, only 0.27 mM of the nanoparticles were used per sample.

1.4.2.3. Excitation Emission Matrix Fluorescence

Excitation Emission Matrix Fluorescence analyses were performed on solutions of HSA and HSA with AgNPs. The spectrofluorometer was a Fluoromax-4 and the working conditions were Excitation wavelength from 225 nm to 500 nm with an increment of 5 nm between two measures. The Emission wavelengths were measure between 220 and 500 nm with an increment of 2 nm. For all these experiments, HSA was dissolved in phosphate buffer and the pH was adjusted to 7.4. The working concentration of HSA was constant at 0.25 µM as it was the concentration at which the protein elicited the best fluorescence signal without saturating the detector. The solutions of

HSA are stable for at least 20 days (Kratz 2008).They were prepared at least 12 h before analysis and kept at 4 ºC until their use. Before each experiment, HSA solution was brought to room temperature and aliquots of 0.93 µM of AgNPs were added to the HSA in order to observe their effects on the EEMF of the protein.

1.4.2.4. Fluorescence Spectra

The fluorescence measures were performed for a specific point at λ: Excitation = 275 nm; Emission = 344 nm. This point corresponded to the maximal of the EEMF peak representing the fluorescence of tryptophan and tyrosine moieties of HSA. Solutions of HSA were prepared in phosphate buffer at pH 7.4 and at working concentrations of 0.25µM.

1.4.2.4.1. Fluorescence of HSA with AgNPs or $AgNO_3$

Working concentrations of AgNPs of 0.0093, 0.093, 0.93, and 9.3 µM (corresponding to 1, 10, 100, and 1000 µg/L) were added to the solution of HSA and the fluorescence signal was measured after 5, 15, 30, 60, 120, and 240 min exposure. The same experiment with same exposure times was performed with $AgNO_3$ at working concentrations of 0.025, 0.25, 2.5, and 25 µM (corresponding to 4.246, 42.46, 424.6, and 4246 µg/L). The signal intensity was normalized based on the intensity of the fluorescence signal of HSA alone at t=5 min. The fluorescence of HSA alone at the same time points as the other samples were used as a control.

1.4.2.4.2. Fluorescence of HSA with cysteine

Cysteine was dissolved in nanopure water and solutions of HSA in phosphate buffer with increasing concentrations of cysteine (0, 1, 10, 100 µM) were prepared. The fluorescence of the solutions was measured after 5 min and 24 h of incubation. The fluorescence was normalized based on the intensity of the fluorescence signal of HSA alone at t=5 min.

1.4.2.4.3. Fluorescence of HSA with AgNPs and cysteine

In this experiment, increasing concentrations of cysteine (0, 1, 10, 100 µM, and 1 mM) and 0.93 µM (100 µg/L) of AgNPs were mixed together. The fluorescence was measured after 5 min and

24 h of exposure. The samples were mixed in two different orders. In one case, cysteine was added to HSA first and the AgNPs were added to the reaction only 5 min after the exposure of cysteine to HSA. In the second case, the AgNPs were added first, while the increasing concentrations of cysteine were poured to the solution only after 5 min of exposure. Fluorescence signals were normalized based on the fluorescence's intensity of HSA with 0.93µM of AgNPs at t=5 minutes and without cysteine.

1.5. RESULTS AND DISCUSSION

1.5.1. Raman spectroscopy

Raman spectroscopy was used in order to analyse which molecular groups of cysteine and HSA were affected by the addition of $AgNO_3$ or AgNPs. Table 2 showed a detailed list of spectral regions were peaks could appear in Raman spectroscopy and to which molecular groups they were associated (Diaz Fleming et al. 2009, Graff and Bukowska 2005, Lopez-Tobar et al. 2013, Podstawka et al. 2004).

Table 2 : Wavenumber regions associated with their corresponding chemical bindings

Chemical bound	Wavenumber region (cm-1)
S-S	550-430
C-S	790-630
C-C	1300-600
CH2	1450-1400
Amide	1700-1550
S-H	2610-2530
C-H	3000-2800

The Raman spectrum of cysteine was performed and peaks were attributed to their corresponding stretching regions and molecular groups as shown in Figure 7. In this figure, the X-axis was the wavenumber in cm^{-1} of the Raman spectrum ranging from 0 to 3500 cm^{-1}. The Y-axis represented the intensity of the signal that was normalized based on the intensity of the peak at 2957 cm^{-1}. This peak was attributed to the C-H bond which is expected to be stable and has few to no variations (Tremblay and Gagne 2002). Region from 430 to 550 cm^{-1} corresponded to the S-S bond. The

region between 600 and 1300 cm^{-1} was linked to the C-C bond. However, C-S bounds were found between 630 and 790 cm^{-1}, thus showing that different molecular groups could be found inside the same wavenumber range. The region at 1400-1450 cm^{-1} represented the CH_2 groups while peaks between 1550 and 1700 cm^{-1} referred to the amide groups. The peak at 2530-2610 cm^{-1} was associated with S-H bound. Finally, the region 2800-3000 cm^{-1} was applied to C-H binding (Diaz Fleming et al. 2009, Graff and Bukowska 2005, Jing and Fang 2007, Lopez-Tobar et al. 2013, Martina et al. 2012).

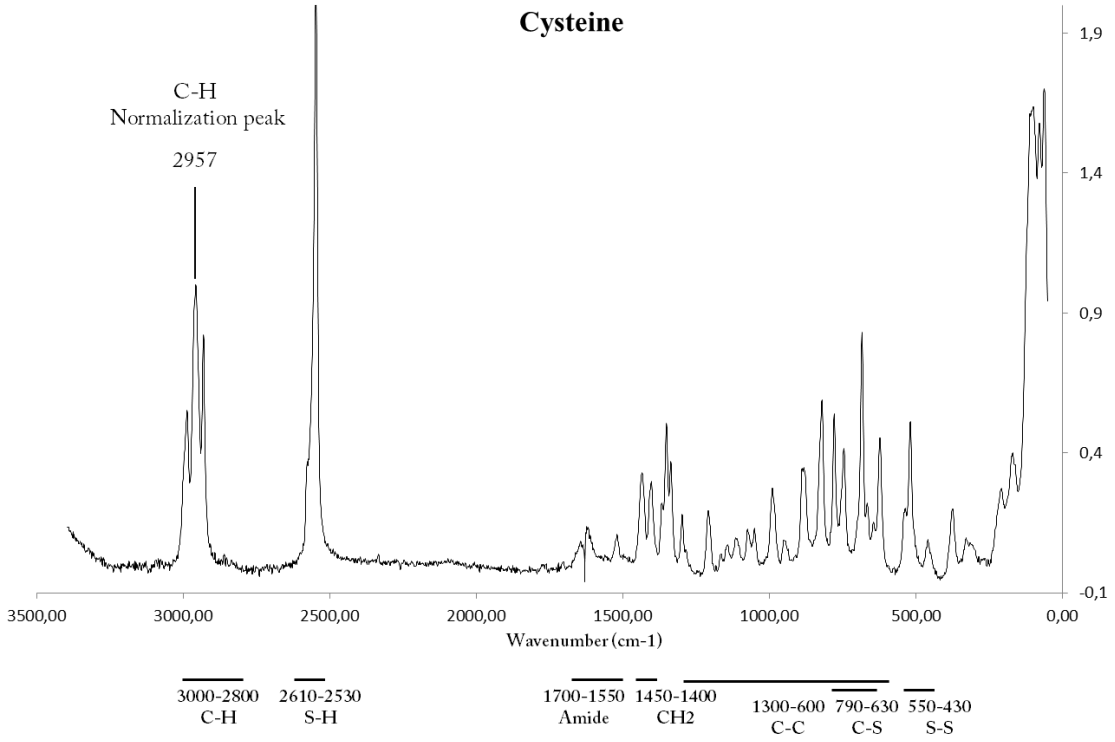

Figure 7 : Raman spectra of cysteine and its stretching region between 0 and 3500 cm^{-1}

The spectrum of cysteine showed the presence of a peak in the S-S region (519 cm^{-1}), and multiple peaks in the C-C and C-S regions (600-1300 cm^{-1}). Two peaks were associated with CH_2 stretching (1401 and 1433 cm^{-1}). The strongest peak was found at 2549 cm^{-1} and represented the S-H bond stretching. Three peaks were found in the C-H region (2930, 2957 and 2987 cm^{-1}).

The peak observed in the S-S region was attributed to the formation of disulfide bound during the formation of cystine from the oxidation of two cysteines. Indeed, it is known that cysteine in

solution is easily oxidized into cystine even in mili-Q water where trace of oxygen could still be present (Diaz Fleming et al. 2009, Lopez-Tobar et al. 2013, Ralph et al. 1994).

Figure 8 compared the spectrum of cysteine alone, in blue, with cysteine in $AgNO_3$ solution, in red. Moreover, the regions where most changes occurred were highlighted. The results showed modifications of the peaks in the C-H region with disappearance of the peak at 2987 cm^{-1} and a shift of the peak at 2930 cm^{-1} to 2917 cm^{-1}. This indicated that the addition of $AgNO_3$ induced modifications of the stretching of bond in the C-H region. The intensity of S-H peak (2549 cm^{-1}) was also reduced in presence of silver ions. Moreover, modifications of the peaks in C-S regions were observed with the appearance of two new peaks at 718 and 703 cm^{-1}. As silver has a high affinity for thiol compounds, these reductions of S-H intensity and alterations of the C-S region were attributed to the binding of Ag ions to the sulfur, which would alter the binding strength of the different groups present on the cysteine (Diaz Fleming et al. 2009, Graff and Bukowska 2005). This figure also showed the appearance of a strong peak at 1045 cm^{-1} in presence of $AgNO_3$. Based on the results of Martina et al. (2012), this peak was attributed to the N-O stretching resulting from the dissociation of Ag^+ and NO_3^-. Moreover, Martina et al. (2012) also observed the appearance of two peaks at 698 and 717 cm^{-1} also arisen from "doubly degenerated N-O in-plane bending". Therefore, the two peaks observed at 718 and 703 cm^{-1} resulted from N-O signal and not from alterations of the C-S region. Finally, it could be seen that the intensity of the S-S peak at 519 cm^{-1} is reduced. Two possible mechanisms were suggested to explain such a reduction. First, by binding to the thiol group, silver reduced the available sulfur groups, thus hindered the dimerization of cysteine. The other hypothesis would be that the presence of silver could cleave the dimers of cysteine through the breaking of the disulfide bound and thus reduce the intensity of the S-S peak (Diaz Fleming et al. 2009, Lee et al. 1991, Siriwardana et al. 2015).

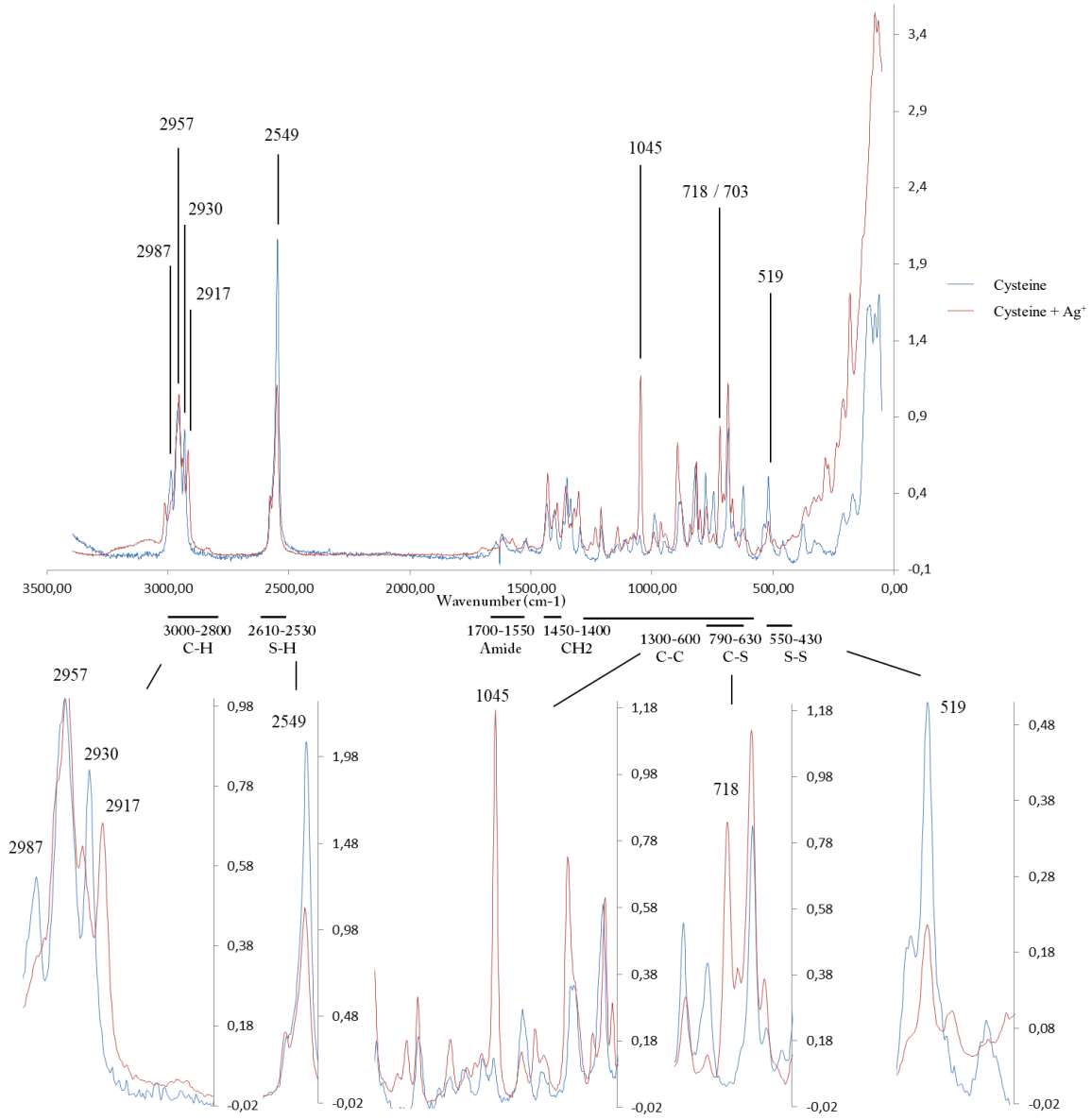

Figure 8: Raman spectra of cysteine alone and cysteine with $AgNO_3$ between 0 and 3500 cm^{-1}. Zoom on altered stretching regions

Raman spectrum of HSA (Figure 9) showed that solution of HSA exhibited no specific peak. However, the spectrum intensity tended to show a slight increase between 1544 and 790 cm^{-1} and a peak-like shape between 3023 and 2828 cm^{-1}possibly attributed to C-H binding. Despite those small trends, the wave-like signals between 3400 and 500 cm^{-1} are attributed to background signals and could therefore not be identified. It was suggested that due to the high molecular weight and the complexity of HSA, no specific molecular binding could be pointed out using this technique.

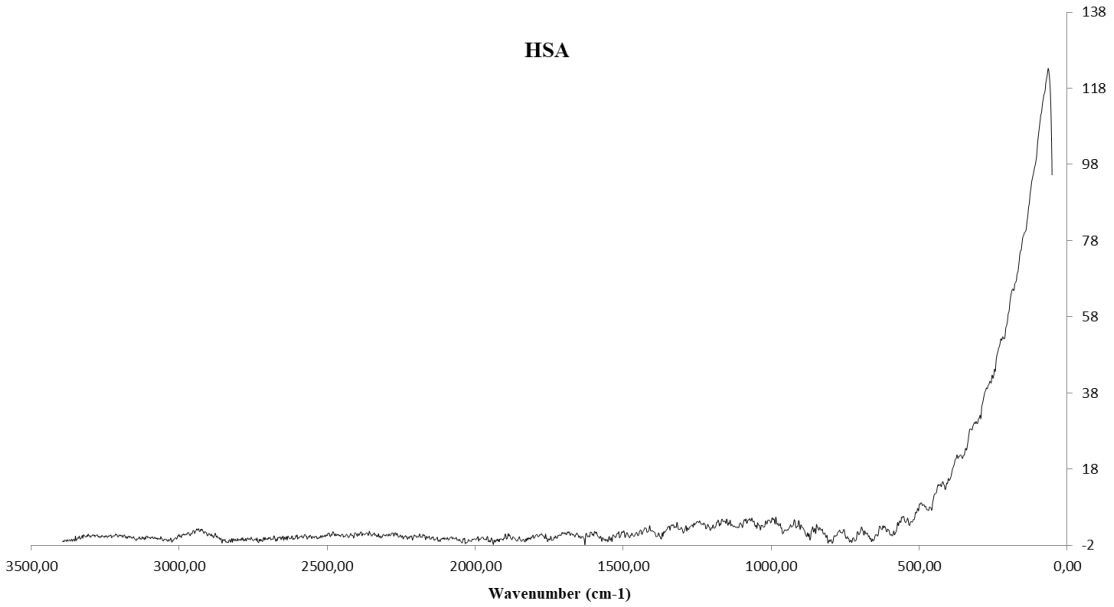

Figure 9: Raman spectrum of HSA between 0 and 3500 cm^{-1}

Raman spectra of HSA (blue) and HSA with $AgNO_3$ (red) were compared in Figure 10. Except for the appearance of three new peaks at 1050, 737, and 717 cm^{-1}, no distinctive changes were observed. As described earlier for the spectra of cysteine with $AgNO_3$, the three peaks are associated with the signal of N-O stretching. The presence of these peaks is supporting the hypothesis that the two peaks observed at 718 and 703 cm^{-1} in the sample of cysteine with $AgNO_3$ did originate from the signal of N-O bond (Martina et al. 2012).

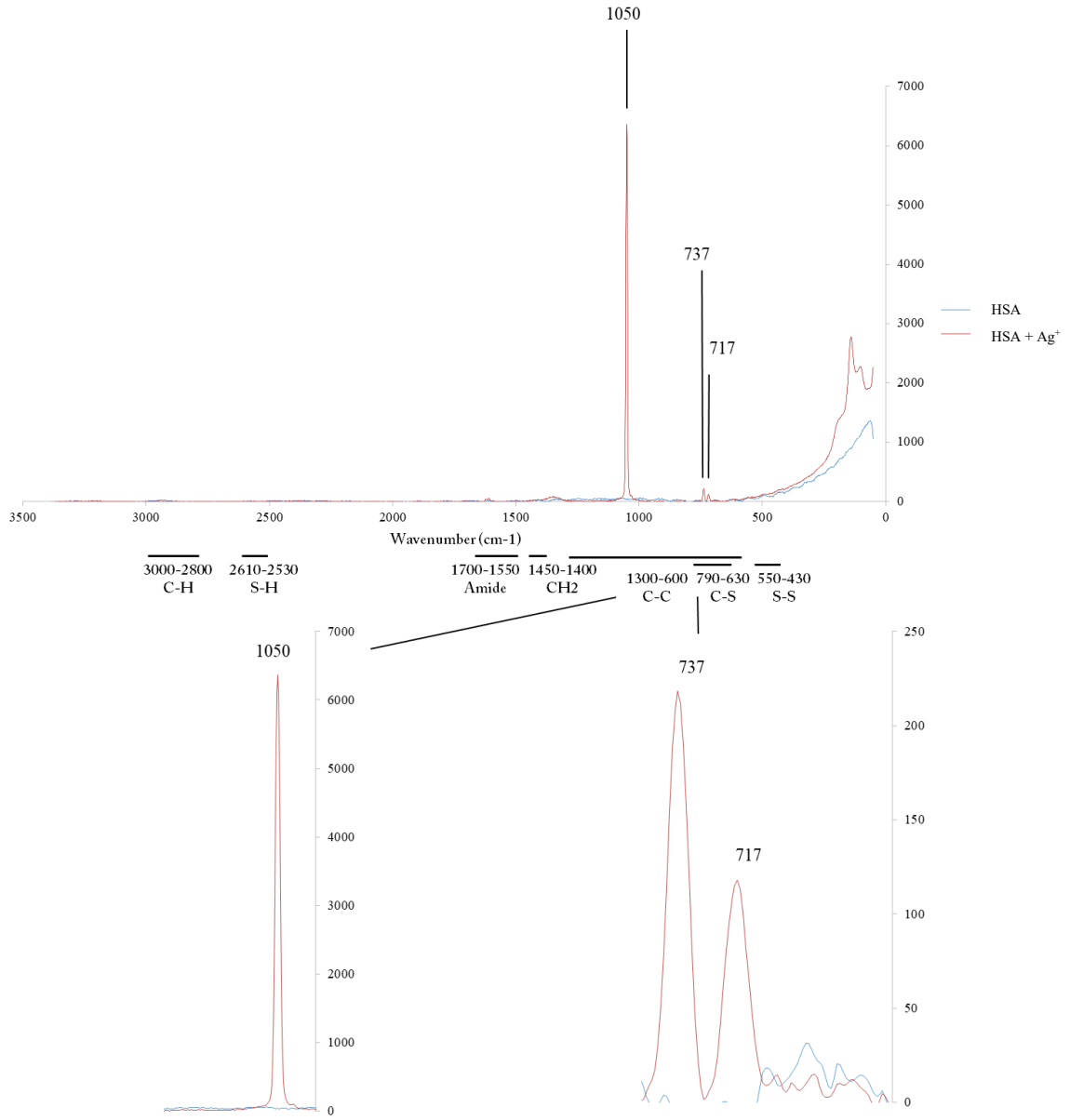

Figure 10: Raman spectra of HSA and HSA with $AgNO_3$ between 0 and 3500 cm^{-1}

It is known that solutions of silver nanoparticles are never totally exempt of silver ions because of the reactivity of AgNP surface in water solution. Thus, it was hard to determine which effects were truly due to silver nanoparticles and which ones were due to the released ions when examining a suspension of AgNPs in presence of soluble sulphur organic compounds. To allow a better understanding of the direct effects of AgNPs, some studies have used cysteine in order to chelate

the free silver ions, therefore removing the ions from the system (Siriwardana et al. 2015, Wigginton et al. 2010).

Thus, before analysing the interactions between HSA, cysteine and $AgNO_3$ or AgNPs, it was important to understand how cysteine and HSA behave together. Figure 11 showed the Raman spectra of cysteine compared to cysteine with HSA. Two major differences were observed. The first one was a reduction of intensity of the peak in S-H region (2546 cm^{-1}) in the sample with cysteine and HSA compared to cysteine alone. The second was the appearance of a strong new peak in the S-S region (504 cm^{-1}). The reduction of the S-H peak was indicative of the reduction of the free thiols, while the appearance of the peak at 504 cm^{-1} tended to indicate the formation of disulfide bounds. It was thus suggested that cysteine and albumin might bind together through the formation of S-S bounds. This has already been described in literature, as albumin is known to be a major transporter of thiol containing compound and is able to bind cysteine (Lewis et al. 1980 cited in Bal et al. 2013, Kragh-Hansen et al. 2002, Kratz 2008). As HSA possesses only one free cysteine the cys34, these results tended to suggest a binding between cysteine and cys34 of HSA.

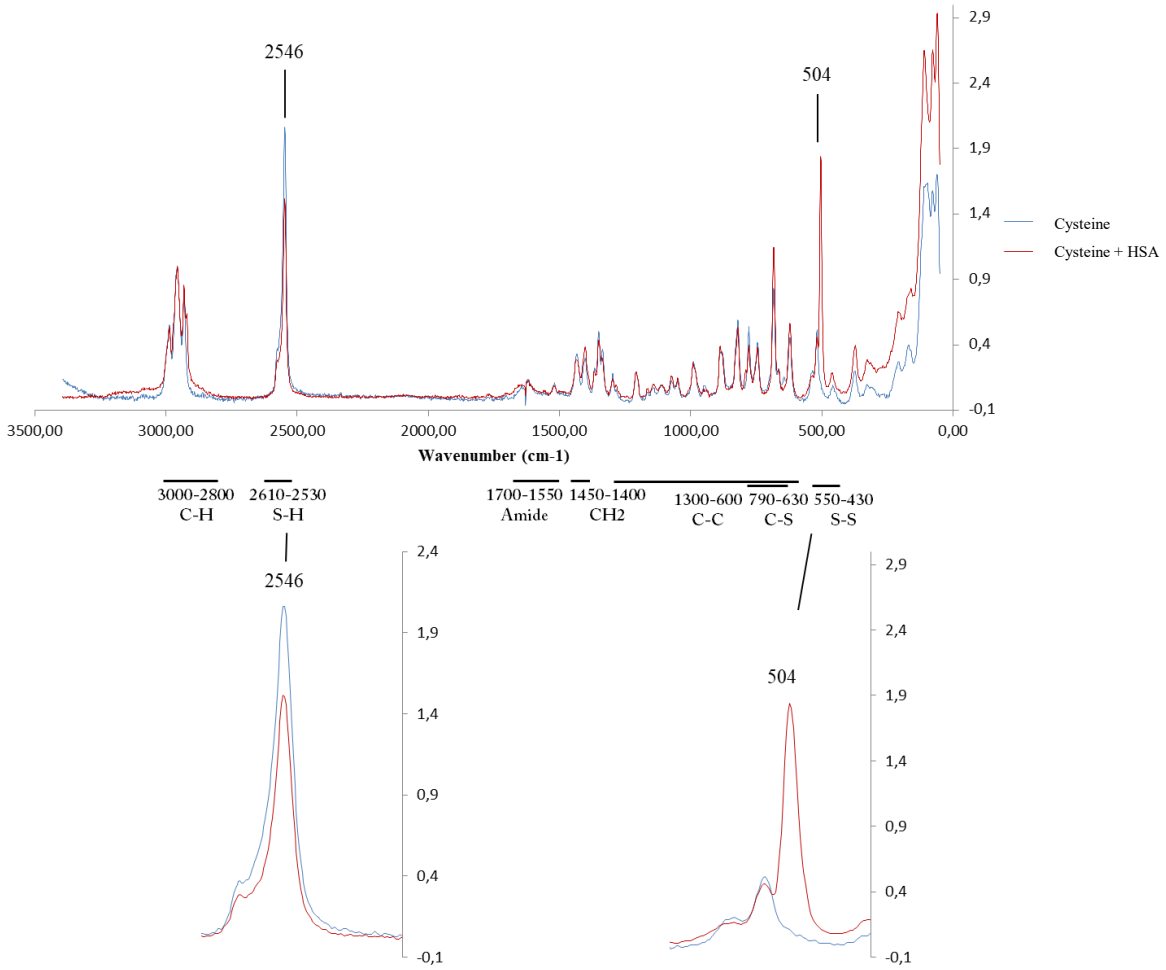

Figure 11: Raman spectra of cysteine and cysteine with HSA between 0 and 3500 cm^{-1}. Zoom on altered regions

Figure 12 compared the spectra of HSA and cysteine (blue) with the spectra of HSA, cysteine, and $AgNO_3$ (red). The results indicated a reduction and slight shift of the S-H peak from 2546 to 2553 cm^{-1} in presence of $AgNO_3$. In the C-H region, the peak at 2976 cm^{-1} was shifted to 3012 cm^{-1}, while the intensity of the peak at 2948 was reduced. Addition of $AgNO_3$ induced the reduction of many peaks in the C-C region such as the peaks at 1400, 1349, 989, 886, 820, 777, 743, and 621 cm^{-1}, while other peaks disappeared or were shifted such as 1304, 937, and 720 cm^{-1}. Also, with the addition of silver nitrate, the peak in the S-S region at 504 cm^{-1} fully disappeared. Reduction of the S-H peak as well as a number of modifications observed in the C-C and C-S regions in

cysteine, HSA, and Ag^+ sample tended to indicate that silver ion was able to bind to cysteine even in the presence of HSA. The reduction and slight shift of the S-H peak at 2553 cm^{-1} was indicative of reduction of the free thiols, which would go along with the postulate that silver ions can bind to the thiol group of cysteine (Alekseev et al. 2012, Bell and Kramer 1999, Cecil 1950, Pakhomov et al. 2010). Moreover, the presence of silver ions in solution of cysteine and HSA elicited a strong reduction of the S-S peak, which showed that Ag^+ ions could either break the disulfide bound between cysteine and HSA or hinder their formation.

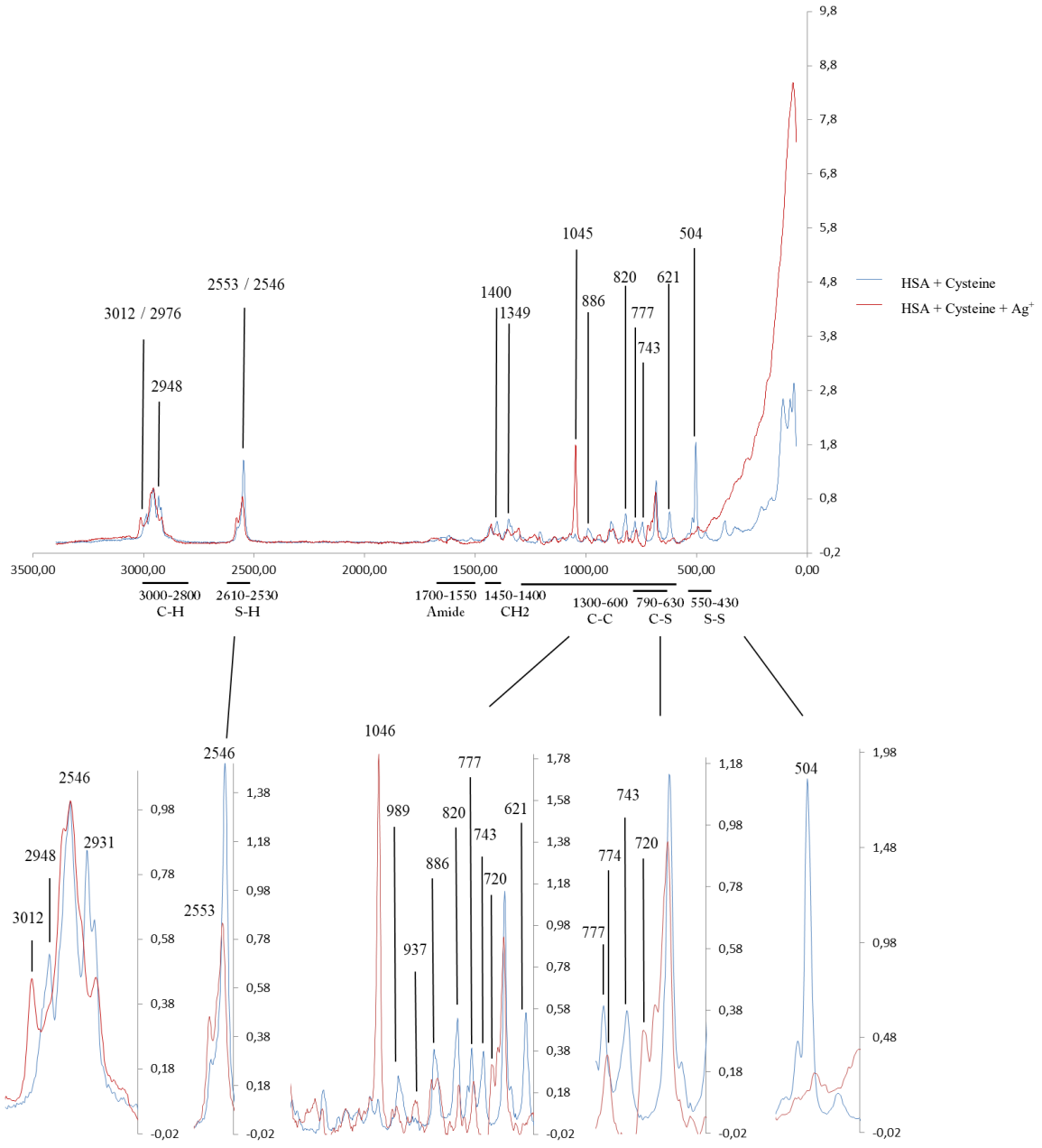

Figure 12: Raman spectra of HSA with cysteine and HSA, with cysteine and $AgNO_3$ between 0 and 3500 cm^{-1}. Zoomed on altered regions

Figure 13 juxtaposes the Raman spectra of cysteine alone (blue) and cysteine with AgNPs (red). The results showed that both spectra were highly similar, except for the appearance of a small peak at 631 cm^{-1} in the region belonging to C-C and C-S bond. Moreover, no strong difference could be observed between the samples of HSA and HSA with AgNPs.

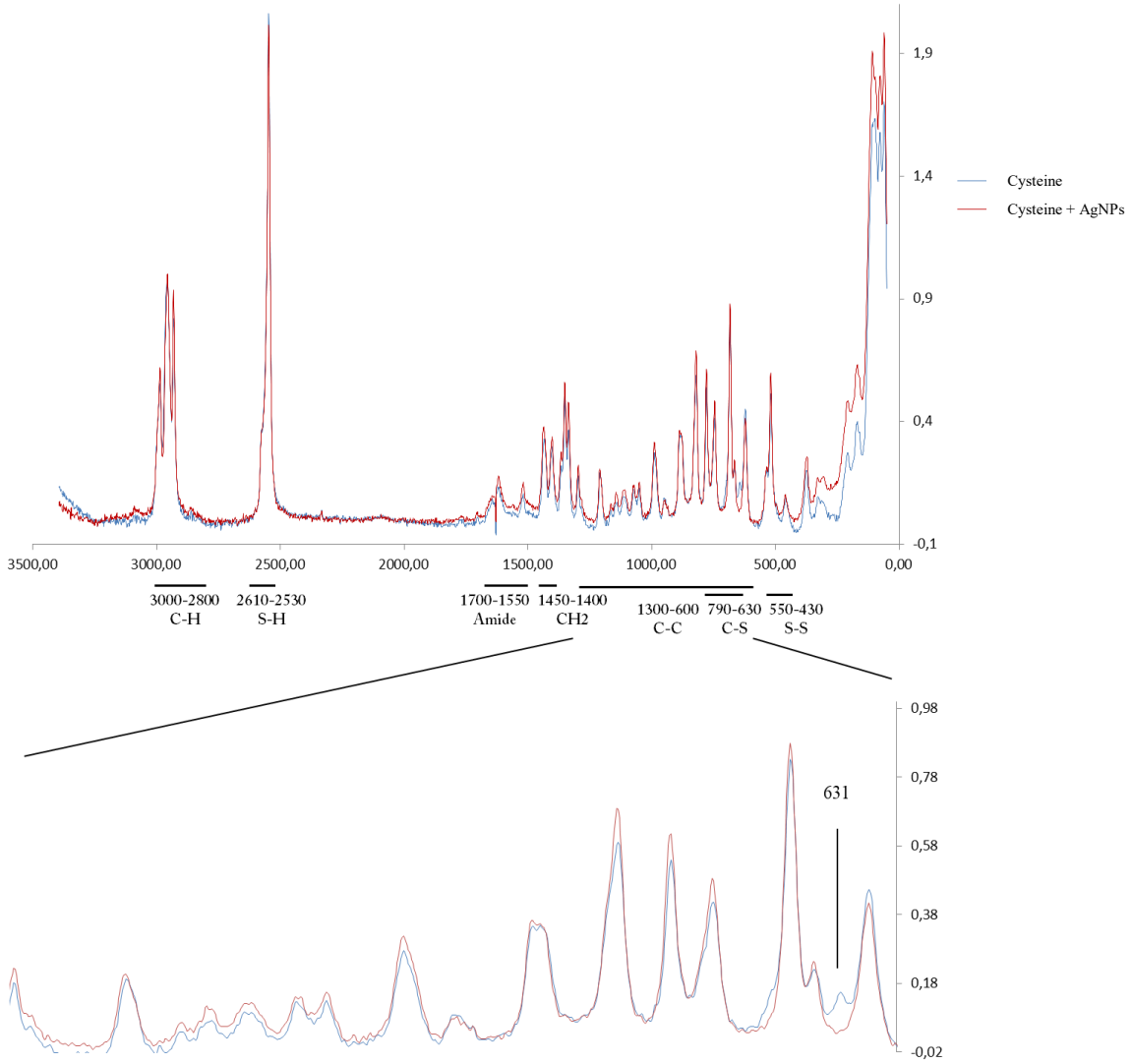

Figure 13: Raman spectra of cysteine and cysteine with AgNPs between 0 and 3500 cm^{-1}

Although AgNPs were expected to enhance the Raman spectroscopy signals (Haynes et al. 2005, Jeanmaire and Van Duyne 1977), our results showed few to no increase of the Raman signal of AgNPs on cysteine or HSA. Lack of SERS signal in presence of AgNPs and gold nanoparticles (AuNPs) has already been observed (Jing and Fang 2007, Lopez-Tobar et al. 2013). Jing and Fang

(2007) observed that in presence of AgNPs alone, no SERS signal could be observed. They attributed this phenomenon to the low energy which did not provide a strong surface plasmon excitation. However, with the addition of chloride they could obtain SERS spectra. Thus, they suggested that the addition of chloride anions could congregate the AgNPs, thus increasing the SERS effect. Also, Lopez-Tobar et al 2013 (Lopez-Tobar et al. 2013) did not observe SERS signal of cystine in presence of gold or silver nanoparticles coated with 0.66 mM of citrate. Nevertheless, when the citrate concentrations were reduced to 0.14 mM, a strong SERS signal was elicited. Thus, they suggested that citrate coating could inhibit the binding of cystine and reduce the SERS signals, or that citrate could hinder SERS signals.

Therefore, such low to null effects of AgNPs on cysteine and HSA are attributed here to two main factors. First, the available concentrations of AgNPs-citrate were low, AgNPs were the limiting reagent and the used concentrations were significantly lower than the one used for cysteine or HSA. Indeed, only 0.27 mM of AgNPs per sample were used, compared to 500µM of cysteine and 250µM of HSA. For comparison silver nitrate was concentrated at 0.25 M. Therefore, the AgNPs could be too diluted to enhance the SERS signals. Also, their low concentration compared to the other compounds may lead to too few interactions with the reactant and thus result in too weak signals compared to the one of cysteine. Furthermore, as AgNPs used in this experiment were coated with citrate it is possible that this coating induced repulsive forces leading to a good dispersion of the AgNPs, but also reduced the binding of cysteine to the nanoparticles, both resulting in low SERS signals, as suggested by Lopez-Tobar et al. (2013).

1.5.2. Fluorescence

In order to analyse the possible changes of HSA conformation in presence of silver ions or AgNPs, EEMF spectrometry was performed. The spectrum of HSA (Figure 14) showed the presence of two bands a and b which, respectively, represented the Raman and Rayleigh scattering. In between these two bands, two peaks were easily visible. Peak 1 was attributed to the fluorescence of the amino acids tyrosine and tryptophan of the protein, while peak 2 represented the backbone of the polypeptide chain of HSA (Ding et al. 2010, Iranfar et al. 2012).

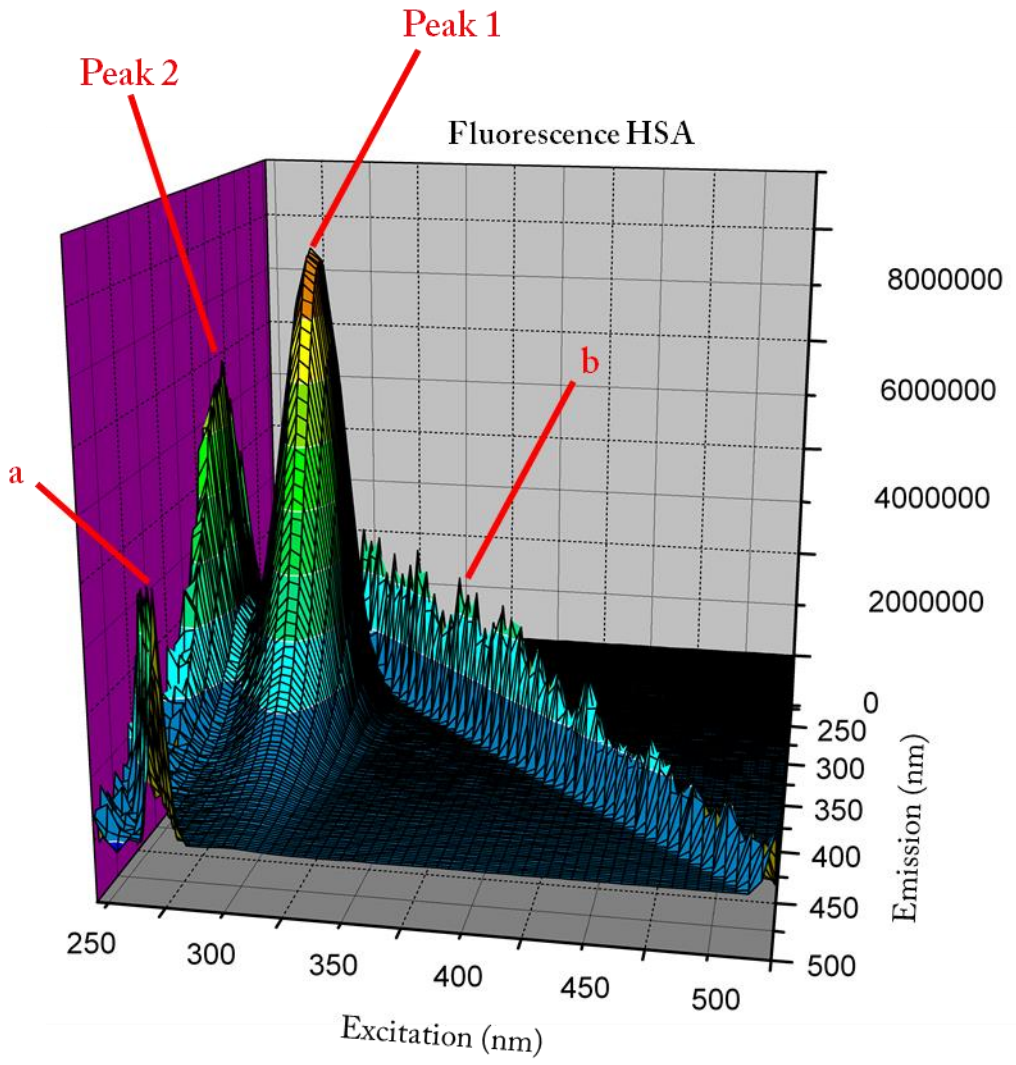

Figure 14: EEMF spectrum of 0.25 µM HSA

In presence of AgNPs, the fluorescence spectrum of HSA was altered. Figure 15 showed that the scattering signal of AgNPs superposed to the signal of fluorescence of peak 2 of HSA. This superposition between the two signals prevented a precise interpretation of intensity variations of peak 2. Therefore, only the maximum of peak 1 (λ: Excitation = 275 nm; Emission = 344 nm) was considered for measuring the variation of intensity of HSA signal in presence of other compounds.

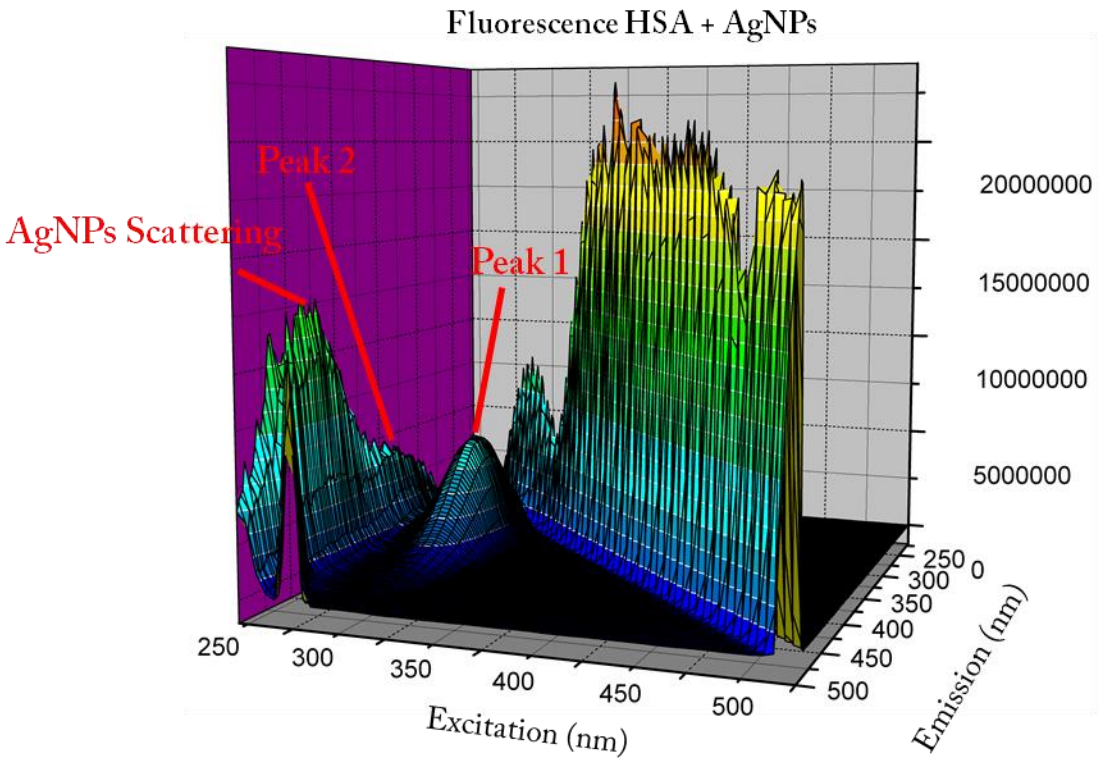

Figure 15: EEMF spectrum of 0.25 µM HSA with 0.93 µM of AgNPs

Further, the Rayleigh scattering was increased in the presence of AgNPs. Such increases of the Rayleigh scattering were also observed by Ali et al. (2015) on HSA with AgNPs and by Guo et al. (2015) on BSA. They both attributed the phenomena to an increase of the size of the protein due to the unfolding of the albumin by AgNPs.

The Figure 16 showed the intensity of fluorescence of HSA at Excitation = 275 nm and Emission = 344 nm, in presence of AgNPs or $AgNO_3$. The fluorescence was measured at different time periods from t=5 min up to t= 480 min. The blue line elicited the intensity of fluorescence of HSA alone; the other full lines represented the samples with HSA in presence of AgNPs, while the dotted lines were attributed to HSA with $AgNO_3$. The fluorescence of the samples was normalized based on the intensity of fluorescence of HSA alone at t=5 min.

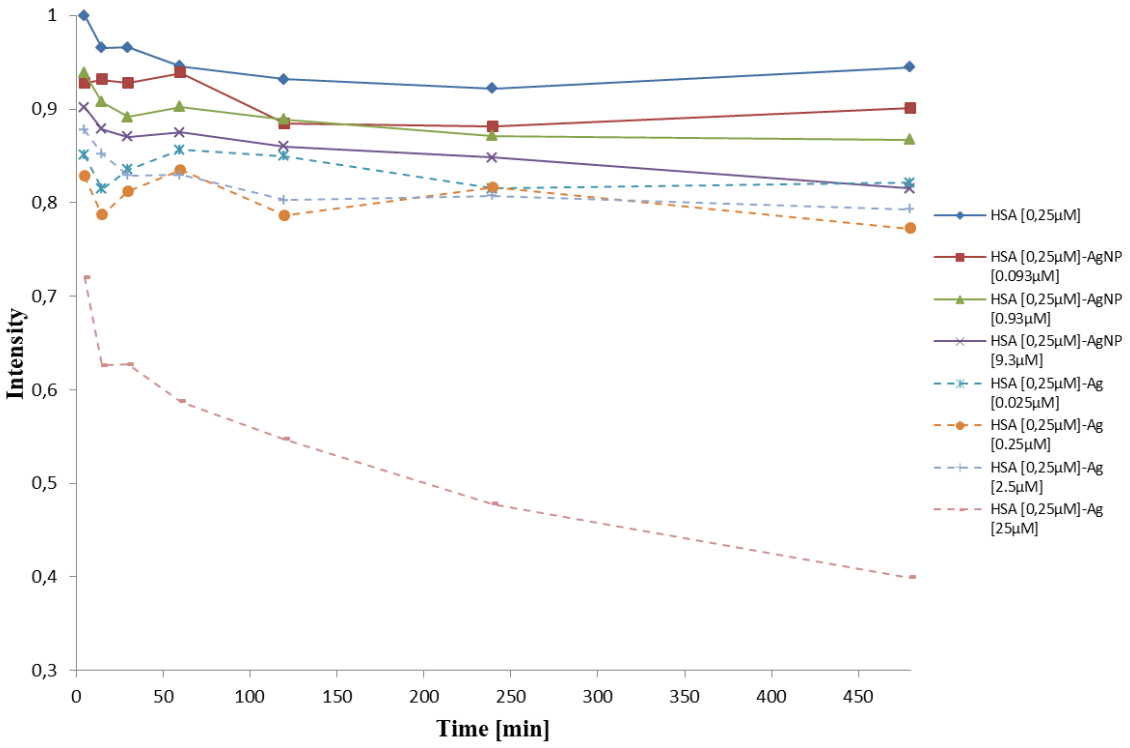

Figure 16: Intensity of fluorescence of HSA (peak 1) in presence of increasing concentrations of AgNPs or $AgNO_3$ over 4h. Excitation of 275 nm and Emission 344 nm

The presence of both silver species induced a quick reduction of HSA fluorescence within the first minutes after their addition in the samples. In presence of AgNPs, the reduction of the fluorescence was fast and after less than one hour, the signal tended to reach plateau. However, although the reduction of signal intensity was also fast with $AgNO_3$, the equilibrium was not reached after one hour. The results also showed that AgNPs elicited less reduction of the signal than $AgNO_3$ for equivalent silver concentrations. Indeed, in the lower concentration of AgNPs (0.093 µM), after five minutes' exposure the intensity of HSA fluorescence was reduced by 4 to 5% and reached a maximum of 12%. With 9.3µM of AgNPs the reduction of fluorescence was approximately 8% at t=5 min and reached a maximum of 16% of reduction after 240 min, the fluorescence then slightly increases at 480min.

For $AgNO_3$ the reduction of HSA fluorescence was stronger than AgNPs as for 0.025 µM of $AgNO_3$ the reduction was of 15% after 5 minutes and went up to a maximum of 19% after 240 minutes. The fluorescence of HSA in presence of both 0.25 and 2.5 µM of $AgNO_3$ were similar

with minimal reduction of fluorescence of 13% for 2.5 µM of silver and a maximal reduction of around 22% with 0.25 µM $AgNO_3$ after 120 minutes' exposure. A concentration of 25 µM of $AgNO_3$ induced the strongest effects on the fluorescence of HSA as after five minutes the fluorescence was reduced by 28% and reached a maximal reduction of 60% after 480 minutes. However, this reduction should be higher if measurement was done for a longer period of time.

Such results suggest that the reaction of silver ions with HSA happened through two different ways. During the first phase, the interaction of silver with the protein was fast and silver could easily reach the reactive sites with which it has a strong affinity. However, once all the easily accessible binding sites were saturated, the remaining ions may have attacked the binding sites that were less accessible and that could have been hidden inside the protein hydrophobic pockets. Indeed, such results have already been observed by for silver ions with HSA by Shen et al. (2003) who reported that HSA could bind numerous silver ions even at a low concentration of HSA. Moreover, they postulated that two types of binding sites existed with different binding affinity. They numbered 14 high affinity binding sites and numerous binding sites of lower affinity. Further, they suggested that the binding of Ag^+ to HSA was not immediately stable and passed through conformational and binding changes before reaching a steady state (Dasgupta et al. 2016, Shen et al. 2003). Cys34 is expected to be one if not the major high affinity binding site for silver on albumin (Marambio-Jones and Hoek 2010b, Shen et al. 2003, Siriwardana et al. 2015). Also, Shen et al. (2003) suggested that the binding of silver ions to high affinity sites could change the conformation of the protein and thus enhance the ability of silver ions to reach new binding sites located in hydrophobic pockets.

Furthermore, the binding of both silver ions as well as AgNPs induced conformational changes such as loss of the secondary structure and reduction in the percentage of alpha helix (Gebregeorgis et al. 2013, Iranfar et al. 2012, Liu et al. 2009, Mariam et al. 2014, Shen et al. 2003).

However, although silver ions elicited stronger reduction of fluorescence than the AgNPs for equivalent concentrations of total silver, it was hard to clearly conclude that silver ions had stronger effects than AgNPs or even that the nanoparticles had an effect at all on HSA. Indeed, it is known that AgNPs solutions are never fully exempt of silver ions due to partial dissolution of the nanoparticles. In fact, the solutions of AgNPs used in these experiments contained less than 1.2%

of free silver ions. These ions may be the reason behind the lower reduction of the signal in the AgNPs samples as they would represent around one percent of the total silver.

Cysteine can be used to chelate silver ions, thus having only the effects of AgNPs (Wigginton et al. 2010). However, first it was important to understand how cysteine alone could affect the fluorescence of HSA. Thus, solutions of HSA 0.25µM were stirred at room temperature with different concentrations of cysteine (1µM, 10µM, and 100µM) and the fluorescence of the samples were measured at t=5 min ant t=24h (Figure 17). Results showed that after a 5-minute exposure, cysteine did not induce reduction of the fluorescence of HSA. On the contrary, the intensity of fluorescence tended to increase after a 5-minute exposure at 100µM of cysteine. After 24h of stirring at room temperature, fluorescence of HSA dropped considerably and reached a value around 45% of its initial intensity. This could be due to the stirring, which could increase the contacts between the HSA, thus enhancing the formation of aggregates. Such process has been previously observed. Albumin can bind together and aggregate, thus reducing the total fluorescence of the sample (Maruyama et al. 2001, Militello et al. 2004). Moreover, the fluorescence of HSA was further reduced when the protein was in presence of cysteine and reached a minimal intensity of 24% compared to HSA alone at t=5 min.

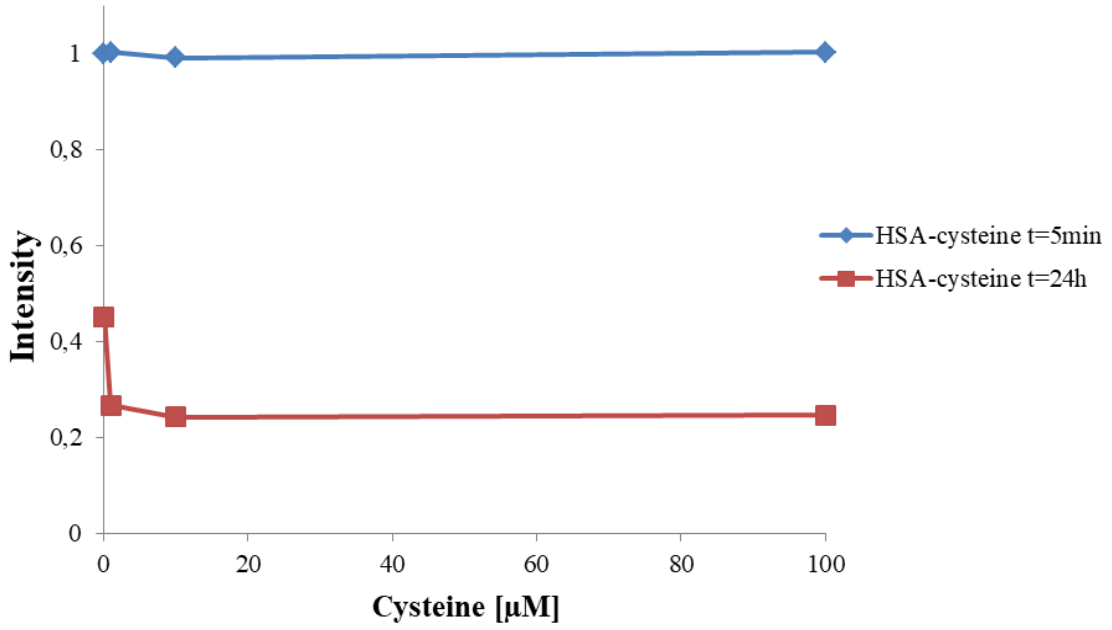

Figure 17: Fluorescence of HSA with increasing concentration of cysteine at t=5min and t=24h. Excitation of 275 nm and Emission 344 nm

Such results tend to support the idea that cysteine had only mild effects on HSA during a short exposure, but after 24h reaction time, the presence of cysteine could affect HSA or its microenvironment near the tryptophan and tyrosine moieties. Furthermore, based on literature and the Raman spectra showed here, cysteine is expected to bind to the free thiols of HSA, which are composed of the only free cysteine, cys34, of the protein (Lewis et al. 1980, Sengupta et al. 2001). Therefore, there was only one available binding site (of high affinity) for the molecules of cysteine per protein of HSA. In this experiment, even at a lowest concentration, cysteine was at least four times more concentrated than the albumin. Thus, a part of the cysteine molecules could rapidly bind to the cys34 group, having low to no effects on the protein as this binding site is expected to bind thiol groups (Bal et al. 2013, Kragh-Hansen et al. 2002, Kratz 2008, Lewis et al. 1980). However, once cys34 sites were saturated, the rest of the cysteine could search for other less available binding sites on HSA, leading to alteration of its conformation and a reduction of the fluorescence signal.

Figure 18 showed the intensity of fluorescence of a solution of 0.25µM HSA in presence of 0.93 µM AgNPs and increasing concentrations of cysteine (1µM, 10µM, 100µM, 1mM)at t=5 min and t=24h. The intensity of signal was normalized with the intensity of the solution of HSA containing0.93 µM of AgNPs and no cysteine at t=5 min. In this experiment, in one case cysteine was added five minutes before the addition of AgNPs (HSAcysAgNP in the figure). These results are represented by the dotted lines. In the other case, the AgNPs were added first (HSAAgNPcys in the figure) to cysteine. This latter case is represented by the full lines. The scratched lines stand for samples containing 0.25 µM HSA, increasing concentrations of cysteine, and 0.6 µM $AgNO_3$. These analyses were performed in order to have a better clue on the reactivity of cysteine and silver with HSA.

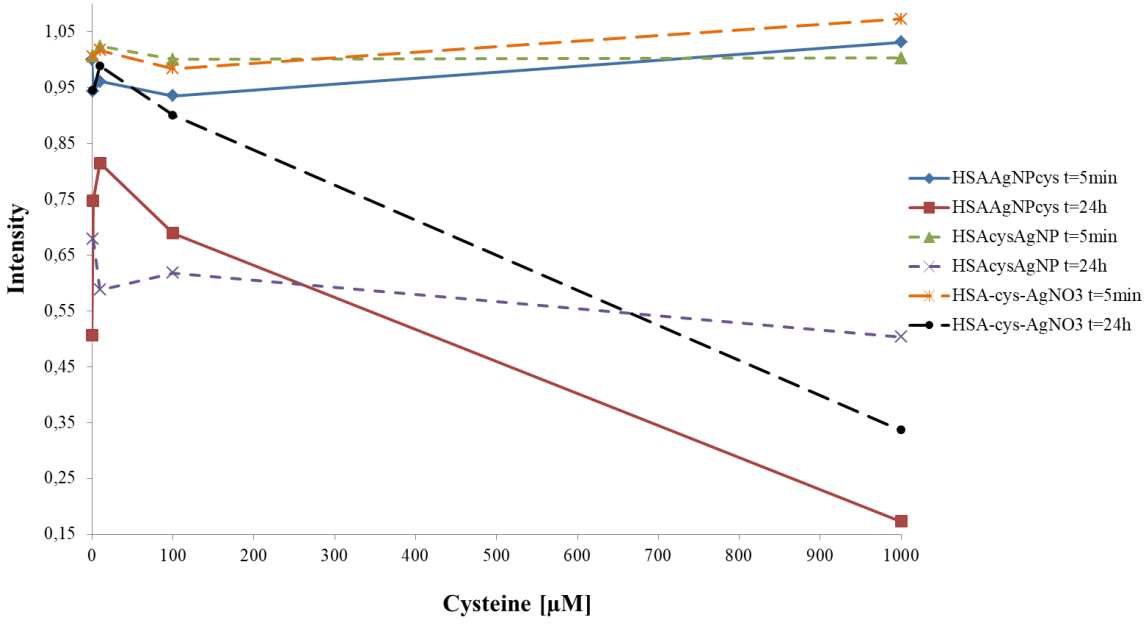

Figure 18: Fluorescence of 0.25 µM of HSA in presence of 0.93 µM of AgNPs and increasing concentrations of cysteine

The green line was attributed to HSAcysAgNPs after five minutes. It could be seen that when cysteine was added first, almost no variation of fluorescence could be observed due to the addition of AgNPs, excepted for a slight increase in fluorescence up to 102% of initial signal of fluorescence, while otherwise the signal was around 100% for all the other tested concentrations. The samples of HSAAgNPcys t=5 min in blue showed a slight reduction of 4-7% of the fluorescence signal in presence of low concentrations of cysteine (1-100µM) compared to the

fluorescence without cysteine. At 1000 µM of cysteine the fluorescence signal increased up to 103% of the initial signal. The purple line represented the fluorescence of HSAcysAgNPs after 24h. Results indicated that after 24h-exposure to AgNPs only, the fluorescence of HSA was reduced up to 50%. When cysteine was added first at 1 µM the fluorescence was restored up to 67% of the initial intensity but was then reduced with increasing concentrations of cysteine up to 61% at 100 µM and reached a minimum of 50% at 1000 µM cysteine. In samples where AgNPs were added first, after 24h-exposure the fluorescence of HSAAgNPcys at 1 µM of cysteine was partially restored up to 74% of intensity. Further, increasing concentrations of cysteine induced more restoration of the fluorescence. Indeed, at 10 µM of cysteine, the fluorescence was of 81%. When the concentrations of cysteine were further increased, the fluorescence intensity started to reduce to 68% for 100 µM and was minimal at 1000 µM where the intensity was 17% of the initial fluorescence at t=5 min.

The orange and black doted scratched lines represented the fluorescence intensity of HSA with cysteine and $AgNO_3$ ($HSAcysAgNO_3$) at respectively 5 min and 24 h exposure. In orange it could be seen that when cysteine was added first, silver ions had similar or slightly lower effects on the reduction of fluorescence of HSA for concentrations of cysteine between 1 and 100 µM. With 1000 µM of cysteine, the fluorescence signal was increased up to 107%. After 24h of exposure and in presence of low concentrations of cysteine, the fluorescence of HSA was restored to 94, 98, and 90% for 1, 10 and 100 µM of cysteine respectively. However, with 1000 µM of cysteine, the fluorescence was significantly reduced to 34%.

As there was less reduction of the fluorescence signal in the $HSAcysAgNO_3$ sample after 24h than with HSAcysAgNP or HSAAgNPcys samples, this indicated that cysteine could efficiently complex the silver ions and remove them from the system. Also, this also proved that AgNPs had a direct and specific effect on HSA which was not dependent on the presence of dissolved silver. This effect was clearly observable after 24h exposure as the fluorescence of $HSAcysAgNO_3$ reached 98%, while the fluorescence of HSAcysAgNPs and HSAAgNPcys were 58% and 81%, respectively, when cysteine was concentrated at 10 µM. These results also suggested different mechanistic ways of actions between the AgNPs and the silver ions on HSA. Indeed, as already described, silver ions can have numerous binding sites on HSA (Shen et al. 2003). However, AgNPs have been shown to have only a few binding sites on albumin. Mariam et al. (2011) as well

as Roy and Das (2014) have found only one binding site for the AgNPs on BSA. While silver ions were expected to bind with the free cys34 of HSA (Marambio-Jones and Hoek 2010b, Shen et al. 2003), Guo et al. (2015) postulated that BSA did not bind AgNPs via the free cystein as treatment with SDS did not lead to reduction of the binding between AgNPs and BSA. Nevertheless, Mariam et al. (2014) observed a reduction of the total free thiols of HSA exposed to AgNPs. Also, silver ions are really small compared to AgNPs, thus they could reach regions that AgNPs are unable. Indeed, silver ions measure 0.165 nm (https://www.webelements.com/silver/atom_sizes.html (checked 21-09-2017)), while AgNPs measured 20nm. Moreover, HSA has a hydrodynamic diameter around 8.5 nm (Kiselev et al. 2001, Mariam et al. 2014). Therefore, silver ions could easily reach hidden hydrophobic pockets of the protein, while HSA could only bind to the AgNPs through its surface. Further, it was demonstrated that albumin bound on the surface of AgNPs through a side-on or end-on model, thus leading to the formation of a corona on the surface of the nanoparticles (Ostermeyer et al. 2013, Ravindran et al. 2012). Also it was postulated that absorption of BSA on the surface of AgNPs happened through multiple steps (Ravindran et al. 2012). Dasgupta et al. (2016) suggested that AgNPs bind to the protein of BSA in proximity of its active site IB. We have estimated that the total surface would be approximately of 57 nm^2 which could bind up to 22 proteins of HSA for 20 nm diameter AgNPs. Different mechanistic ways of action between the silver ions and the AgNPs were also observed by Mariam et al. (2014) who observed that AgNPs affected the esterase activity of HSA although silver ions elicited no effects on this activity of HSA.

Moreover, at t=5 min, the results of HSAcysAgNPs were highly similar to the ones of HSAcysAgNO_3, while an important difference was observed after 24h. Therefore, it is suggested that both AgNPs and AgNO_3 present in a solution of AgNPs could impact the fluorescence of albumin. Within the first minutes of reaction, silver ions present in the solution of AgNPs would interact with easily accessible sites of the protein, while after longer incubating time the AgNPs would directly affect the structure of the protein.

Furthermore, the results demonstrated that the order the reactant was added to HSA had a significant effect on the reactivity of the system. When cysteine was added before AgNPs, the signal was stronger at five minutes than when AgNPs were added first. This indicates that cysteine interfered and reduced the fast interactions between HSA and silver source. This could have

happened either because cysteine bound to the same sites than silver or because the amino acid interacted with the AgNPs or the silver ions, thus inhibiting their potential actions with the protein (Siriwardana et al. 2015, Wigginton et al. 2010). Further, cysteine is known to be used as a coating material for AgNPs, but could also lead to the formation of aggregates of AgNPs (Choi et al. 2003, Mandal et al. 2001, Varghese et al. 2009). Thus, the aggregation of AgNPs could hinder their effects on HSA. However, when AgNPs were added first, the nanoparticles or the silver ions could have directly interacted with HSA, thus altering its conformation resulting in reduced fluorescence. After 24h exposure, HSAAgNPscys samples elicited a stronger fluorescence signal compared to HSAcysAgNPs, except for the samples at 1000 µM of cysteine. The maximal gap between the two samples was of 22% of fluorescence intensity at 10 µM of cysteine.

With high concentrations of cysteine (100, 1000µM), the fluorescence signal was strongly reduced after 24h of exposure. This indicated that high amount of cysteine led to modification of the microenvironment of tyrosine and tryptophan moieties or to degradation of the albumin.

1.6. CONCLUSION

In this work, we demonstrated the affinity between Human Serum Albumin and silver either under its ionic or nanoparticulate form. Raman spectroscopy confirmed the affinity of silver for the thiol group of the cysteine. Our experiments also highlighted that the binding of AgNPs or silver ions on HSA modifies the structure of the protein. The particularity of this study was that it compared the direct effects of silver ions to the effects of AgNPs (without silver ions) on HSA. The results showed that although AgNPs had direct effects on HSA, the impacts of silver ions were stronger, resulting in a higher reduction of the fluorescence of the protein. We suggested that one reason for such difference could be due to the presence of multiple binding sites for silver ions as it is a small ion that can enter inside the skeleton of the protein. Thus the silver ions could reach sites that may be inaccessible for the AgNPs as they are bigger than the protein, which could result in steric hindrance.

CHAPITRE 2

NANOTOXICITÉ DES NANOPARTICULES D'ARGENT : DES DÉVERSEMENTS ENVIRONNEMENTAUX AUX EFFETS SUR LES ORGANISMES

2.1 RÉSUMÉ EN FRANCAIS

L'utilisation croissante des AgNPs dans les produits d'utilisation courante mène au relâchement de ces mêmes particules dans l'environnement. Suite à cette libération dans les milieux aquatiques, les AgNPs vont pouvoir être soumises à de nombreuses transformations. Elles peuvent en effet interagir avec de la matière organique et/ou inorganique, mais également être sujet à des phénomènes tels que l'oxydo-réduction, la dissolution, la dégradation par la lumière, de même que des phénomènes d'agrégation. Ces transformations vont modifier la chimie de surface des nanoparticules et ainsi modifier leur comportement physique, chimique et toxicologique dans l'environnement. De plus, ces phénomènes ne sont pas indépendants les uns des autres et peuvent par conséquent se produire simultanément, complexifiant ainsi d'avantage les comportements des AgNPs dans l'environnement.

De par leur libération dans l'environnement et les nombreuses transformations qu'elles peuvent y subir, les AgNPs posent de sérieuses préoccupations écologiques aux scientifiques. En effet, il est important de déterminer de manière précise les effets potentiels des AgNPs sur les organismes vivants. L'article ci-dessous est une revue de littérature qui regroupe les principaux effets des AgNPs sur les grandes classes d'organismes marins (bactéries, microalgues, zooplancton, macro-invertébrés, et poissons).

Il a été montré que les effets bactéricides des AgNPs sont principalement dus à des impacts sur les membranes cellulaires avec formation de pores et augmentation de la perméabilité cellulaire; la liaison avec des protéines intracellulaires et transmembranaires suivit de leur altération, ainsi que l'inhibition des activités enzymatiques; altération des voies métaboliques; inhibition de l'activité ribosomique; dégradation de l'ADN; augmentation de la production d'espèces réactives oxygénées (ROS) et l'augmentation de la peroxydation des lipides. Ces effets présents chez les cellules procaryotes ont tous pu être également retrouvés dans les cellules eucaryotes, indiquant des effets potentiellement néfastes des AgNPs sur ces organismes. De plus, il a été observé que les daphnies pouvaient activement filtrer les AgNPs présentes dans leur proximité et ainsi accumuler des

concentrations importantes des particules. Il a également été montré que les AgNPs pouvaient s'adsorber à la surface des carapaces des daphnies et dans leur système digestif. La présence de nanoparticules d'argent peut altérer la reproduction ainsi que les capacités natatoires des daphnies, mais aussi augmenter leur mortalité. Chez les macroorganismes tels que les macro-invertébrés et les poissons, il a pu être démontré que les AgNPs peuvent fortement altérer le développement des juvéniles et embryons. Ceci induit l'apparition de malformations physiologiques importantes telles que des problèmes de calcification de coquilles ; malformations des yeux et des nageoires ; diminution de l'épaisseur de l'épiderme ; et malformations d'organes interne. De plus, des altérations du métabolisme ont été mesurées, ainsi que des mortalités accrues et des réductions de reproduction.

Cet article sous forme de revue de littérature a donc permis de compiler et comparer les impacts négatifs des déversements de nanoparticules d'argent dans l'environnement ainsi que leurs effets et potentiels de bioaccumulation le long de la chaine trophique. La conclusion de cette revue est que malgré les faibles concentrations d'AgNPs actuellement présentes dans l'environnement, les effets potentiels des AgNPs existent bel et bien de telle sorte que ces nanoparticules représentent un intérêt particulier dans les domaines de l'écotoxicologie.

Cette revue de littérature a été écrite en commun avec Mathieu Millour, Kim Doiron, Adriano Magesky, Karine Lemarchand, Émilien Pelletier, et Jean-Pierre Gagné. Ma contribution à cet article fût une contribution générale à toutes les parties. En effet, je me suis attelé à rédiger une première version de la revue dans lequel apparaissait tous les sous chapitres et à donner une vision générale du problème des nanoparticules dans l'environnement. J'ai également travaillé plus spécifiquement sur les sous chapitres concernant les microalgues ainsi que le zooplancton. Mathieu Millour a travaillé spécifiquement sur les transformations physico-chimiques des AgNPs une fois présentes dans l'environnement ; Kim Doiron et Karine Lemarchand ont apporté leurs expertise et rédigé la section concernant les effets des AgNPs sur les bactéries/communautés bactériennes ; Adriano Magesky et Émilien Pelletier ont travaillé sur les effets des AgNPs sur les invertébrés, notamment sur les oursins ; et Jean-Pierre Gagné a rédigé l'introduction ainsi que la conclusion de la revue. Tous les auteurs ont participé à la relecture complète ainsi qu'à la correction de l'article.

2.2. NANOTOXICITY OF SILVER NANOPARTICLES: FROM ENVIRONMENTAL SPILL TO EFFECTS ON ORGANISMS

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2.3. INTRODUCTION

Nanotechnologies are a remarkable source of modern innovation leading to the production of cutting edge products with high potential for new processes and goods. From a recent survey, we know that 622 companies from 32 countries manufacture 1814 products claiming the presence of engineered nanomaterials (ENMs) or engineered nanoparticles (ENPs) in their goods Vance et al. (2015). Silver nanoparticles (AgNPs) are the most popular advertised nanomaterial with 438 products (Vance et al. 2015). Each year, thousands tons of ENMs are produced. These ENMs are very small objects having at least one dimension between 1 and 100 nm (ISO 2008). These tiny structures can be embedded in solid products to form nanostructured materials or surface bound particles. However, in most of consumer products they are present as minuscule suspended particles in a variety of fluids or dispersed in powders (Vance et al. 2015). During the life cycle of ENMs, which includes production, use and disposal (Caballero-Guzman and Nowack 2016, Som et al. 2010), it is estimated that ten of the major ENMs; SiO_2, CeO_2, carbon nanotubes, nanoclays, Al_2O_3, Cu, Fe, ZnO, TiO_2 and Ag, could release about 190 000, 50 000, 8 000 and 69 000 metric tons per year to landfill, soil, air and water, respectively (Keller and Lazareva 2014). Even if important uncertainties exist on the production and real mass flows of nanoproducts released in the environment and the difficulty to measure their concentrations in environmental matrices (Keller and Lazareva 2014, Piccinno et al. 2012, Vance et al. 2015, Holden et al. 2014), proofs of leaching of ENMs have been published. For instance, it is well known that nanoparticles liberation can occur from textiles, coating or from solid nanocomposites products (Benn and Westerhoff 2008, Froggett et al. 2014, Kaegi et al. 2010, Mitrano et al. 2015). What would happen if important spills of ENMs occur in wastewater, river, lake or estuary? What is the fate and toxic action of those ENMs dropped in environment? Today, our knowledge concerning their environmental risks and their nanotoxicity is limited.

As shown in Figure 1, throughout their life cycle, ENMs undergo variations in their particle size distributions from production to disposal with a general increase in size explained by various processes explained below (Caballero-Guzman and Nowack 2016). This is because the production of ENMs is processed under monitored parameters exerting control over the small size of native ENMs. However, the inclusion of ENMs into products and their use, weathering and aging under variable conditions result in aggregation or transformation at the time of disposal. ENMs can be

released from nanomaterials during their entire life cycle, but should be more important at the use phase (Caballero-Guzman and Nowack 2016, Gottschalk and Nowack 2011). After their release, ENMs can be processed in recycling systems, incinerators, wastewater treatment plants or delivered to environmental compartments, landfill, air, soil, water (Caballero-Guzman and Nowack 2016). At this moment, exposure and risk begin to be a reality for bacteria, microalgae, zooplankton, fish and ultimately human beings (Figure 19). Throughout their journey, ENMs, can experience numerous changes in their physicochemical speciation and environmental exposure to organisms.

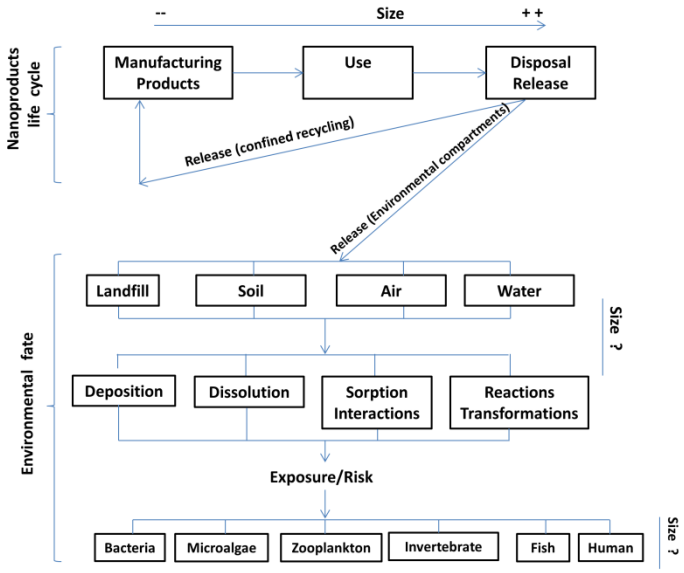

Figure 19: Nanomaterial life cycle, environmental fate and exposure/risk of organisms to native nanomaterials or by products

It is impossible to describe all biophysicochemical interactions affecting ENMs in complex aqueous media or near biological interfaces, but Nel et al (2009a) have described a useful conceptual framework to guide us in understanding what happens at the nano-bio interface and in providing clues to mechanisms of nanomaterial toxicity. Following Nel et al (2009a) the nano–bio interface comprises three dynamically interacting components: (i) the nanoparticle surface, the characteristics of which are determined by its physicochemical composition; (ii) the solid–liquid interface and the changes that occur when the particle interacts with components in the surrounding medium; (iii) the solid–liquid interface's contact zone with biological substrates. The main parameters affecting the nano-bio interactions are related to the nature of ENMs, the composition

of the suspending media, the solid-liquid interface present in the environmental compartments and the interactions occurring at the nano-bio interface as shown in Table 3.

At the production step, the chemical composition, size, surface coating, geometry, crystallinity, porosity, roughness, hydrophobicity or hydrophilicity are fixed. The suspending media where ENMs are dispersed determine the pH, ionic strength, zeta potential, aggregation, state of dispersion and stability, dissolution characteristics, and the presence of large organic molecules (such as humic substances, proteins, polysaccharides) or detergents (Nel et al. 2009a). The particle attributes foster the interactions with the medium through: (i) promoting the adsorption of ions, the sorption of proteins, natural organic materials (NOM) or detergents; (ii) double layer formation; (iii) dissolution; or (iv) minimizing free surface energy by surface restructuring (Khan et al. 2011, Nel et al. 2009a). These effects can results from forces arising from attractive van der Waals (VDW) and (generally) repulsive electrostatic double layer interactions, plus short range forces arising from charge, steric, depletion and solvent interactions (Nel et al. 2009a). Nel et al (2009a) suggested that hydrodynamic, electrodynamic, electrostatic, solvent, steric and polymer bridging interactions are the main forces controlling the interfacial interactions between nanomaterials and biological systems.

In this review, we focused our interest to silver nanoparticles (AgNPs), the most widespread used nanomaterial (Vance et al. 2015). AgNPs have important applications in food packaging materials, odor-resistant textiles, paints, cosmetics, medical devices, water disinfectants, because of their potential bactericidal effects (SCENIHR 2014). Multiple uses of AgNPs make inevitable their release and transport in wastewaters and their migration toward rivers and coastal waters explaining why they are listed as aquatic emergent contaminants (SCENIHR 2014, USEPA 2009). Little is yet known about the fate of AgNPs in natural waters and their potential environmental impacts and toxicity on aquatic organisms from bacterial communities to vertebrates. This review aims to provide a critical assessment of the current understanding of the fate of AgNPs in natural waters and to link this behavior to toxic effects observed in some prokaryotes and eukaryotes organisms. Our survey is describing the behavior of AgNPs in aqueous media, and then discuss the toxic effects reported for bacteria, microalgae, zooplankton, invertebrates and fish. A special attention is given to marine organisms when informations were available.

Table 3: Main biophysicochemical parameters affecting nanomaterials speciation and the interface between nanomaterials, suspending media and biological systems (modified from Nel et al. (2009a))

Nanoparticle speciation
Size, size distribution, shape, surface area
Surface charge, energy, roughness and porosity
Chemical properties (element, valence, functional groups/coating)
Crystallinity and defect
Hydrophobicity and hydrophilicity
Solubility
Suspending media
Water molecules
Acids and bases
Salts and multivalent ions
Natural organic matter (NOM such as humic substances, proteins, lipids, etc.)
Oxygen, sulfur compounds
Surfactants
Polymers
Polyelectrolytes
Solid-liquid interface
Surface hydration and dehydration
Surface reconstruction and release of free energy
Ion adsorption and charge neutralization
Electrical double-layer formation, zeta potential, isoelectric point
Sorption organic matter, steric molecules or toxins
Electrostatic, steric and electrosteric interactions
Aggregation, dispersion and dissolution
Hydrophilic and hydrophobic interactions
Nano-bio interface
Membrane interaction: specific and nonspecific forces
Receptor-ligand binding interactions
Membrane wrapping: resistive and promotive forces
Biomolecule interactions (lipids, proteins, DNA) leading to structural and functional effects
Free energy transfer to biomolecules
Oxidant injury to biomolecules.
Mitochondrial and lysosomal damage, decrease ATP

2.4. PHYSICAL AND CHEMICAL BEHAVIOR AND FATE OF SILVER NANOPARTICLES IN AQUATIC SYSTEMS

As mentioned above, some critical physical and chemical transformations of AgNPs occur in aquatic environments (Figure 20). Two important processes are commonly observed: (i) single particle or individual behavior such as oxidation and sulfidation; (ii) multiple particles or group behavior as autoaggregation or heteroaggregation. Multiple sorption processes or interactions between nanomaterials and NOM are located at the confluence of single and group behavior. This NOM can be formed by dissolved small molecules or large geomolecules generated *in situ* or by particulate detritus released by biological activities. The way nanoparticles behave in the environment is controlled by their own properties (size, geometry, coating, hydrophobicity, etc.), and by the characteristics of the receiving environment: i.e. redox conditions (oxic or hypoxic/anoxic), ionic strength, pH, NOM and suspended particulate matter (SPM) (SCENIHR 2014).

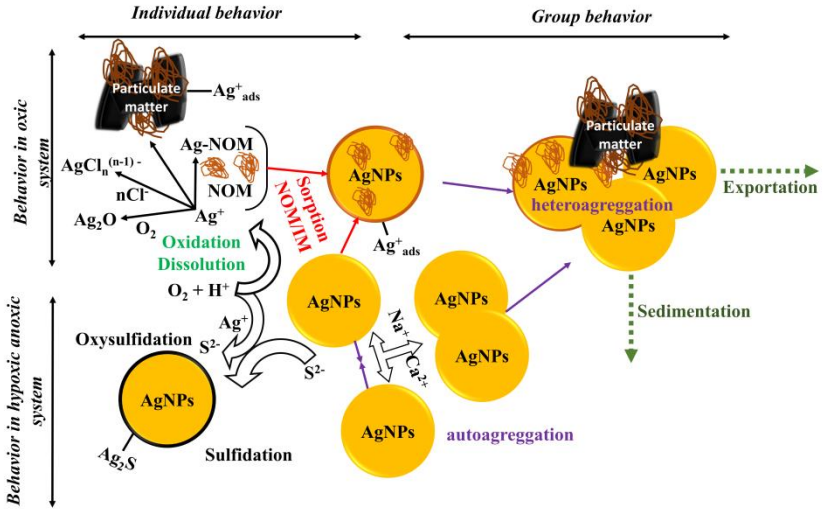

Figure 20: Complex behavior of silver nanoparticles in aquatic systems

2.4.1. Single nanoparticle and individual behavior

2.4.1.1. Oxidation – Dissolution

The presence of dissolved oxygen can promote surface oxidation of AgNP (Liu and Hurt 2010a, Zhang et al. 2016a), according to equation 1.

$$AgNP + ¼\ O_{2\,(aq)} + H^{+} = Ag^{+}_{(aq)} + ½\ H_2O \quad (1)$$

This oxidation-dissolution process is directly influenced by pH of the solution, being higher under acidic conditions (Peretyazhko 2014, Zhang et al. 2016a). Intermediate reaction with formation of H_2O_2 can enhance the dissolution (Zhang et al. 2016a). Dissolution of silver nanoparticles is also affected by the size of AgNPs (Dobias and Bernier-Latmani 2013, Lowry et al. 2012a, Peretyazhko 2014, Zhang et al. 2016a). This can be explained by the higher reactivity of small AgNPs due to their higher surface/volume ratio. However, many studies have shown that this process is generally slow under natural conditions. Authors reported 2-3% AgNP dissolution after 14 days and only 30% after four months (Dobias and Bernier-Latmani 2013, Li et al. 2012, Zhang et al. 2016a).

Some studies (Dobias and Bernier-Latmani 2013, Li et al. 2010c) mentioned strong variations in silver ions levels during the first minutes of experiments where a quick increase was followed by a strong decrease. Sotiriou et al. (2012) hypothesized a desorption or dissolution of silver oxide layers from AgNP surface. This layer could result from hydrolysis or oxidation of AgNP surface (Elimelech et al. 1998, Zhang et al. 2016a). Desorption of chemisorbed silver at the surface of AgNPs (Dobias and Bernier-Latmani 2013, Li et al. 2012, Sotiriou et al. 2012) was affected by the presence of sodium-halogen salts (Espinoza et al. 2012). For instance, this process is not observed at NaCl concentrations below 27 mM, but enhanced at higher concentrations (Espinoza et al. 2012).

Silver ions released from AgNPs can interact with inorganic or organic compounds present in aqueous media. In an oxic system, silver ions interact with chlorides, carbonates or sulfates (Levard et al. 2012). Due to the concentration of these anions in natural waters, the formation of silver chlorocomplexes ($AgCl_n^{(n-1)-}$) is thermodynamically favored (Levard et al. 2012) and these complexes ($AgCl^-$ and $AgCl_2^-$, etc.) are strongly influenced by the Cl/Ag ratio (Levard et al. 2013). These formed salts can be sorbed around AgNPs modifying their coating or can precipitate to form new silver nanomaterials ($K_{spAgCl} = 1.77 \times 10^{-10}$ (Levard et al. 2012)). Moreover, silver ions can

also interacts with NOM to form organic silver nanomaterials (see also section 2.2.2 for AgNPs interactions with NOM) (Adegboyega et al. 2013, Akaighe et al. 2012, Akaighe et al. 2011) or sorb on SPM as clay (Zhou et al. 2012).

2.4.1.2. Sulfidation

Sulfidation of AgNPs is a process mainly observed in hypoxic and anoxic systems where levels of sulfur ions (S^{2-}) are high (Levard et al. 2012). This transformation occurs mostly in wastewater treatment plants (Kaegi et al. 2011, Levard et al. 2012), some-S-rich soils (Colman et al. 2013) and sediments (Dale et al. 2013, Lowry et al. 2012a). Sulfur ions can interact with AgNPs by two pathways. In the first one, the oxysulfidation, S^{2-} interacts with silver ions in the presence of oxygen and sulfur, under hypoxic conditions (Liu et al. 2011). Due to the low solubility of Ag_2S ($K_{sp} = 5.92 \times 10^{-51}$ (Levard et al. 2012)), this salt can precipitate to form new silver nanomaterials, and/or sorb around AgNPs (Levard et al. 2011a, Liu et al. 2011). The second process could be a direct sulfidation inducing the formation of a new Ag_2S coating over the surface of AgNPs (Baalousha et al. 2015, Liu et al. 2011). This process is known to occur preferentially in anoxic environments with high sulfur levels (Liu et al. 2011). These two processes are strongly influenced by the original coating of AgNPs (Baalousha et al. 2015, Liu et al. 2011), and depending on this coating, the sulfidation can be limited and some properties of native AgNPs can be preserved, as reported for citrate-coated AgNPs (cit-AgNPs) (Baalousha et al. 2015).

2.4.2. Multiple particles or group behavior

2.4.2.1. Aggregation

Aggregation is defined as a process where dispersed particles tend to interact together to form an assemblage of particles named aggregates (McNaught and Wilkinson 2014). In aqueous solution, AgNPs move randomly due to the Brownian motion (Elimelech et al. 1998, Petosa et al. 2010), and can collide and interact together to form larger particles or aggregates. Depending on various physicochemical parameters of the medium these interactions can be efficient or not.

Interactions between free-moving particles in liquid have been modeled by Derjaguin, Landau, Verwey and Overbeek (DLVO) (Derjaguin and Landau 1941, Petosa et al. 2010, Verwey and

Overbeek 1948, Zhang et al. 2016a). The DLVO theory indicates that the attractive and repulsive forces, respectively van der Waals forces and electrical double layer repulsion, are controlling the aggregative behavior. Stability of AgNPs in suspension is observed when the repulsive forces are stronger than attractive ones, as seen in nanopure water for cit-AgNPs (Millour et al. 2015). The addition of dissolved salts to cit-AgNPs solution induced a destabilization of the steady-state of the suspension (Millour et al. 2015, Baalousha et al. 2013, El Badawy et al. 2010). When the ionic strength increases, the thickness of electrical double layer decreases and favors the close encounter of AgNPs (Millour et al. 2015, Petosa et al. 2010). In these conditions, the repulsive forces decrease and tend to be weaker than attractive forces. The presence of dissolved salts also modulates surface charges of AgNPs (Millour et al. 2015, Petosa et al. 2010) as measured with the zeta potentials. The surface charges are screened by counterions (Millour et al. 2015, Petosa et al. 2010), as cations tend to interact with negatively charged AgNPs whereas anions are attracted by positive AgNP surface. The pH of the medium can also influence the stability of AgNPs through surface charges or isoelectric points (Petosa et al. 2010) by adding H^+ or OH^- ions. The collision between two AgNPs resulting in aggregation is not observed asfully efficient when the ions concentration is too low to diminish electrical double layer repulsion (slow or reaction limited aggregation), but could be fully efficient when ions are sufficiently concentrated to impact electrical double layer repulsion (fast or diffusion limited aggregation) (Millour et al. 2015, Petosa et al. 2010). The transition between these two steps is determined by the critical coagulation concentration (CCC) (Millour et al. 2015, Petosa et al. 2010). Moreover, the valence of counterions influence strongly the CCC (Petosa et al. 2010). The CCC decreases with valence increasing. The CCC determined for AgNPs in water are generally higher than 50 mM in presence of monovalent salts and smaller than 60 mM for divalent salts depending on the organic coating of AgNPs (Baalousha et al. 2013, Chen and Zhang 2012, Huynh and Chen 2011).

Using DLVO model, we can assume that the CCC is correlated to zeta potentials, as observed for cit-AgNPs, polyethyleneimine-AgNPs or bare-AgNPs (El Badawy et al. 2010, Philippe and Schaumann 2014). However, this is not the case with AgNPs coated with polymers as polyvinylpyrrolidone. With this coating, AgNPs have low zeta potentials, but the CCC is observed at higher ion concentrations (Chen and Zhang 2012, El Badawy et al. 2010, Huynh and Chen 2011). NOM sorption can also modulate the aggregation and CCC values (Baalousha et al. 2013, Chen and Zhang 2012, Huynh and Chen 2011, Millour et al. 2015). The effects of NOM is to

modify the forces involved to reach the aggregation equilibrium. In this case, it becomes important to use the extended-DLVO theory to describe aggregative behavior, because additional interactions due to polymers or NOM are taken in account (Grasso et al. 2002, Petosa et al. 2010), including hydrogen bonding, hydrophobic interaction, hydration pressure, non-charge transfer Lewis acid base interactions and steric interactions. These complex interactions and effects of medium are fully describe in an exhaustive review by Grasso et al. (2002).

Multiple interactions of AgNPs with other particles or compounds (Table 1 and Figure 2) are observed with NOM as humic substances (Chen and Zhang 2012, Huynh and Chen 2011, Millour et al. 2015, Wang et al. 2016), clay minerals (Liu et al. 2015, Zhou et al. 2012), exopolysaccharides and extracellular polymeric substances (Khan et al. 2011, Lau et al. 2013), as well as proteins and other biological compounds (Nel et al. 2009a, Philippe and Schaumann 2014). Depending on particles interacting together, two modes of aggregation are observed. Autoaggregation describes aggregation of many identical particles whereas heteroaggregation occurs between particles of different nature (Yu and Borkovec 2002). In natural aquatic environment, heteroaggregation of AgNPs with other particles or colloids is the primary and most important process, because AgNP concentrations are between $ng{\cdot}L^{-1}$ to few $\mu g{\cdot}L^{-1}$(Coll et al. 2015, Gottschalk et al. 2013) whereas natural particles, colloids or dissolved NOM are at $mg{\cdot}L^{-1}$ level (Klaine et al. 2008, Tremblay and Gagné 2009). Moreover, interactions between AgNPs and colloids are modulated by physicochemical conditions of aquatic environments. In natural waters, with a complex mixture of ions, aggregation of AgNPs or other particles are driven by additive or synergistic effects of each salt and controlled by the dominant salt (Baalousha et al. 2013). For negatively charged-AgNPs, one can assume that divalent cations drive the aggregation in freshwater whereas in seawater, monovalent cations are activating the process. This is caused by the average concentrations of major cations in world rivers: Na^+ (0.23 mM), Ca^{2+} (0.33 mM), and Mg^{2+} (0.15 mM), and Na^+ (468 mM), Ca^{2+} (10.2 mM), and Mg^{2+} (53.2 mM) in seawater (Bianchi 2007). For anions, concentrations are HCO_3^- at 0.86 mM, Cl^- 0.16 mM and SO_4^{2-} 0.069 mM in freshwater, 2.38, 545 and 28.2 mM, in seawater, respectively. The toxicity mechanisms of AgNPs and their environmental fate can be different depending on the size of aggregates (Millour et al. 2015, Nel et al. 2009a, Zhu et al. 2012). Small aggregates can interact directly with cellular surface and be internalized while large aggregates cannot penetrate directly the cell membrane. Moreover, large aggregates can settle rapidly, whereas small ones can stay a long time in suspension increasing

possible interactions with NOM, detritus, or living cells or even be transported far from their discharge point in the environment.

2.4.2.2. Interaction or sorption with natural organic matter

Sorption is a process where a substance (sorbate) interacts with a solid (sorbent) by adsorption, absorption and desorption (McNaught and Wilkinson 2014, Gagné et al. 2011). Adsorption occurs when sorbate interact just with the surface of sorbent whereas absorption appears when sorbate is internalized by sorbent. Desorption described the process where the sorbate separates to the sorbent. Sorption is an exothermic and spontaneous process (Somorjai 1994). This process implies some possible interactions with nanomaterials: (i) physical interactions as van der Waals and hydrophobic interactions; (ii) electrostatic interactions as charge transfer complex, ligand exchange, ionic bridging and hydrogen bound; and (iii) chemical interactions as covalent bound (Gagné et al. 2011, Philippe and Schaumann 2014, Wang et al. 2016). As described above, surface of AgNPs can be a sorbent for salts or/and organic matter inducing their transformation. Moreover, transformed AgNPs can also interact with heterogeneous surfaces like cell membranes. Depending on the surface involved in the interaction the fate of AgNPs can differ.

NOM is ubiquitous in pristine natural waters, wastewaters, industrial waters or inside biological cells. Humic substances are particularly present in freshwater with more than 50% of NOM composition (Thurman 1985, Tremblay and Gagné 2009), but polysaccharides, lipids, proteins and other organic compounds are also present (Philippe and Schaumann 2014) as well as in living cells. Interactions between NOM and AgNPs in aqueous environment are mostly studied with humic substances and more specifically with humic acids (Chen and Zhang 2012, Doiron et al. 2014, Huynh and Chen 2011, Kim et al. 2013, Philippe and Schaumann 2014, Wang et al. 2016). However, to increase the knowledge of NOM-AgNPs interactions, it is important to describe effects with fulvic acids. In aquatic systems the presence of fulvic acids is more important than humic acids (ratio fulvic/humic acids is between 2 to 10 (Tremblay and Gagné 2009)). Moreover, proteins, lipids, polysaccharides are more representative of biological systems and should receive more attention. Considering the composition of NOM, the interactions of AgNPs with NOM in natural waters or into cells exposed to nanomaterials could be different due to the nature and the

relative proportion of the organic matter found in each medium. It is important to note that bare nanomaterials do not exist under biological conditions due to protein adsorption, which at any time provides an organic coating (Soenen et al. 2015). Thus, it is clear that toxicity of nanomaterials to bacteria, algae, invertebrates and fish is modulated by the presence of NOM (Grillo et al. 2015, Petersen et al. 2014, Wang et al. 2016). Presence of inorganic and some organic surfaces, as SPM, can decrease the bioavailability of AgNPs although organic surfaces, such as biofilms, could favor interactions of AgNPs with organisms and induce toxicity.

2.5. EFFECTS OF AGNPS ON AQUATIC ORGANISMS

2.5.1. Nanoparticles and prokaryotes

Heterotrophic prokaryotes constitute a key component in the functioning of aquatic microbial food web and serve integral environmental functions (Azam and Malfatti 2007, Pomeroy et al. 2007). Through its capacity to degrade organic matter and regenerate nutrients, this biological compartment plays an essential role in the transfer of matter and energy to higher trophic levels (Azam et al. 1983). Thereby, the potential impact of bactericidal AgNPs on aquatic heterotrophic bacteria might strongly affect their capacity to provide ecosystem services listed above. In addition, due to their high growth rates, their short generation times (Del Giorgio and Cole 1998), and the vast diversity of their metabolic activities, bacterial communities constitute very interesting biological models to assess, in real time, the toxicological effects of AgNPs in aquatic ecosystems (Ghiglione et al. 2016). Many studies have described the effects of AgNPs on model microorganisms such as bacterial pathogens of the genera *Vibrio*, *Escherichia* and *Pseudomonas*(Dibrov et al. 2002, Holt and Bard 2005, Xu et al. 2004, Dorobantu et al. 2015). Some results are now available on their potential effects on complex bacterial communities, in terms of diversity and abundance, in different environmental compartments (soil, sediment, and water) but they are generally reporting conflicting results (Maurer-Jones et al. 2013). As an example, a 30-day experiment conducted on the bacterial diversity of estuarine sediments did not demonstrated a correlation between the presence of AgNPs and change in the bacterial community (Bradford et al. 2009). However, other researchers (Choi et al. 2008, Choi and Hu 2008) came to the conclusion that the presence of AgNPs reduced the natural nitrifying by about 90%, which means a drastic change in the bacterial community. Similarly, Fabrega et al. (2011b) have shown

that the presence of AgNPs influenced the bacterial community within marine biofilms by reducing the development and succession of normal bacterial biofilm within 4 days of growth. Modification of the taxonomic diversity of natural bacterial communities was also demonstrated in estuarine waters at low and environmentally relevant AgNP concentrations (5 and 50 $\mu g \cdot L^{-1}$) (Doiron et al. 2012). These contrasting results demonstrate that additional knowledge of nanoparticle physicochemistry, as well as biotic and abiotic factors affecting AgNPs behavior over time (see section 2) is needed to fully understand the mode of action of AgNPs on prokaryotic cells inhabiting aquatic environments (Fabrega et al. 2011a).

2.5.1.1. Potential antimicrobial mechanisms of AgNPs

As AgNPs are expected to affect bacteria by many different ways simultaneously, the development of a specific resistance against AgNPs is quite impossible (Silver et al. 2006, Rai et al. 2012). Given the worldwide emergence of multidrug-resistant (MDR) bacteria and the need to synthesize new antimicrobials, the use of AgNPs as antimicrobial agent has become of great interest in many applications ranging from anti-odour products to food packaging (Rai et al. 2012, SCENIHR 2014). In the last decade, many studies have reported efficient bactericidal activities of AgNPs against bacterial models, human and phyto-pathogens as well as MDR pathogens (Marambio-Jones and Hoek 2010a, Lara et al. 2010, Mishra and Singh 2015). Bacterial models used in nanotoxicity studies varied greatly and have included common research species such as *Escherichia coli*, *Bacillus subtilis*,*Vibrio splendidus* and *Pseudomonas aeruginosa* along with bacteria that play key roles within environmental compartments, such as *Nitrosomonas europaea*, a nitrifying bacteria with a key role in wastewater treatment (Dorobantu et al. 2015, Yang et al. 2013, Doiron et al. 2014, Maurer-Jones et al. 2013). Interestingly, AgNPs are not only active against free cells, but have also demonstrated a great efficiency against bacterial biofilms (Abdel Rahim and Ali Mohamed 2015a, Flores et al. 2013, Radzig et al. 2013). Moreover, the combined use of AgNPs and classical antimicrobial compounds tends to enhance their bactericidal potential in a synergistic way (Abdel Rahim and Ali Mohamed 2015a, Lara et al. 2010, Shahverdi et al. 2007, Deng et al. 2016).

Despite the increasing use of AgNPs as an antimicrobial agent, the scientific community still debates about the nature of the nanosilver antimicrobial mechanisms. Although silver ion (Ag^{+})

released by AgNPs dissolution close to membranes or within bacterial cells is likely the predominant antimicrobial mechanism of AgNPs (Martínez-Gutierrez et al. 2012, Zhang et al. 2016a, Xiu et al. 2012), the debate on the existence of a "particle-specific" effect of nanosilver is still open (Marambio-Jones and Hoek 2010a, Zhou et al. 2015, Zhang et al. 2016a). Antimicrobial properties of AgNPs are negatively correlated with the particle size and shape; small size (< 10 nm) AgNPs being the particles most likely to be taken up by microorganisms and affecting cellular viability through their rapid dissolution in silver ions within cells (Choi et al. 2008, Choi and Hu 2008, Morones et al. 2005, Shrivastava et al. 2007, Sondi and Salopek-Sondi 2004, Kumari et al. 2016). Generally speaking, nanosilver exposure can seriously damage cellular structures (such as cell membranes and cell walls), cellular components (nucleic acids, lipids and proteins) and inhibit critical cellular processes (ATP-associated metabolism, DNA replication, transcriptional activities) (Prabhu and Poulose 2012, Yang et al. 2013, Marambio-Jones and Hoek 2010a, Morones et al. 2005, Zhang et al. 2016a, Zhou et al. 2015). Some recent studies reported that the contribution of particulate (ion-free) nanosilver and reactive oxygen species generation (Choi et al. 2008) to the overall toxicity of nanosilver must not be neglected and need to be carefully explored (Zhang et al. 2016a, Fabrega et al. 2009). Many studies concluded that silver ion release is plausibly the major antimicrobial mechanism of nanosilver. Nevertheless, others suggested that silver ion release from AgNPs dissolution might not be the exclusive toxicity factor of nanosilver. These authors reported direct and indirect antimicrobial activities of particulate nanosilver as well as antimicrobial effects related to the generation of reactive oxygen species (ROS) under aerobic conditions (Zhang et al. 2016a). The main AgNPs-related damages are presented in Table 4.

Table 4: Main AgNPs-related damages on bacterial cells (for review see Zhang et al. (2016a) and Zhou et al. (2015))

Membrane or wall damage
Accumulation of AgNPs on cell membranes;
Membrane proteins denaturation by AgNPs binding on sulfur-containing proteins;
Increased membrane permeability allowing penetration of AgNPs inside the cells
Membrane destabilization or disruption
Cell lysis
Inhibition of wall synthesis

Cellular components alteration
Penetration and dissolution of AgNPs inside cells generating local concentration of silver ions.
Enzymes denaturation
Inhibition of proteins and nucleic acids synthesis
Interaction with nucleic acids (mutations)

Physiological damages
Catalytic generation of ROS inside the cell
Inhibition of respiratory process
Inhibition or decrease of ATP-mediated metabolism
Inhibition of DNA replication
Inhibition of transcriptional activity
Inhibition of transduction by phosphotyrosine and tyrosine phosphorylation modulation
Dissipation of the proton motive force
Reduction of growth rate
Increase of the lag phase
Enhanced phosphate efflux

Cell membranes might be the main biological target of nanosilver. Many studies reported interactions of AgNPs with bacterial membranes leading to an accumulation of nanoparticles on the surface of bacterial cells, a local release of silver ions generating membrane damages (pits formation) and finally an increased membrane permeability by membrane protein denaturation (Flores et al. 2013, Krishnaraj et al. 2010, Li et al. 2010b, Lok et al. 2007, Lok et al. 2006, Morones et al. 2005, Raffi et al. 2008, Sondi and Salopek-Sondi 2004). Membrane alterations allow the penetration of small AgNPs within the cells and the release of lipopolysaccharides and cellular components in the extracellular medium, resulting at least to microbial cell lysis (Krishnaraj et al. 2010, Lara et al. 2010, Morones et al. 2005, Sondi and Salopek-Sondi 2004). Interaction of AgNPs with cell membranes is essentially due to their binding on sulfur-containing proteins or enzymes that interfere with their proper functions (Zhou et al. 2015). In addition, positively charged nanoparticles might alter state and organisation of lipids and phospholipids, generating serious damages to the structure, function and permeability of cellular membranes (Zhang et al. 2016a).

2.5.1.2. Effects of AgNPs on complex natural bacterial communities

Although the antimicrobial capabilities of AgNPs have been widely reported on bacterial monocultures, their impacts on ecologically important microbial communities are still not well

understood. Monoculture bacteria nanotoxicity studies revealed the mechanisms of nanoparticle-induced toxicity (e.g., ROS production, dissolution of AgNPs to toxic ions within cells, membrane damages) and the significance of nanoparticle physical properties such as particle and aggregate sizes and surface modifications in determining toxicity. Natural isolate studies, on the other hand, involve complex mixtures of bacterial species and have generally focused on understanding toxicity in more complex and ecologically relevant environments, often at the bacterial community level (Maurer-Jones et al. 2013)

AgNP toxicity was reported to depend on concentration, size, shape, and coating-type of the particles (Kumari et al. 2016). In natural environments, AgNP antimicrobial mechanisms reported on monocultures might also be modulated by biotic (e.g. AgNP bioaccumulation) and abiotic (e.g. salinity, presence of NOM, aggregation) factors that might modify shape and size of nanoparticles, as previously described in section 2 of this chapter.

Presently, an increasing number of studies focused on the effects of AgNPs on various microbial communities in different environments (from activated sludge to estuarine water) (Alito and Gunsch 2014, Yang et al. 2014, Samarajeewa et al. 2017, Zhang et al. 2016a, Blakelock et al. 2016, Echavarri-Bravo et al. 2015, Dorobantu et al. 2015, Antizar-Ladislao et al. 2015). Unfortunately, results are conflicting and difficult to compare due to the high variety of nanoparticle types, sizes and coatings and the wide range of concentrations used from few $\mu g \cdot L^{-1}$ to more than 1 $g \cdot L^{-1}$(Zhang et al. 2016a). The concentration of AgNPs in the environment is one of the main drivers of antimicrobial effects on bacterial communities and some studies revealed a clear dose-effect response (Calabrese 2005). In monocultures, this dose-response effect resulted in the observation of an hormesis response at low AgNPs concentrations (less than 20 $\mu g \cdot L^{-1}$) (Doiron et al. 2014), and in the inhibition of bacterial growth (i.e. increase of the lag phase or total growth inhibition) at higher AgNPs (Irwin et al. 2010, Martínez-Castañón et al. 2008, Morones et al. 2005).

Most of the AgNPs released from consumer products enter sewer systems and wastewater treatment plants. As a consequence, wastewater and activated sludge microbial communities are particularly exposed to nanosilver (Kim et al. 2011, Shon et al. 2007, Sun et al. 2013). The investigation of the AgNPs bacterial toxicity in wastewater is critical for risk assessment since autotrophic nitrifying organisms allowing water depuration are among the most sensitive

prokaryotes to AgNPs (Choi et al. 2008, Choi and Hu 2008). The potential perturbations of nanosilver to microbial mediated nitrogen cycling might be important to consider in many environments since nitrifiers could be highly affected by the presence of AgNPs (Langdon et al. 2014, Masrahi et al. 2014) in terms of enzymatic activities (Colman et al. 2013), nitrification activities (Jeong et al. 2014), and microbial taxonomic and functional diversity (Samarajeewa et al. 2017, Shah et al. 2014, Yang et al. 2014).

Only a limited number of studies have been conducted to evaluate the impacts of AgNPs on complex microbial communities after AgNPs release by wastewater effluents to aquatic ecosystems (Bradford et al. 2009, Echavarri-Bravo et al. 2015). In 2012, Das et al. demonstrated that AgNPs rapidly, but temporarily, inhibited natural bacterioplankton production in freshwater. Similarly, Colman et al. (2012) observed a decrease in microbial respiration of pelagic communities, but found no impact on benthic communities. However, Blakelock et al. (2016) did not report any toxic effects of AgNPs on bacterioplankton abundance, biomass, production or chlorophyll-a content in a boreal lake. These recent results contradict the observations of Pradhan et al. (2011), who have demonstrated that the presence of AgNPs induced a decrease in microbial biomass and in community richness in freshwater streams. Only scarce data are available concerning the effects of AgNPs on estuarine or marine microbial communities and they reported highly conflicting results. In 2009, Bradford et al. did not observe any effects of AgNPs on the taxonomic diversity of microbial communities inhabiting estuarine sediments whereas Antizar-Ladislao et al. (2015) recently reported an increase in total bacterial abundance characterized by a richness modification and the loss of key species such as *Pelobacter propionicus* within the bacterial community. Similar observations were reported by Doiron et al. (2012) in estuarine waters where the addition of low concentrations of AgNPs(5 to 50 $\mu g \cdot L^{-1}$) induced a rapid, but temporary, reduction of total bacteria abundance and a significant reduction of planktonic bacteria richness. Echavarri-Bravo et al. (2015) have also reported short term modifications of the functional diversity of impacted estuarine benthic bacterial communities followed by a recovery after 120 hours at 1 $mg \cdot L^{-1}$ of AgNPs.

Two different hypotheses have been proposed to explain the rapid recovery of the bacterial community in fresh and estuarine waters: a selection of silver resistant strains within the community and/or AgNPs pressure alleviation on microbial communities due to aggregation,

sedimentation or environmental modifications of nanoparticles structure along time. (Das et al. 2012, Doiron et al. 2014, Doiron et al. 2012, Fabrega et al. 2009). Even if the mechanisms involved in the reduction of AgNPs toxicity along time are presently not well understood, it was proposed that natural organic matter compounds, such as humic substances, could sorb on the surface of AgNPs (Fabrega et al. 2009, Doiron et al. 2014), lessen the dissolution into silver ions and, consequently, reduce the overall toxicity of AgNPs to bacterial cells. As a result, the inconsistency of the results on AgNPs toxicity to environmental microbial communities may be related to their interactions with different environmental components (e.g. NOM, salts, see section 2 for detailed information). In addition, impacts of AgNPs on complex microbial communities could be modulated not only by the structure of AgNP aggregates but also by the spatial distribution of microorganisms (free cells versus attached cells or biofilms) (Sheng and Liu 2011).

2.5.2. Effects of AgNPs on phytoplankton

2.5.2.1. Effects and mechanisms of AgNPs on phytoplankton

Although AgNPs are expected to have minor effects on human health and only affect prokaryotes, there are increasing evidences that AgNPs can be a threat to eukaryotic aquatic organisms. Indeed, it was postulated that silver is the second most toxic metal for aquatic organisms, after mercury (Moreno-Garrido et al. 2015). In this regard, a great interest has been given to the effects and mechanisms of AgNPs on eukaryotic organisms.

2.5.2.1.1. Interactions of AgNPs with microalgae

Phytoplankton assemblages play a major role in the aquatic food web as these microscopic plants are the main source of energy for many aquatic herbivores. Although toxic concentrations of AgNPs are generally higher for phytoplankton than bacteria many studies have shown impacts of AgNPs on microalgae leading to phenomenon such as growth inhibition (Das et al. 2012, Moreno-Garrido et al. 2015, Navarro et al. 2008), reduction of algal viability (He et al. 2012, Huang et al. 2016a) and morphological malformations (He et al. 2012, Moreno-Garrido et al. 2015).

Toxic effects of AgNPs on microalgae cells membranes are quite similar to those described on bacterial membranes (see section 3.1.1). First, AgNPs may partly dissolve in the media, thus

releasing silver ions that could diffuse through the algal surface and penetrate the cells, thus affecting vital enzymes (Miao et al. 2009). Second, AgNPs within the diffusion layer of microalgae or directly attached to the cell surface may dissolve, providing additional silver ions directly to the surface of algae (Dash et al. 2012, Zouzelka et al. 2016). Third, as cell pores of microalgae are in the range of 5 to 20 nm (Bisalputra and Weier 1963), AgNPs smaller than 20 nm could penetrate through cellular membrane pores (Zouzelka et al. 2016). However, larger AgNPs and aggregates can destabilize the cell membrane to form new pores increasing the cellular permeability (Zouzelka et al. 2016). This would facilitate AgNPs entry inside the cytoplasm, where they can exert their toxic effects. Moreover, entry of AgNPs inside algal cells through ion channels or endocytosis has been suggested by Zhang et al. (2016b). All these mechanisms are non-mutually exclusive and they can take place simultaneously providing synergistic effects (Burchardt et al. 2012).

2.5.2.1.2. Toxic effects of AgNPs on microalgae

Many studies have highlighted direct interactions (sorption) of AgNPs with outer shell of microalgal cells (Huang et al. 2016a, Oukarroum et al. 2012a). Formation of aggregates on cell membranes may inhibit cell growth (Navarro et al. 2008), and alters the cellular acquisition of essential nutrients by clogging to the walls (Bundschuh et al. 2016). Moreover, such sorption of AgNPs onto the cell surface may reduce the photosynthesis due to shading effects of AgNPs (Huang et al. 2016a). Further, Moreno-Garrido et al. (2015) suggested that adsorption of nanoparticles on and in microalgae could increase their cellular weight and remove the microalgae from the photic zone, thus affecting the photosynthesis.

After penetrating inside the cell cytoplasm, AgNPs or released silver ions may induce various toxic effects. They can interact with thiol and phosphate containing proteins and enzymes, thus altering their activity and function (Mitrano et al. 2015, Zouzelka et al. 2016), disturb management of cellular phosphate, collapse of the proton pump, denaturation of ribosomes (Moreno-Garrido et al. 2015), inhibit transcriptomic processes of the mRNA, penetrate the nucleus through the nuclear pores to induce DNA damage, and inhibit its repair process (Dash et al. 2012, Zhang et al. 2016b). Furthermore, the increase of ROS production by microalgae is one of the most important response to AgNPs and/or Ag^{+} ions exposure and has been described in numerous studies (He et al. 2012, Huang et al. 2016a). Qian et al. (2016) observed that *C. vulgaris* is able to efficiently detoxify

AgNPs-induced ROS via the induction of antioxidant enzymes thus reducing the toxicity of AgNPs. Nevertheless, other studies observed that high levels of intracellular ROS may impair cell viability (Carlson et al. 2008a, Oukarroum et al. 2013) inducing lipid peroxidation, increasing cell membrane permeability, DNA and RNA damages, and activating apoptotic pathways (He et al. 2012).

2.5.2.1.3. Effects of AgNPs on mitochondria and chloroplasts

Contrary to bacteria, eukaryotic cells possess specific organelles such as mitochondria and chloroplasts. As both these organelles are considered as ancient bacteria, AgNPs are expected to interact with them and inhibit their functions. NPs can induce mitochondrial damages via a number of processes (Leonardo et al. 2016, Stensberg et al. 2014). Maurer and Meyer (2016) categorised four main modes of mitochondrial toxicity. The first one was the induction of ROS which are associated with impairment of mitochondrial function that could reduce cell viability after exposure to AgNPs (Carlson et al. 2008a). Moreover, mitochondria as well as chloroplast membranes contain polyunsaturated lipids which are especially prone to oxidant attack by ROS (von Moos and Slaveykova 2014). The second mode of action was the loss of mitochondrial inner membrane potential. Depolarization is the most frequently studied mitochondrial outcome as a result of AgNP exposure (Maurer and Meyer 2016). Third, AgNP exposure may reduce the mitochondrial activity. The fourth suggested mode was inhibition of the electron transport chain. Alteration of mitochondria could lead to a loss of membrane potential, inhibition of enzymes involved in oxidative phosphorylation, induction of more ROS, and induction of apoptotic pathways among others (Maurer and Meyer 2016).

Chloroplasts are also affected by AgNPs. Indeed, reduction of chlorophyll a, carotenoids and inhibition of the photosynthesis are commonly observed in microalgae exposed to AgNPs (Zhang et al. 2016c, Zou et al. 2016). Besides the shading effect of AgNPs, other mechanisms have been described to explain the reduction of the photosynthesis. Reduction of chlorophyll-a content and direct alteration of the chloroplast by inhibition of the photosystem II (PSII) electron transport and induction of PSI photosynthetic membrane damage by AgNPs or silver ions inside the cell have been shown to play a major role in photosynthesis inhibition (Huang et al. 2016a, Navarro et al. 2008).

2.5.2.1.4. Silver detoxification process of microalgae

While some authors suggested that AgNPs toxicity is only due to the release of silver ion (Moreno-Garrido et al. 2015), other postulated that the toxicity is due to combination of both AgNPs and silver ions (Bundschuh et al. 2016). Interestingly, it has been demonstrated that microalgae are able to reduce toxic metals and produce ENPs based on the dissolved ions (Moreno-Garrido et al. 2015). Leonardo et al. (2016), suggested that detoxification of silver in green alga *Coccomyxa actinabiotis* may be due to interaction with cytosolic S bearing compounds that reduce the toxicity of Ag and transport it to the chloroplast where they are reorganized into small, stable nanoparticles that apparently do not interact with other cellular components.

In the aquatic environment, presence of NOM is known to reduce AgNPs toxicity (Huang et al. 2016b). The sorption of NOM on algal cells and AgNPs surface, could promoted the repulsive charges due to increased negative surface, by NOM a negatively polyelectrolyte in natural water (Philippe and Schaumann 2014, Thurman 1985), preventing their direct interaction or uptake of AgNPs (Li et al. 2016a). The excretion of exopolysaccharides and NOM by phytoplanktonic biofilms, could potentially reduce AgNPs toxicity by complexation of silver ions (González et al. 2016, Li et al. 2016a) or by coating and stabilization of ENPs (Liu and Hurt 2010a, Gao et al. 2009). Li et al. (2016a) found that amide, carboxyl, and phosphorus functional groups of extracellular polymeric substances were involved in the binding and removing of AgNPs and/or dissolved silver. Such processes have been found to be accelerated under light conditions and may affect speciation and transformation of silver in natural waters (Zhang et al. 2016d). This was also suggested by Schiavo et al. (2017) as they proposed that the release of exopolymeric substances could also play a role in higher AgNPs tolerance. The reduction of silver, and the formation of AgNPs ions outside and/or inside cells can explain why microalgae are less sensitive to AgNPs than other organisms such as bacteria or zooplanktons.

2.5.2.2. Various sensitivity to AgNPs depending on the species

Not all microalga species elicit the same sensitivity to AgNPs, thus their effects may vary from one species to another (Kwak et al. 2016, Das et al. 2014). Kwak et al. (2016) have observed that

the 72h EC_{50} of the two phytoplanktonic algae, *Chlamydomonas reinhardtii* and *Chlorococcum infusionum* were 13.53 and 0.68 $mg{\cdot}L^{-1}$, respectively. Angel et al. (2013), showed that toxicity of cit-AgNPs was much higher against freshwater alga *Pseudokirchneriella subcapitata* (72h EC_{50} 3.0 $\mu g{\cdot}L^{-1}$) than against marine diatom *Phaeodactylum tricornutum* (72h EC_{50} 2380 $\mu g{\cdot}L^{-1}$). Planktonic cyanobacteria such as *Microcystis aeruginosa* appeared much more sensitive to AgNPs than eukaryotic phytoplankton like *Chlorella vulgaris* (Park et al. 2010, Chen et al. 2016). *Dunaliella tertiolecta* has been shown to produce much more ROS than *Chlorella vulgaris* when exposed to AgNPs (Oukarroum et al. 2012b), which could explain difference in sensitivity. The composition of the cell membranes and cell walls could at least partially explain the difference in sensitivity to AgNPs (Piccapietra et al. 2012, Zouzelka et al. 2016) as microalgae cell walls are thick and rigid, which may induce a better resistance to the entry of AgNPs inside the cells. In this regard, Piccapietra et al. (2012) suggested that algal cell walls and membranes could affect the bioavailability of AgNPs by limiting their uptake. In accordance to these results, Schiavo et al. (2017) found that *P. tricornutum* are less sensitive to AgNPs than *T. suecica*. They attributed this difference in sensitivity, at least in part to the presence of a resistant silicified cell wall in the diatom species.

In the above section, we described main toxic effects, modes of action and detoxification process of AgNPs in microalgae. We also pointed out that AgNPs can sorb onto cell surface as well as penetrate inside the cell cytoplasm. As microalgae play a major role in the food chain it is likely that AgNPs-contaminated microalgae may contaminate other organisms due to trophic transfer.

2.5.3. Effects of AgNPs on zooplankton

Zooplankton are heterotrophic planktonic organisms playing an important role in the marine and freshwater food web as they feed on microalgae and are a resource for consumers on higher trophic levels. Due to their role in the food web, interactions of zooplankton with environmental contaminants are highly important. In this regard, a great interest has been given on the effects of AgNPs on zooplankton, small crustaceans found in the sea and freshwater. Different studies reported on the impacts of AgNPs on zooplankton and observed that the main toxicity endpoints concerning AgNPs on these organisms are mostly increased mortality (Arulvasu et al. 2014, Chae

and An 2016), reduced reproduction (Arulvasu et al. 2014, Sakka et al. 2016), and swimming alteration (Chae and An 2016, Cupi et al. 2015).

Based on the high sensitivity of *Daphnia magna* towards AgNPs, Asghari et al. (2012) suggested, according to the Globally Harmonized System of classification and labelling of chemicals (UNECE 2013), that AgNPs should be classified as "category acute 1" to aquatic organisms such as *D. magna* as their 48h EC_{50} is less than 1 $mg{\cdot}L^{-1}$.

2.5.3.1. Physical interactions of AgNPs to zooplankton

AgNPs have been found to accumulate on the carapace and filtering appendages (Asghari et al. 2012, Shanthi et al. 2016), and also inside the gut of crustaceans (Asghari et al. 2012, Vijayakumar et al. 2016). Authors observed that attachment of ENPs to the carapace of zooplankton may cause a reduction or even an inhibition of molting. Exposure of *D. magna* to AgNPs induced a production of bubbles under the carapace and pigmentation of the brood chamber which was not observed for soluble $AgNO_3$ exposure (Asghari et al. 2012, Shanthi et al. 2016). The presence of AgNPs on filtering appendages and inside the gut indicated that one of the major interactions of AgNPs with zooplankton is taught to be through ingestion. Indeed, *D. magna* is known to uptake AgNPs mainly via filtering appendages, while transport of silver from the surrounding media to its body includes diffusion at the cell membrane, endocytosis at the cell surface and adhesion to the gut internal wall (Bhatt and Tripathi 2011). It has been suggested that AgNP uptake in *D. magna* is mostly through ingestion (Kalman et al. 2015). Recently, Sakka et al. (2016) demonstrated that AgNPs size may impact their uptake through filtration as only particles in the range of 300-500 nm are actively filtered by the *Daphnia*, thus may have higher ingestion than smaller ENPs (Kwon et al. 2014). On contrast, Ribeiro et al. (2014) hypothesized that attachment of AgNPs on filtrating appendages may decrease the filtering activity and feeding rate.

As previously described, AgNPs exposure can induce a reduction of swimming ability, which was attributed to three non-exclusive major mechanisms. The first one is the binding of AgNPs to the carapace and filtering appendages of zooplankton (Asghari et al. 2012). The second one is massive ingestion of AgNPs that may alter their floating ability, thus leading to its sinking. The third hypothesis is that, as disruption of energy production has been observed (Cupi et al. 2015,

Stensberg et al. 2014) reduced energy content may induce reduced swimming ability (Cupi et al. 2015).

2.5.3.2. Toxic effects of AgNPs to zooplankton

Investigating AgNP toxicity, Stensberg et al. (2014) observed alteration of the proton efflux of mitochondria and mitochondrial membrane damages induced by AgNPs, while Cupi et al. (2015) also observed alteration of Na^+, K^+ ATPase activity. Studies on the effects of AgNPs on zooplankton demonstrated heart rate reduction, disturbance in energy metabolism as well as oxidative stress (Li et al. 2015, Vijayakumar et al. 2016). Furthermore, elevated lactate levels were observed in all AgNP treatments, while it was not the case for ionic Ag treated groups, thus providing evidence that AgNPs may enhance anaerobic metabolism, probably caused by increased movement and perturbed breathing. Reduction of lysine, arginine and glucose levels were also observed in both AgNP and $AgNO_3$ treatments. Such a reduction of amino acids was also observed for Cu^{2+} toxicity and was suggested to be a response to the induction of defense and repair mechanisms, such as the synthesis of stress proteins and DNA repair enzymes (Smolders et al. 2005). Other alterations due to AgNP exposure have been demonstrated like; altered morphology of the eyes (Becaro et al. 2015), formation of curved shell spine, developmental arrest in neonates, rupture of the carapace (Shanthi et al. 2016), translocation across the intestinal epithelium (Mattsson et al. 2016), destruction of digestive organs (Chae and An 2016), production of ROS, enzymatic activity (Ulm et al. 2015), DNA damages, induction of apoptosis (Arulvasu et al. 2014), and induction of gene expression. Such AgNP effects have already been described in the previous sections 3.1 and 3.2, and are mostly applicable to all kind of eukaryotic cells. Moreover, AgNPs also inhibited multixenobiotic resistance (MXR) transporter activity in *D. magna* (Georgantzopoulou et al. 2016). This inhibition reduced the excretion of toxicants out of *Daphnia* cells, thus AgNPs could also affect the survival of this crustacean by increasing the effects of other pollutants and acting synergistically with the contaminants. Nevertheless, zooplankton were shown to be able to, at least partially, eliminate bioaccumulated silver. As already observed for other species, elimination of silver was biphasic with a first fast elimination through feces, and a second slower diffuse excretion not clearly identified (Kalman et al. 2015, Khan et al. 2015).

Further, Ribeiro et al. (2017) postulated that bigger NP have a much faster clearance than smaller NPs.

Although acute and chronic experiments on the exposure of AgNPs to zooplankton have been reported, only few research teams worked on impacts of AgNPs on successive generations of zooplanktonic organisms. Völker et al. (2013) found that AgNPs could negatively affect different *Daphnia* sp. survival and reproduction, as well as their intrinsic rates of population increase on multiple generations. They showed some evidences of bioaccumulation of AgNPs along the different generations and observed potential induction of tolerance to AgNPs through generations potentially due to induction of metallothionein-like proteins.

As seen for other aquatic species in this chapter, it is still under debate whether AgNPs induced toxicity to zooplanktonic species is resulting directly from AgNPs or is driven by the released silver ions. While some authors suggested that the toxicity was due to the silver ions, Poynton et al. (2012) analysed the gene expression profiles of *Daphnia* exposed to silver ions or to AgNPs and highlighted that the major biological processes disrupted by AgNPs included protein metabolism and signal transduction, while $AgNO_3$ mainly caused a downregulation of developmental processes, particularly in the sensory development. So, it could be an addition of effects from both chemical forms.

2.5.3.3. Influence of environmental conditions on AgNPs toxicity to zooplankton

Examining the effects of environmental conditions, Macken et al. (2012) showed that salinity may increase the toxicity of certain types of AgNPs and suggested that the increase in chloride ions or other inorganic ligands within the media may change the behavior and bioavailability of the AgNPs to planktonic crustaceans. As described in section 3.2.2, the presence of natural organic matter could reduce the toxicity of AgNPs to zooplankton (Cupi et al. 2016). Blinova et al. (2013) supported this hypothesis by demonstrating that the toxicity of AgNPs was decreased in natural water compared to artificial water. They attributed this difference to interactions of AgNPs with organic/inorganic matter present in natural waters that may modify silver speciation and bioavailability/toxicity, but did not clearly identified chemical reactions that could be involved. This finding was confirmed by Cupi et al. (2015) who observed a reduction of AgNP toxicity against *D. magna* in presence of NOM. Indeed, in their experiment, *D. magna* had alteration of

swimming ability when exposed to AgNPs only, however when exposed to AgNPs and natural organic matter, no swimming alteration was observed. AgNPs have also been found to modify the toxicity of other toxicants. Bioaccumulation and toxicity of cadmium on *D. magna* increased in the presence of AgNPs, while Cu bioaccumulation decreased in presence of AgNPs (Kim et al. 2016). In this regard, Lopes et al. (2016)found that binary mixture of AgNPs and ZnONPs showed a synergistic toxicity pattern on the immobilization and feeding inhibition of *D. magna*, thus they suggested that AgNPs together with other chemicals could induce synergistic effects although mechanisms have not been suggested. Moreover, it has been highlighted that most laboratory studies were using continuous exposure to AgNPs, which is not likely to be environmental conditions. Sørensen et al. (2016) performed a 3h pulse exposure of 10-50 $\mu g \cdot L^{-1}$ AgNPs to *D. magna* and observed that after 21 days, no significant mortality, body length nor any effect on molting were observed.

2.5.4. Behavior and impact of AgNPs on eukaryotic macroorganisms

2.5.4.1. Acute toxic and sub-lethal effects of AgNPs on young and mature marine invertebrates

Marine invertebrates have been largely used as biological models to unravel the main sublethal effects caused by AgNPs on marine fauna (Table 5). Among all published studies, 57% had the adult stage exposed to nude or coated silver nanoparticles. Gametes and/or early stages of development (embryos, larvae and juveniles) of oysters, sea urchins, polychaetes, copepods, coral, branchiopods, jellyfish, barnacles, gastropods and bivalves accounted for 46% of the studied models. Interestingly, 65% of all the reported experiments have been using AgNPs with a large range of sizes from 20 to 100 nm. Moreover, two third of the studies described toxic effects of organic coated AgNPs. As shown in section 2 and 3.2, it is well known that the size of AgNPs is important to their cytotoxicity, but this point is not fully recognized by authors. For instance, smaller AgNPs (< 20 nm) induced more oxidative stress, DNA damage and lethality in mammalian models than larger ones when compared at higher doses (Kim and Ryu 2013). Although small nanoparticles have the potential to be strongly harmful to cells, little is known about their toxicity mechanisms with invertebrates in both adult and early stages.

Table 5: Summary of studies on the effects of AgNPs on marine invertebrates

Biological models	AgNPs size in ultrapure water	Physiological effects observed	References
Oyster (*Crassostrea virginica*)	15 ± 6 nm	Embryonic development impaired at low concentrations; high metallothionein mRNA levels in both embryos and adult oysters; lysosomal integrity affected in hepatopancreas tissues	Ringwood et al. (2010)
Polychaeta (*Nereis diversicolor*)	30 ± 5 nm	Internalization of AgNPs by gut epithelium; association of nanoAg with inorganic granules, organelles and heat denatured proteins	García-Alonso et al. (2011)
Polychaeta (*Nereis diversicolor*)	<100 nm	Highest genotoxicity effects compared to dissolved silver and micron-silver (2-3.5 µm)	Cong et al. (2011)
Mussel (*Mytilus edulis*)	20-35 nm	Accumulation of >60% of Ag in soft tissues in nanoAg and Ag^{+} treatments with maximum concentrations located in the digestive organ; low bulk activity of Ag in the extrapallial fluid	Zuykov et al. (2011)
Estuarine snail (*Peringia ulvae*)	16.5 ± 4.5 nm	Fast elimination of Ag from AgNPs in the first days of depuration compared to dissolved silver	Khan et al. (2012)
Oyster (*Crassostrea virginica*)	26 ± 1.2 nm	Reduction of phagocytosis by 1.64 fold in exposed hemolymph	Abbott Chalew et al. (2012)
Copepod (*Tisbe battagliai*)	56.9 ± 7.8 nm	Mortality increased in a concentration-dependent manner for AgNPs	Macken et al. (2012)
Sea urchin (*Paracentrotus lividus*)	5-35 nm	Agglomerated AgNPs found in larvae tissue; Ag oxidized species found with S and O/N ligands in larvae; loss of calcite	Piticharoenphun et al. (2012)
Brine Shrimp (*Artemia salina*), sea urchin (*Paracentrotus lividus*), barnacle (*Amphibalanus amphitrite*)	1-10 nm	Mortality (0.1 $g{\cdot}L^{-1}$), abnormal development (0.001 $g{\cdot}L^{-1}$), skeletal abnormality (0.001 $g{\cdot}L^{-1}$) for sea urchins; high mortality of barnacle nauplii for all tested solutions; larvae of brine shrimp less sensitive to nanoAg	Falugi et al. (2012)
Mussel (*Mytilus galloprovincialis*)	<100 nm	Alteration of proteins associated to stress response, cytoskeleton and cell structure (actin and α-tubulin)	Gomes et al. (2013b)
Mussel (*Mytilus galloprovincialis*)	<100 nm	DNA damage in hemolymph cells	Gomes et al. (2013a)
Oyster (*Crassostrea virginica*)	20-30 nm	Total protein levels increased in hepatopancreas tissues pointing to tissue damaging and metabolic impairment	McCarthy et al. (2013)
Sea urchin (*Paracentrotus lividus*)	1-10 nm	Developmental anomalies, larval enzymatic activity (acetylcholinesterase and propionylcholinesterase) affected; potential neurotoxic damage	Gambardella et al. (2013)
Sea urchin (*Paracentrotus lividus*)	5-35 nm	Development arrest before gastrula stage; strong morphological changes on skeletal parts	Šiller et al. (2013)

Scallop (*Chlamys islandica*)	10-20 nm; 70-80 nm	Strong accumulation (3800 $mL.g^{-1}$) of AgNPs in short time followed by efficient depuration process	Al-Sid-Cheikh et al. (2013)
Polychaeta (*Laeonereis acuta*)	33.5 nm and 205.6 nm	Symbiotic bacteria reduced; posterior region of the worms with lower antioxidant capacity	Marques et al. (2013)
Bivalve (*Scrobicularia plana*)	40-45 nm	Response of catalase, glutathione S-transferase, superoxide dismutase; feeding behavior impaired	Buffet et al. (2013)
Gastropod (*Littorina littorea*)	45 ± 33 nm	Presence of silver in the head, gills and foot; little measurable Ag accumulation in the kidney or stomach when introduced by Ag nano-form	Li et al. (2013)
Polychaeta *(Platynereis dumerilii*)	16.5 nm (cit-AgNPs), 13.1 nm (HA-AgNPs)	Mortality, abnormal development and high uptake rate particularly for HA-AgNPs	García-Alonso et al. (2014)
Coral (*Acropora japonica*)	57.2 ± 3.6 nm	Larval deformation and metamorphosis failure under 50 $\mu g \cdot L^{-1}$ exposures; high mortality of gametes, larvae and polyps with 500 $\mu g \cdot L^{-1}$,growth impairment of polyps	Suwa et al. (2014)
Mussel (*Mytilus galloprovincialis*)	41.68 ± 9.62 nm	Oxidative stress; antioxidant enzymes activation; lipid peroxidation higher in gills; metallothionein seem to be the main detoxification mechanism of nanoAg in the gills	Gomes et al. (2014)
Amphipod (*Ampelisca abdita*), mysid (*Americamysis bahia*) and polychaete (*Nereis virens*)	30 nm	No toxicity for amphipods and mysids at concentrations up to 75 $mg \cdot Kg^{-1}$ dw; worms exposed to cit-AgNPs had a similar distribution among Ag metal, AgCl and Ag_2S; whereas those exposed to PVP-AgNPs contained mostly Ag metal and AgCl	Wang et al. (2014)
Polychaete (*Nereis (Hediste) diversicolor*)	20-80 nm	Lysosomal membrane permeability and DNA damage of *Nereis* coelomocytes increased in a concentration-dependent manner, AgNPs were more toxic than Ag^+	Cong et al. (2014)
Polychaeta (*Hediste diversicolor*) and bivalve (*Scrobicularia plana*)	40-45 nm	DNA damages in the digestive gland of *S. plana*, and higher presence of phenoloxidase and lysozyme activities in both species.	Buffet et al. (2014)
Brine Shrimp (*Artemia salina*)	30–40 nm	With increasing concentrations of nanoAg, the mortality rate, aggregation in gut region, apoptosis and DNA damage increased in nauplii; *Artemia* cysts hatching decreased	Arulvasu et al. (2014)
Sea urchins (*Arbacia lixula, Paracentrotus lividus* and *Sphaerechinus granularis*)	59.67 ± 3.06 nm	Larval deformities and developmental arrest	Burić et al. (2015)
Polychaeta (*Nereis diversicolor*)	63 ± 27 nm; 202 ± 56 mm (bulk)	Significant increases in total glutathione concentrations, superoxide dismutase depletion; inhibition of catalase	Cozzari et al. (2015)
Barnacle (*Balanus amphitrite*), gasteropod (*Crepidula onyx*) and	30–50 nm	Retardation in growth and development; reduction of larval settlement rate; internalization of nanoAg by vacuoles of epithelial cell in the digestive tract of *C. onyx*	Chan and Chiu (2015)

polychaete (*Hydroides elegans*)			
Crustaceans (*Amphibalanus amphitrite* and *Artemia salina*), jellyfish (*Aurelia aurita*) and sea urchin (*Paracentrotus lividus*)	990 nm	Mortality, cnidaria immobility and frequency of pulsations alteration, crustacean swimming speed alteration and sea urchin sperm motility change	Gambardella et al. (2015a)
Sea urchin (*Paracentrotus lividus*)	1-10 nm	Altered fibronectin suggesting a role in alteration of skeletogenic cells migration; different degree of anomalies related to Golgi apparatus modification; AgNPs interfered with biomineralization	Gambardella et al. (2015b)
Mussel (*Mytilus galloprovincialis*)	<10 nm; 20 nm, 80 nm and 2 µm (bulk)	Higher sensitivity of gill cells compared to hemocytes, ROS production, catalase activity, DNA damage in both cells; activation of lysosomal AcP activity, disruption of actin cytoskeleton, stimulation of phagocytosis in hemocytes; increase of MXR transport activity and inhibition of Na-K-ATPase in gill cells	Katsumiti et al. (2015)
Mussel (*Mytilus galloprovincialis*)	42 ± 10 nm	Up-regulation of cytochrome CYP4y1 and Cathepsin; down-regulation of catalase, glutathione transferase, caspase, HSP70 and elongation factor	Bebianno et al. (2015)
Gastropod (*Potamopyrgus antipodarum*) and polychaeta (*Capitella teleta, Capitella* sp. S)	10-15 nm	Low Ag accumulation; no apparent toxicity to snails; survival and growth affected (worms)	Ramskov et al. (2015)
Sea urchin (*Strongylocentrotus droebachiensis*)	15 ± 5 nm	Developmental deformities in different embryonic stages, vacuolization of blastocoelar cells, lack of skeleton, abnormal clusters of primary mesenchyma cells	Magesky and Pelletier (2015)
Sea urchin (*Strongylocentrotus droebachiensis*)	15 ± 5 nm	Failure of metamorphosis completion, high larval mortality during recovery period; lethargy, oedema and immobility in juveniles; cellular immune reaction in both larvae and juveniles	Magesky et al. (2016)
Bivalve (*Scrobicularia plana*)	2-10 nm (± 5 nm in average)	Burrowing impairments; significant bioaccumulation of Ag in cytosolic fraction of the soft tissues of the digestive gland; reduced anti-oxidative capacities and impairment of metabolic activity	Bertrand et al. (2016)
Sea urchin (*Strongylocentrotus droebachiensis*)	14 ± 6 nm	Increasing levels of HSP60 and HSP70 expression, coelomic contamination and cell immune response	Magesky et al. (2017)

Another key stone to understand AgNP effects on marine organisms is how their physico-chemical behavior (see section 2) may influence toxicity during short or long term experiments. Even though AgNP behavior in seawater has been described (Levard et al. 2012), there is much more to be understood concerning their agglomeration and dissolution inside the multicellular organisms. As an example, poly(allylamine)-coated AgNPs (PAAm-AgNPs, ~15 nm) tend to form small aggregates in seawater due to high ionic strength, but the presence of amine groups along the polymer chains prevent the formation of large aggregates that could tend to precipitate (Magesky et al. 2016). AgNPs might be able to go through coelomic circulation as small aggregates and/or single particles or even as larger aggregates that may dissociate inside the coelomic fluid (Magesky et al. 2016).

Behavioral responses such as altered swimming, lethargy, immobility and burrowing impairments have been reported as the first signs of nanosilver intoxication in zooplankton (see section 3.3), crustaceans, sea urchins, jellyfish and polychaetes (Bertrand et al. 2016, Gambardella et al. 2015a, Magesky et al. 2016). Considered as quick assessment endpoints, lethargy and disrupted swimming can also indicate different early stages of sea urchin development being more or less affected by AgNPs and dissolved silver contamination (Magesky and Pelletier 2015). However, main physiological effects observed in embryos and larvae exposed to AgNPs greatly reflect the impairment of fate and activity of mesenchyme cells at different steps during the development. Activated in primary mesenchyme cells (PMCs) entering the blastocoel from sea urchin blastula, acetylcholinesterase (AchE) regulates the balance between cell proliferation and cell death; cell adhesion and migration (Drews 1975). Additionally, acetylcholinesterase (AchE) and propionylcholinesterase (PrChE) enzymatic activities can be differently affected by very small AgNPs (1-10 nm) in developing embryos of *Paracentrotus lividus* (Gambardella et al. 2013). While AchE activity was impaired by AgNPs at several concentrations (1.0^{-4} to 1.0 $mg \cdot L^{-1}$), PrChE levels were generally enhanced by them at similar concentrations. AchE activity regulates neurotransmission and some other crucial biological functions in larvae whereas PrChE plays a role in the nervous and neuromuscular systems (Falugi and Aluigi 2012, Talesa et al. 1993). Because both enzymes have very specific roles during sea urchin development, the onset of developmental anomalies corresponds to the change in AchE and PrChE expression. Taking into account potential neurotoxic damages caused by small AgNPs (1-10 nm) in embryos (Gambardella et al. 2013), morphological studies focusing in neurogenesis in embryonic stages, prisma and early

pluteus larvae might reveal how AgNPs could interfere with neurotransmitters expression, neurons organization and swimming behavior.

Crucial events in early life of sea urchins such as skeleton mineralization, secondary mesenchyma cells differentiation, primary mesenchyma cells and red spherulocytes migrations could be disrupted by AgNPs in different species (Burić et al. 2015, Falugi et al. 2012, Gambardella et al. 2015a, Magesky and Pelletier 2015). Sublethal effects caused by cit-AgNPs (5-35 nm) on developing embryos of *Paracentrotus lividus* are stronger and cannot be correlated only with ions release (0.030 $mg \cdot L^{-1}$ between 30-40h) at 18-19°C while in *Strongylocentrotus droebachiensis* the nanotoxicity effects arose from early blastula to midgastrula stage probably as a consequence of fast release of free Ag ions (~37.5% between 48-96h) at 5°C (Magesky and Pelletier 2015, Šiller et al. 2013). Comparatively, apoptosis and mortality of branchiopod *Artemiasalina* nauplii emerged after 24h/48h short exposures to cit-AgNPs (30-40 nm) in a concentration-dependent manner (Arulvasu et al. 2014). Polychaete *Platynereis dumerilii* exposed to AgNPs endured malformations and mortality during early development (García-Alonso et al. 2014, Suwa et al. 2014). Humic acid-capped AgNPs (HA-AgNPs, 13.1 nm) were more toxic in terms of mortality and abnormality rate than cit-AgNPs (16.5 nm), and dissolved silver for all life stages of *P. dumerilii*, except for fertilized eggs. HA-AgNPs also showed the highest uptake rate (about 20 $\mu g \cdot g^{-1}$) in adult worms compared to cit-AgNPs, and dissolved silver (5 $\mu g \cdot g^{-1}$) (García-Alonso et al. 2014). Negative impacts on fertilization, larval survival, larval metamorphosis and primary polyp growth of the lecithotrophic species *Acropora japonica* emerged after exposure to silver nanocolloids (57.2 ± 3.6 nm) at concentration higher than 50 $\mu g \cdot L^{-1}$ (Suwa et al. 2014). According to the authors, some degree of AgNP ionization in addition to concentration and uptake of particles were probably the main factors leading to nanotoxicity. More information about mechanisms of toxicity of AgNPs in lecithotrophic species as well as their defensive mechanisms against silver is still needed.

There is a growing body of evidence indicating that metamorphosis of both planktotrophic and lecithotrophic species can be particularly affected by nano-contamination. With total silver concentration relatively high over a 24h exposure (nominal concentration of 50 $\mu g\ L^{-1}$), only 14% of larvae of *A. japonica* went through metamorphic process (Suwa et al. 2014). Because coral species appear to be sensitive to metal contamination (Reichelt-Brushett and Harrison 2005); low

concentrations of trace metals such as silver might trigger strong toxicity effects. In addition, polyvinylpyrrolidone-coated AgNPs (PVP-AgNPs), oleic acid-coated AgNPs (OAg-NPs) (30-50 nm) and dissolved silver reduced the larval growth rate of gastropod *Crepidula onyx* as well as settlement rate in barnacle *Balanus amphitrite* and *C. onyx* by a similar extent and a concentration-dependent manner (Chan and Chiu 2015). Both organic-coated AgNPs were even more disruptive to growth and larval settlement rate of polychaete *Hydroides elegans* when compared to dissolved silver, which is pointing toward the effects of the nanoparticle itself. As another example, fed metamorphic echinoplutei of *S. droebachiensis* chronically exposed to PAAm-AgNPs (15 ± 5 nm, 100 $\mu g \cdot L^{-1}$) did not undergo metamorphosis and showed strong mortality rate during recovery period (Magesky et al. 2016). Conversely, Ag^{+}-treated larvae experienced high mortality and a slow and constant morphogenic process throughout juvenile stage during exposure period. With large nano-aggregates (130-400 nm) flowing through larval blastocoel, some internally distributed AgNPs could not only harm tissular integrity, but also act as a source of Ag ions. Recognized as foreign bodies inside the coelom, AgNPs triggered cellular immune response evolving phagocytic amoebocyte, spherulocytes and petaloid phagocytes (Magesky et al. 2016). Even though AgNPs internalized by sea urchin larvae might be oxidized and complexed with S and O/N ligands (Piticharoenphun et al. 2012), the metabolic cost allowed to nano-detoxification during metamorphosis is likely to be very high and difficult to overcome over time.

Molluscs and polychaetes mostly in adult stage have been extensively used as biological models to study AgNP effects, exploring silver biodistribution and bioaccumulation, and defensive mechanisms against silver (Abbott Chalew et al. 2012, Al-Sid-Cheikh et al. 2013, Bebianno et al. 2015, Buffet et al. 2013, Buffet et al. 2014, Cozzari et al. 2015, Gomes et al. 2013a, Gomes et al. 2013b, Gomes et al. 2014, Katsumiti et al. 2015, Khan et al. 2012, Li et al. 2013, Marques et al. 2013, Ramskov et al. 2015). After PAAm-AgNPs (20-35 nm) exposure, bivalve *Mytilus edulis* can accumulate more than 60% of Ag (both nano and ionic forms) in their soft tissues with maximum concentrations located in the digestive organ and between 0.07 and 7% of total Ag in the extrapallial fluid (Zuykov et al. 2011). In *Mytilus galloprovincialis* exposed to AgNPs (42 ± 10 nm), metallothionein expression as well as the anti-oxidative enzymatic system represented by SOD, CAT and glutathione peroxidase were activated against lipid peroxidation along a 15-day exposure (Gomes et al. 2014). Interestingly, the antioxidant enzymatic activity remained either unchanged or decreased with time exposure in the digestive organ, suggesting

differential and less pronounced responses by these defensive mechanisms compared to gills. Comparatively, the digestive organ of oyster (*Crassostrea virginica*) contaminated by cit-AgNPs (15 ± 6 nm and 20-30 nm) showed lysosomal impairment, tissue damaging and metabolic disruption, but also high metallothionein mRNA levels (McCarthy et al. 2013, Ringwood et al. 2010).

Increasing salinity associated with PVP-AgNPs exposure, Macken et al. (2012) observed high mortality rate in young copepods *Tisbe battagliai*. Notwithstanding, working with polyoxyethylene glycerol trioleate stabilized-AgNPs (PGT-AgNPs) and tween 20 stabilized-AgNPs (2-10 ± 5 nm), Bertrand et al. (2016) showed that Ag^+ might be a predominant source of toxicity at different salinities (15 and 30 psu) in clam *Scrobicularia plana*. More importantly, dissolution rate of AgNPs appeared faster under lower salinity, ~81.7% (15 psu) compared to ~68.1% (30 psu) of Ag^+. In this study, an increased amount of Ag was accumulated as cytosolic fraction (as soluble Ag and AgNPs) of the digestive organ with no measured change of metallothionein levels. In addition, the decreasing salinity condition seemed to enhance oxidative mechanisms, apoptosis and energetic reserve depletion. According to Berthet et al. (1992), silver sulphide loaded amoebocytes can strongly increase in the vesiculous tissue and in the gills of *Crassostreagigas* chronically exposed to dissolved silver in brackish water. Silver might be trapped within insoluble fractions as relatively nontoxic Ag_2S in basement membranes for extrusion.

Similar mechanisms have also been described for polychaetes chronically exposed to different coated AgNPs. As an example, *Nereis diversicolor* exposed to sediment amended with cit-AgNPs (30 ± 5 nm) internalized nanoAg in gut epithelium over 10 days (García-Alonso et al. 2011). Strikingly, Ag accumulated from cit-AgNPs was strongly associated with metal-rich granules, organelles, and heat sensitive proteins while dissolved silver was basically localized to the metallothionein fraction. *N. diversicolor* is tolerant of heavy metals and able to control its body metal concentration using different mechanisms: (1) physiological detoxification pathway with formation of metal-containing extracellular granules, (2) formation of mineralized lysosomes, (3) excretion of metals via coelomocytes, (4) synthesis and turnover of metal-binding proteins (Cong et al. 2011). Yet, one-month exposure to PVP-AgNPs (20-80 nm) caused lysosomal membrane permeability and DNA damage in *N. diversicolor* coelomocytes (Cong et al. 2014). It is interesting

to note that AgNPs surface capping agents might also influence distinct pathways of accumulation, biotransformation and depuration in polychaetes. For instance, *Nereis virens* exposed to cit-AgNPs (30 nm) had a relatively similar distribution among Ag metal, AgCl and Ag_2S whereas those exposed to PVP-AgNPs (30 nm) and dissolved silver mostly accumulated Ag metal and AgCl (Wang et al. 2014). These findings indicated that different distributions of Ag species might be due to chemical transformation of AgNPs, *in vivo* bioreduction or the preferential uptake or excretion of particular Ag chemical forms.

2.5.4.2. Toxicity to fish early developental stages and adults

2.5.4.2.1. Early toxicity assays and observations

The toxic effects of AgNPs on fish species attracted the attention of a large number of aquatic toxicologists, and early works demonstrated that early life stages were, as expected, much more sensitive than later stages and adults when exposed to dissolved and nanoparticulate silver (Asharani et al. 2008, Bilberg et al. 2010, Bilberg et al. 2011, Choi et al. 2010, Cowart et al. 2011, Farkas et al. 2010, Wu et al. 2010, Lee et al. 2007). Most of these studies involved embryos at different stages, larvae and juveniles. Lee et al. (2007) mentioned that individual AgNPs were observed inside embryos at each developmental stage, and toxicity of Ag nanoparticles and types of abnormalities of zebrafish highly depended on the dose of Ag nanoparticles. Rates of passive diffusion and accumulation of nanoparticles in embryos are likely responsible for the dose-dependent abnormalities. Malformation such as edema, spinal and fin fold abnormalities were observed as well as heart malformation and eye defects (Lee et al. 2007). Results suggested that while ingestion was common, gills were the main sites of AgNP uptake. AgNPs were recognized is a source of toxic Ag ions, while nanoparticles themselves contributed partially to toxicity to fish (Kwok et al. 2012). It was suggested by Wu et al. (2010) that most of the abnormalities detected in juvenile and larval medaka (*Oryzias latipes)* were related to homeostasis malfunctions. Additionally, authors found that the most severe abnormalities were associated with yolk edema. As edema are known to be caused by disturbance in osmoregulation (Kiener et al. 2008), their observation was in accordance with the postulate suggesting that many of the deformities observed in fish after exposure to a toxic compound were related to the disrupted circulatory system and osmoregulation (Peters et al. 2007). Severe circulatory abnormalities were associated with the

occurrence of pericardial edema and tubular heart (Wu and Zhou 2012). PVP-AgNPs have been shown to induce sluggish circulation, hemostasis, hemocytes overfilling in blood vessels, hemorrhages in different parts of the larval body and global basophilia, indicating once again that AgNPs can lead to modification of the circulatory system (Griffitt et al. 2012, Wu et al. 2010, Wu and Zhou 2013).

A number of early studies highlighted the fact that high concentrations of AgNPs tend to interact and bind with fish gills inducing major undesired effects (Bilberg et al. 2010, Kwok et al. 2012, Griffitt et al. 2012, Griffitt et al. 2013, Wu and Zhou 2013). Among them, Kwok et al. (2012) demonstrated that PVP- and cit-AgNPs distribution on the gills of medaka embryos were ubiquitous, while smaller amount were present in other organs like liver and brain. Based on their results, they concluded that although ingestion was common, the main uptake site for AgNPs in medaka remained the gills. A significant thickening of the epithelial gill tissues was observed in sheepshead minnows *Cyprinodon vagiegatus* (Griffitt et al. 2012). Wu and Zhou 2013 (Wu and Zhou 2013) showed that in medaka at high concentrations of AgNPs (0.5 mg L^{-1}), lesions to the branchia became severe and desquamation of lamellar epithelium and disruption of cartilaginous rod were prominent. Moreover, Wu and Zhou (2013) suggested that, as gills are surrounded by aqueous media and mucus, AgNPs may be trapped and retained in the mucus thus preventing their uptake. According to this hypothesis, their results showed that exposure of medaka to PVP-AgNPs led to increased hyperplasia and mucus production, which in turn could affect the uptake of AgNPs by the gills. Bilberg et al. (2010) observed impairment of the tolerance to hypoxia in Eurasian perch *Perca fluviatilis* exposed to AgNPs, proposed that AgNPs may, by interacting with the gills, reduce their diffusion conductance, thus leading to hypoxia during low water oxygen tension.

Although AgNPs can be ingested by adult fish and found in their guts; the other organ after the gills accumulating most of silver was the liver (Griffitt et al. 2012, Choi et al. 2010, Wu and Zhou 2013). Liver abnormalities included spongiosis, cyst, disruption of hepatic cord and apoptotic changes, hepatocyte enlargement, loosened liver parenchyma, disorganisation of hepatocytes, focal necrosis, and focal lymphocytic infiltration were reported. Choi et al. (2010) attributed the observed alterations to the presence of silver ions released by AgNPs in liver. However, the real contribution from AgNPs and dissolved silver was not clear and is still subject to controversy as already mentioned for other aquatic organisms. When paying attention to bioaccumulation only,

fish treated with AgNPs had significant higher silver burden in tissue than both control and soluble silver treated fish, which could indicate a specific AgNP toxicity and probable AgNP bioaccumulation (Farkas et al. 2010). An *in vitro* study on hepatocytes of rainbow trout demonstrated that cit-AgNPs caused significant reduction in membrane integrity and metabolic activity in a concentration dependent manner. At high AgNP concentrations, a significant reduction of mitochondrial activity happened (Farkas et al. 2010).

At an intracellular level, Wise et al. (2010) found that cit-AgNPs could induce chromosomal aberration such as chromatid lesion or chromatid exchanges, aneuploidy and damages in metaphase leading to cell cycle arrest. Chromatin condensation, pyknosis and DNA damages, probably due to double strand break, were also observed (Choi et al. 2010). The presence of agglomerated AgNPs in cytoplasmic location such as the vicinity of the plasma membrane and the nuclear membrane was also demonstrated (Choi et al. 2010). Furthermore, the expression of a variety of genes and proteins were modified by exposure to AgNPs (Choi et al. 2010). The expression of metallothionein 2 (MT2), a protein involved in metal detoxification (Hogstrand et al. 1996), has been found in zebrafish after exposure to AgNPs (Choi et al. 2010).

Dose dependant decrease in antioxidant enzymes in liver of medaka were reported as well as depletion in glutathione in the liver, gill and brain, and increased lipid peroxidation in their liver and gills (Wu and Zhou 2012, 2013). Authors suggested that the oxidative stress induced by AgNP could be associated with a large number of histological changes in the fish. The observed decrease in catalase and superoxide dismutase (SOD) activities suggested an excessive consumption of these antioxidant enzymes. The reduction of SOD combined with limited catalysis activity could lead to a failure in the catalysis of oxyradicals, thus indicating that the antioxidant defenses in medaka liver were depressed and that a potential enhancement of ROS was produced. Griffitt et al. (2013) found that the expression of a variety of zebrafish genes was affected by exposure to AgNPs. As reported in section 3.3, many genes were related with DNA repair, cellular restructuring, and developmental processes. Their results tend to indicate a direct effect of AgNPs more than an effect of released silver ions. These observations were consistent with the hypothesis that metallic NPs, including AgNPs, could cause toxicity through enhanced production of ROS, which will in turn induce oxidative DNA damages, resulting in upregulation of genes involved in DNA repairing processes. Finally, it should be mentioned that Farkas et al. (2010) did not observe

a reduction of AgNPs cytotoxicity on the hepatocytes in presence of dissolved organic carbon (DOC). Their results showed that neither size changes nor presence of DOC appeared to influence the AgNP toxicity to a significant degree, suggesting that AgNP might be toxic even under ecologically relevant exposure. Nevertheless, Kim et al. (2013) observed that a reduction in AgNP toxicity was related to the role of coating of HA to the surface of AgNPs and bridging between the NPs. The role of AgNPs coating and DOC is still unclear and subject to recent research efforts.

Possible impacts of AgNP on fish reproductive organs have also been highlighted as it has been shown that they could alter the expression of genes in *Cyprinodon vagiegatus* gonads (Griffitt et al. 2012) as well as modulate the expression of a variety of genes responsible for the steroidogenesis in marine medaka *Oryzias melastigma* primary ovarian cell culture (Degger et al. 2015). Authors evaluated the effects of two differently coated AgNP particles: (Oleic Acid) OA-nAg and PVP-AgNPs on fish ovarian tissues, using $AgNO_3$ as a positive control. They observed that the greatly enhanced silver toxicity using PVP-AgNPs may be due to difference in aggregation and suggested, for the first time, that AgNPs can affect specific genes regulating steroidogenesis, involving AgNP as a potential endocrine disruptor.

2.5.4.2.2. Recent progress in AgNPs toxicity mechanisms in fish

Recently published papers on effects of silver nanoparticles have been selected and summarized in Table 6. The first striking point is the broad use of zebrafish as a model to study AgNP toxicity, first for practical reasons (small size, low cost, diverse adaptability, short breeding cycle, high fecundity, and transparent embryos) and second for its high sensitivity to a large range of toxicants and possible application of transgenic technology (Dai et al. 2014). Otherwise, nine other species have been used (mostly early-life stages) which provide a large diversity in species used and ensure a better understanding of variable effects between species. The second point to be mentioned is the use of more realistic concentrations by a number of authors (Clark et al. 2016, Oprsal et al. 2015, Murray et al. 2016, Bruneau et al. 2016, Hawkins et al. 2015, Garcia-Reyero et al. 2015) in an attempt to derive conclusions that could be applied to field risk assessment.

Table 6: A selection of recently published papers on the effects of AgNPs on some fish species

Species	Exposure and main observed effects	References
Acipenser baerii	Larval Siberian sturgeon exposed to AgNPs for acute and 21d-exposure. 96h LC50 was 1.41 ± 0.24 mg L^{-1}. AgNPs negatively influenced survival, body length and mass and morphology and physiology of the epidermis, gills, and liver of Siberian sturgeon larvae. Pathological changes are irregular structure and pyknotic nuclei of epidermis, aplasia and/or fusion of lamellae, telangiectasis, epithelial necrosis and lifting of the gills, dilation of sinusoidal space, overfilled blood vessels, and pyknotic nuclei of the liver.	Ostaszewska et al. (2016)
Acipenser persicus Acipenser stellatus	Sturgeon fertilized eggs and embryos exposed to AgNPs (16.6 nm) at concentrations from 0.025 to 8 mg L^{-1}.96h LC50 values in both species varied between 0.163 and 0.158 mg L^{-1}. AgNPs induced a concentration-dependent toxicity in both species during early life stages.	Banan et al. (2016)
Carassius auratus	Goldfish exposed to AgNPs (5 nm) from 0.1 to 5 mg L^{-1} over 14-d period. Same pathologies for AgNPs and $AgNO_3$ including hyperplasia and lamellar fusion of the gills, and hemosiderosis and pyknotic nuclei of the liver.	Abarghoei et al. (2016)
Coregonus palaea	Whitefish embryos at late-eyed-stage exposed to low concentrations (0.5 or 100 μg L^{-1}) of differently sized AgNPs. The induced hatching did not vary with nanoparticle size and was stronger in the silver nitrate group. Results suggest that the evolutionary potential for adaptation to these types of pollutants may be low.	Clark et al. (2016)
Cyprinus carpio	Embryos of common carp exposed repeatedly for 6 h to AgNPs (5–50 μm L^{-1}) either under conditions varying short-time exposure, concentration and maximum agglomerate size of 200 or 400 nm. Importance of AgNP agglomerates in nanosilver adverse effects. Agglomerates locally enhanced toxicity particularly with larger 400 nm ones	Oprsal et al. (2015)
Danio rerio	Embryos exposed to AgNPs (4 nm, 10 nm) from 0.5 to 23 mg L^{-1}. AgNPs could affect the neural development of zebrafish embryos, and the toxicity of AgNPs may be partially attributed to the comparatively higher uptake in the head area. Potential neurotoxicity of AgNPs and could be extended to other aquatic organisms.	Xin et al. (2015)
	Adult zebrafish: estimated 96 h LC50 value 16.76 mg L^{-1}. Long-term exposure (14 d) indicate that AgNPs could inhibit the activities of Na+/K+-ATPase and AChE, thus interfering with the proper ionoregulation and neuroregulation.	Katuli et al. (2014)
	Zebrafish exposed to cit-AgNPs (20 and 110 nm) for 4 h, 4 days, or 4 days plus a 7 day depuration period. Both particle types retained in the intestines even after depuration. Striking size-dependent differences in the ultrastructural features and histopathology in the target organs in response to the particulates.	Osborne et al. (2015)
	Embryos exposed to AgNPs (10 and 40 nm) at concentration range from 0.2 to 1 mg L^{-1}. Specification of hematopoietic progenitor cells and differentiation of erythroids detected at different developmental stages. Erythrogenesis found to be inhibited in developmental-stage-specific and cell-specific manners. AgNPs affected erythrogenesis mostly by their particles other than their releasing ions.	Cui et al. (2016)
	Embryos exposed to small (9 nm) and larger (30 nm) AgNPs at concentrations ranging from 0 to 20 mg L^{-1}. Smaller AgNPs induce increased mortality rates and decreased hatchability rates than the larger particles in a dose-dependent manner. Striking discrepancies in the phenotypic defects that were induced by AgNPs and $AgNO_3$.	Mosselhy et al. (2016)
Oncorhynchus mykiss	Fish exposed to environmentally-relevant (average 0.28 μg L^{-1}) and higher (average 47.60 μg L^{-1}) AgNP concentrations. Significant increase in blood plasma cortisol for both treatments and Ag detected in fish muscle tissue. Low AgNP concentrations did not affect growth or condition under the experimental conditions investigated.	Murray et al. (2016)

	Juvenile rainbow trout exposed to commercial AgNPs (22 nm) at 40 µg L^{-1} in diluted municipal treated wastewater.Induced morphological modifications without visible nanoparticle bioaccumulation. AgNPs in wastewater were bioavailable to fish despite of their formation of aggregates.	Bruneau et al. (2016)
Oryzias latipes	Medaka larvae exposed to a total of ten AgNPs of different coating and diameters. Aggregation behavior and toxicity of various AgNps were dependent on coating materials. 96h LC50 values correlated with AgNP aggregate sizes. Gene expression patterns suggested toxicity related to disruption of sodium regulation and not to oxidative stress.	Kwok et al. (2016)
Pimephales romelas	Fathead minnows exposed to $AgNO_3$ (1.3 or 3.7 µg L^{-1} as Ag^{+}), cit-AgNPs (15 or 39 µg L^{-1}), and PVP-AgNPs (11 or 50 µg L^{-1}). Results indicated that both $AgNO_3$ and AgNP created similar disruptions in gill structure and ionic regulation, possibly due to the ionic Ag portion of each treatment.	Hawkins et al. (2015)
	Fathead minnows 96h-exposure to 20 nm PVP-AgNPs (50.3 µg L^{-1}) or cit-AgNPs (56.0 µg L^{-1}) or $AgNO_3$ (3.81 µg/L). All silver treatments affected mucus production in fish gills and also lead to common and unique transcriptional changes in expressed genes.	Garcia-Reyero et al. (2015)

Molecular mechanisms for AgNPs toxicity were reviewed by McShan et al. (2014) and more specifically examined in zebrafish embryos by van Aerle et al. (2013) and Japanese medaka by Kwok et al. (2016). The main mechanism generally accepted is that AgNPs generate ionic silver (Ag^+) causing oxidative stress through the generation of reactive oxygen species and cause damage to cellular components including DNA damage, activation of antioxidant enzymes, depletion of antioxidant molecules, and disabling of proteins, and damage to the cell membrane (McShan et al. 2014). Using zebrafish embryos as a model organism, van Aerle et al. (2013) examined genotoxicity of very small AgNPs (10 nm) in comparison with large micrometer sized Ag particles (0.6-1.6 μm) and soluble Ag^+. Their results were in accordance with accepted mechanisms as "most transcriptomic alterations caused by AgNPs and its ionic and bulk counterparts were common across all treatments, supporting the hypothesis that the toxicity of AgNPs is principally associated with the toxicity of Ag^+ at the surface of the particles or dissolved in the water (van Aerle et al. 2013). However, they found some gene changes unique to each treatment, first attributed to the proportion of biologically available Ag^+ in the different treatments, but also suggesting that there may also be some particle-specific effects. As a consequence of these findings, the understanding of the bioavailability of dissolved Ag^+ originating from AgNPs should be prioritized in risk assessment studies.

Recently, Kwok and co-workers (2016) examined the toxicity of AgNPs with different coatings and sizes using medaka larvae as a biological model in order to understand how coating could affect AgNP toxicity in vertebrates. Authors found that toxicity was independent of particle size, but related to size of aggregates in test medium, as also observed by Oprsal et al. (2015) and Osborne et al. (2015). They also confirmed that toxicity was heavily influenced by the coating material most probably due to difference in aggregation following the coating chemistry. Interestingly, AgNP exposure did not cause a significant response in three out of four oxidative marker genes examined, a result not in accordance with accepted mechanism described above. Rather, Kwok et al. (2016) observed that AgNPs to cause disruption of Na regulation as often observed with Ag ions with up-regulation of NKA leading to a decrease of whole body Na levels. Toxicity of AgNPs did not correlate with dissolved Ag concentration in the test medium and may instead be related to localized release of Ag^+ on the surface of fish.

AgNP effects are highly variable following fish species, development stages and, among all, exposure protocols and Ag concentrations. Dissolution and aggregation chemistry of coated and uncoated-AgNPs within exposure media is of prime importance, but this fact is no yet fully recognized by authors which leads to more confusion and conflicting results.

2.6. CONCLUSION

Many conflicting results are presented in the scientific literature about toxicity of AgNPs or other nanomaterials. To partially explain or reconciled these results, we must conside that experimental protocols rarely use the same nanomaterials and concentrations and differ systematically in the exposure time. However, a conceptual framework is needed to help to explain the results. Silver nanomaterials are interesting probes to sketch the myriad behaviors of ENMs delivered in complex aqueous systems. They are also very interesting compounds as a chemical model to unravel the spectrum of adverse biological outcomes caused to organisms by an exposition to ENMs. In this review, we examined the behavior of AgNPs when introduced into waters with different chemical compositions where microorganisms such as bacteria and microalgae, and macroorganisms like zooplankton, invertebrates and fish are living and reproducing.

To understand biological effects induced by the exposure to nanomaterials such as AgNPs, environmental chemists and toxicologists have to consider critical information about physicochemical properties of the engineered AgNPs used in laboratory exposure or released in the environment, such as chemical composition, size, surface coating, geometry, crystallinity, porosity, roughness, hydrophobicity or hydrophilicity. We also need detailed data on the composition of the aqueous media, including pH, ionic strength, zeta potential, oxygen, sulfide, dissolved ions, buffers, presence of organic molecules such as humic substances, proteins, polysaccharides, and detergents. These pool of data are essential to determine the stability of native ENMs and their inevitable transformation in complex aqueous media. At the end, ENMs can coexist under a more or less broaden particle size distribution. All tiny particles and aggregates present in experimental media and natural receiving waters are susceptible to produce a wide range of cellular responses

depending of the particle size, duration of contact and metal concentration achieved in the organisms. Many important damages could result to cells at the membrane and/or wall level. Moreover, alteration on cellular components and modification to physiological process such as production of ROS can occur in bacteria, microalgae and higher organisms. Sometime, first signs of nanosilver intoxication can be detected in crustaceans, sea urchins, jellyfish and polychaetes when altered swimming, lethargy, immobility and burrowing impairments are observed. Alterations can likewise reach gametes and/or early stages of development (embryos, larvae and juvenile). About fish, gills could be the target of choice to concentrate ENMs, but other organs such as liver or brain show levels of ENMs. The transport of AgNPs to internal tissues elicits oxidative stress and alteration of normal physiological functions. Because AgNPs or other ENMs show a large range of biological injuries to aquatic organisms it is mandatory to continue to develop a conceptual framework to better understand and assess the exposure and risk from using nanomaterials in upcoming decades.

CONCLUSION GÉNÉRALE

En conclusion, ce mémoire est constitué de deux parties. La première partie comprend des études de laboratoire visant à mieux mettre en évidence les interactions de l'argent ionique ainsi que des AgNPs avec les composés soufrés organiques tels que de petits acides aminés comme la cystéine mais également avec l'albumine, la protéine la plus abondante du sérum sanguin. Les résultats obtenus ont confirmé la présence d'interactions entre l'argent ionique et l'atome de soufre présente sur la cystéine, mais aussi entre l'argent ionique et l'albumine. Des interactions entre la protéine d'albumine et l'argent ionique et nanoparticulaire ont également été observées. L'importance du soufre dans les interactions entre argent et cystéine a été confirmée par spectroscopie RAMAN, qui a mis en avant les modifications des liaisons chimiques comprenant du soufre lors du mélange de cystéine et d'argent ionique. Cette étude se démarque des précédentes recherches en ce qu'elle compare directement les effets des ions Ag^+ seuls avec les effets des AgNPs. Les résultats obtenus montrent des réductions de la fluorescence de l'albumine bien plus importantes en présence d'ions Ag^+ qu'en présence de quantité équivalente en AgNPs. En effet, la réduction de la fluorescence de l'albumine était près de 10 à 15% plus importante pour les ions Ag^+ que les AgNPs lorsque les concentrations en argent étaient basses. À haute concentration en argent, lorsqu'il était sous forme nanoparticulaire, la réduction de la fluorescence se stabilise rapidement dans le temps et atteint une réduction proche de 20%. En revanche lorsque l'argent était présent sous forme ionique, à forte concentration la réduction de la fluorescence suit une courbe asymptotique avec une réduction maximale de la fluorescence de l'albumine de l'ordre de 60% du signal original. Outre le fait que ces résultats montrent des effets plus forts des ions Ag^+ sur la fluorescence de l'albumine, la différence des courbes observées (une courbe se stabilisant rapidement pour les AgNPs et une courbe pouvant être asymptotique pour les ions Ag^+ à haute concentration) tend à indiquer des interactions différentes avec l'albumine entre l'argent ionique et nanoparticulaire. Cependant, l'utilisation de cystéine réduite afin de chélater les ions Ag^+ libre présents dans une solution d'AgNPs nous a permis de démontrer un effet direct des nanoparticules d'argent sur la fluorescence de l'albumine. En effet, dans toute solution d'AgNPs il subsiste toujours un certain pourcentage d'ions Ag^+ issus de la dissolution des nanoparticules. Il était donc difficile de déterminer précisément si les effets observés

étaient dus aux nanoparticules ou à la présence d'ions argent. À ce jour et à notre connaissance, cette étude est la première à comparer et à démontrer un impact direct des AgNPs sur la fluorescence de l'albumine. Des effets additifs des ions et des nanoparticules d'argent sur la fluorescence de l'albumine ont également pu être avancés.

La deuxième partie de ce mémoire présentée sous forme d'une revue de littérature, a souligné les risques écologiques que peuvent représenter les nanoparticules d'argent, ainsi que la grande diversité d'organismes que ces nanoparticules peuvent affecter aussi bien au niveau cellulaire et métabolique, qu'au niveau du développement et de la physiologie des organismes.

Dans le futur, et afin de compléter cette étude, il serait intéressant de déterminer les changements de conformation des protéines d'albumine lors de leurs interactions avec des nanoparticules ou des ions argent. De plus, puisque l'albumine est capable de se lier aux AgNPs, il serait pertinent de d'analyser les effets des AgNPs recouvertes avec de l'albumine au niveau cellulaire ; activation de récepteurs, activations de voie métaboliques, endocytose favorisée ou non, passage par les voies lysosomales ou non, et les implications que cela peut engendrer.

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