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Collegio di Ingegneria Chimica e dei Materiali

**Corso di Laurea Magistrale
in Ingegneria Chimica e dei Processi Sostenibili**

Tesi di Laurea Magistrale

**Behaviour of biocompatible nanoparticles in
biological fluids and in living cancer cells**



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Index

Riassunto.....	I
1.Introduction and objectives.....	1
Bibliography Chapter 1.....	3
2.Background theory.....	4
2.1 Smart Drug Delivery.....	4
2.2 Nanoparticles for anticancer therapy.....	7
2.2.1 Nanocarriers.....	7
2.2.2 Inorganic Nanoparticles.....	8
2.3 Zinc Oxide Nanoparticles.....	9
2.3.1 Synthesis.....	11
2.3.2 Biocompatibility.....	11
2.3.3 Selectivity & Citotoxicity.....	12
2.3.4 Biostability.....	13
2.4 Hela Cells.....	14
Bibliography Chapter 2.....	15
3. Materials and methods.....	19
3.1 Synthesis and functionalization of Zinc Oxide nanoparticles.....	19
3.1.1 Sol-Gel Synthesis.....	19
3.1.2 Microwave-assisted sol-gel synthesis.....	20
3.1.3 Functionalization.....	20
3.2 Zinc Oxide nanoparticles characterization.....	21
3.2.1 X-Ray Diffraction-XRD.....	21
3.2.2 Field-Emission Scanning Electron Microscopy-FESEM.....	23
3.2.3 Dynamic Light Scattering-DLS.....	23
3.3 Biodegradation tests.....	25
3.3.1 Composition of solutions.....	25
3.3.2 Biodegradation tests.....	27
3.3.3 Characterization.....	27
3.4 Coupling of lipids.....	27
3.4.1 Solvent Exchange.....	28
3.4.2 Fusion.....	28

3.4.3 Colocalization tests.....	29
3.5 Cellular uptake.....	32
Bibliography Chapter 3.....	33
4.Results and discussion.....	34
4.1 Characterization of bare and lipid-coated zinc oxide nanoparticles.....	34
4.1.1 Morphological and structural characterization.....	34
4.1.2 Colloidal stability.....	36
4.2 Biodegradation tests.....	37
4.2.1 Morphology and structure.....	37
4.2.2 Dissolution behaviour.....	44
4.2.3 Surface composition.....	45
4.2.4 Aggregation and biostability.....	48
4.3 Colocalization tests.....	50
4.4 Cellular uptake.....	53
Bibliography Chapter 4.....	59
5.Conclusions.....	60
Bibliography Chapter 5.....	62
List of Figures.....	63
List of Tables.....	65

Riassunto

Introduzione e Obiettivi

Il cancro rimane una delle più importanti cause di mortalità con oltre 10 milioni di nuovi casi ogni anno nel mondo. Oggigiorno i trattamenti più comuni contro il cancro sono la chemioterapia, la radioterapia, l'immunoterapia e la chirurgia o una combinazione di queste, ma essi presentano diversi effetti collaterali, tra cui una distribuzione non specifica di agenti antitumorali, concentrazioni inadeguate di farmaci che raggiungono il sito tumorale, citotossicità intollerabile e limitata capacità di monitorare le risposte terapeutiche. Negli ultimi anni l'applicazione delle nanotecnologie per la terapia del cancro ha ricevuto notevole interesse. Come con qualsiasi terapia, gli aspetti principali sono l'efficacia, la sicurezza e la biocompatibilità in modo da raggiungere la concentrazione desiderata di agente terapeutico nei siti tumorali, distruggendo le cellule cancerose e riducendo al minimo il danno alle cellule sane. La cosiddetta "Cancer Nanotechnology", un'area interdisciplinare di ricerca in ingegneria, medicina e scienza, fornisce un approccio interessante e unico, che dovrebbe portare a importanti vantaggi attraverso la detenzione, la diagnosi e il trattamento. I ricercatori sono interessati ad avere un sistema terapeutico integrato che fornisca una terapia e allo stesso tempo diagnosi e monitoraggio: questo approccio chiamato "theranostics" aiuterebbe a determinare la linea corretta di trattamento per un singolo paziente. Lo sviluppo di nanomateriali biodegradabili e biocompatibili per scopi di somministrazione di farmaci è la chiave per rivoluzionare le terapie e la diagnostica sul cancro. Questi nanosistemi hanno molte proprietà che li distinguono dalle altre terapie antitumorali: essi possono avere proprietà terapeutiche intrinseche o possono essere progettati per trasportare la sostanza attiva; inoltre, possono essere attaccati a ligandi di targeting multivalenti, che danno alta affinità e specificità per le cellule bersaglio; possono trasportare più molecole di farmaci che consentano contemporaneamente la terapia combinatoria del cancro e possano bypassare i tradizionali meccanismi di resistenza ai farmaci. A causa delle loro proprietà chimico-fisiche, negli ultimi anni sono state studiate le nanoparticelle di ossido metallico per la somministrazione di farmaci. Come agenti "teranostici", le nanoparticelle di ossido metallico hanno vantaggi rispetto ad altre nanoparticelle a causa della loro intrinseca attività antitumorale. Inoltre, esse sono biocompatibili in natura e possono essere facilmente escrete dal corpo. La superficie delle nanoparticelle di ossido metallico può essere modificata per colpire specificamente le cellule tumorali e per l'imaging può essere applicato un colorante fluorescente o agenti di imaging ottico.

Tra le nanoparticelle di ossido metallico, le nanoparticelle di ossido di zinco hanno ricevuto notevole interesse nella ricerca: sono considerate relativamente biocompatibili e test "in vitro" hanno mostrato una citotossicità preferenziale contro le cellule cancerose. Per eseguire la transizione dagli studi preclinici alle applicazioni "in vivo", è importante comprendere i meccanismi di stabilità e tossicità nell'ambiente biologico. La struttura di questa Tesi Magistrale può essere così schematizzata: la prima parte si concentra sulla sintesi di nanoparticelle di ossido di zinco, la loro funzionalizzazione con gruppi amminopropilici e la loro caratterizzazione dal punto di vista morfologico e

chimico-fisico. In secondo luogo, per testare la stabilità dell'ossido di zinco, sono stati eseguiti test di biodegradazione in PBS (phosphate-buffered saline), una soluzione tampone, comunemente utilizzata nella ricerca biologica per il lavaggio delle cellule, DPBS (Dulbecco's phosphate-buffered saline), che ha una concentrazione di fosfato inferiore a quella standard di PBS, e ALF (artificial lysosomal fluid), che simula le condizioni intercellulari nelle cellule che si verificano durante l'endocitosi. L'analisi dei risultati ottenuti ha permesso di ottenere informazioni sullo stato di aggregazione e dissoluzione delle nanoparticelle di ossido di zinco e sulla loro interazione con i componenti dei fluidi. La possibilità di accoppiare le nanoparticelle di ZnO con liposomi commerciali è stata valutata per migliorare la stabilità e la biocompatibilità. Infine, sono stati presi in considerazione l'internalizzazione e il comportamento intracellulare di nanoparticelle di ZnO tal quali e rivestite di liposomi nella linea cellulare tumorale delle cellule HeLa, appartenenti all'epitelio della cervice uterina.

Stato dell'arte

Smart drug delivery

Il termine "drug delivery" si riferisce a sistemi e tecnologie che trasportano un composto farmaceutico nell'organismo per raggiungere l'effetto terapeutico desiderato. Nei metodi di somministrazione di farmaci convenzionali, il farmaco viene distribuito in tutto il corpo attraverso la circolazione sanguigna. Per la maggior parte degli agenti terapeutici, solo una piccola parte del farmaco raggiunge l'organo interessato, il che implica diversi effetti collaterali, una bio-distribuzione non specifica, un rilascio incontrollato di farmaci, dosi più elevate e minore efficacia della terapia. Per superare questi limiti, sono stati sviluppati sistemi intelligenti di somministrazione di farmaci per consentire l'accumulo di farmaci solo nelle cellule bersaglio, nei tessuti e negli organi di interesse. In questo modo è possibile aumentare l'indice terapeutico (definito come il rapporto tra la dose massima tollerata e la dose efficace più bassa), il che significa meno fluttuazioni nei livelli di farmaco circolante, una minore frequenza dei dosaggi assunti dal paziente e maggiore sicurezza del farmaco. Questi sistemi sono su scala nanometrica, idealmente tra 10 e 200 nm, allo stesso ordine di grandezza delle biomolecole con cui devono interagire, in modo che sia possibile ridurre la tossicità aspecifica e aumentare l'efficacia della terapia. Numerosi studi si stanno concentrando sulla possibilità di effettuare terapie antitumorali basate sull'uso di nanoparticelle composte da una grande varietà di materiali (polimeri, dendrimeri, liposomi, nanotubi di carbonio e particelle metalliche), che possiedono proprietà antitumorali intrinseche o che possono essere utilizzati come trasportatori di medicinali tradizionali. In linea di principio, le nanoparticelle possono raggiungere i tessuti tumorali mediante il targeting passivo o attivo. Il targeting passivo si riferisce all'accumulo di un farmaco o di un trasportatore di farmaci (nanoparticelle) in un sito desiderato a causa di fattori chimico-fisici o farmacologici. Esso sfrutta le dimensioni intrinseche delle nanoparticelle e le proprietà uniche della vascolarizzazione del tumore, come l'effetto EPR (enhanced permeability and retention) e il microambiente tumorale. In generale, tuttavia, l'EPR è un meccanismo di targeting non specifico e le nanoparticelle durante la circolazione sanguigna possono anche accumularsi in altri tessuti sani, tipicamente milza, reni

e fegato. Il targeting attivo, invece, aumenta l'accumulo di nanoparticelle nei siti tumorali e comporta l'ancoraggio di un ligando specifico mirato in grado di riconoscere un recettore specifico, in modo da attraversare barriere biologiche basate su processi di riconoscimento molecolare o fornire un farmaco a siti patologici. A causa della mutagenesi intrinseca dei tessuti tumorali, della variabilità da paziente a paziente, delle difficoltà di identificare specifici antigeni in diverse patologie tumorali, è evidente la difficoltà di sviluppare un ligando mirato altamente specifico. Sia nel caso di targeting passivo e sia nel caso di targeting attivo, le nanoparticelle vengono internalizzate dalle cellule tumorali principalmente attraverso il processo di endocitosi (Figura 2.1): una volta che le nanoparticelle sono entrate negli endosomi e il pH diventa acido, questi maturano a lisosomi e a causa dell'ambiente acido il farmaco viene rilasciato. Le nanoparticelle realizzate con la strategia del targeting attivo mostrano risultati molto positivi, anche se sono ancora nello stadio preclinico.

Nanoparticelle per terapia antitumorale

Nanocarriers

Il termine "nanoparticella" è definito dalla IUPAC come una particella di qualsiasi forma con dimensioni nell'intervallo 1-100 nm (Figura 2.2). Questi nanomateriali hanno proprietà che generalmente non hanno i materiali di bulk e che possono essere sviluppati per applicazioni biomediche: ad esempio, l'elevata area superficiale e la reattività superficiale consentono di massimizzare la capacità di carico dell'agente terapeutico. Questi nanosistemi di somministrazione di farmaci sono essenzialmente costituiti da due componenti: l'agente terapeutico e il carrier, progettati per resistere all'ambiente biologico e proteggere il farmaco fino al raggiungimento del sito desiderato. Tuttavia, ci sono diversi casi in cui l'agente terapeutico coincide con il carrier, cioè quando le nanoparticelle possono essere attivate per produrre un effetto terapeutico nelle cellule tumorali: ad esempio, le nanoparticelle metalliche, magnetiche e ossidometalliche possono essere attivate da uno stimolo esterno (rispettivamente luce, campo magnetico, ultrasuoni...) e sviluppare alte temperature localizzate nel tessuto tumorale (ipertermia, nei primi due casi) o specie tossiche (specie reattive dell'ossigeno, ROS o ioni citotossici nel caso delle nanoparticelle di ossido metallico).

Le nanoparticelle e le micelle polimeriche, i liposomi e i nanotubi di carbonio sono carrier comuni usati come sistemi di drug delivery.

Nanoparticelle inorganiche

Le nanoparticelle inorganiche mostrano proprietà fisiche e chimiche che dipendono dalla loro dimensione, forma e composizione, hanno ampi rapporti superficie-volume ed il loro comportamento è fortemente influenzato dalla loro funzionalizzazione superficiale. Recentemente i ricercatori sono stati interessati nello sviluppo di nanoparticelle con caratteristiche antitumorali intrinsecche (non caricate con l'agente terapeutico e antitumorale) o mediate da stimoli esterni: nanoparticelle di ossidi metallici fanno parte di questa categoria. Esse sono ancora in fase preclinica, anche se mostrano interessanti potenzialità: per i

ricercatori è importante capire in dettaglio la loro biocompatibilità, citotossicità e stabilità in ambiente biologico.

Nanoparticelle di ossido di zinco

L'ossido di zinco (ZnO) è un materiale semiconduttore con promettenti proprietà catalitiche, elettroniche e ottiche: possiede un grande potenziale a causa della sua elevata mobilità elettronica, elevata conduttività termica, ampio band gap (~ 3.3 eV) ed esibisce assorbimento UV nell'intervallo 200-350 nm ed emissione nel vicino UV. In condizioni ambientali l'ossido di zinco è termodinamicamente stabile sotto forma di wurtzite (Figura 2.3), una struttura esagonale compatta, in cui ogni atomo di zinco è circondato da un tetraedro di quattro atomi di ossigeno e viceversa; la struttura risultante è composta da una combinazione di piani formati da ossigeno e atomi di zinco. Negli ultimi decenni, le nanoparticelle di ossido di zinco hanno ricevuto notevole attenzione: ad esempio, esse hanno eccellenti proprietà di assorbimento dell'ultravioletto e trasparenza per la luce visibile, rendendo queste nanoparticelle eccellenti agenti di protezione solare. Inoltre, sono anche efficienti sistemi di trasporto di farmaci: non interagiscono con la maggior parte delle molecole attive disponibili e le nanoparticelle di ossido di zinco superiori a 100 nm sono considerate relativamente biocompatibili, meno tossiche rispetto ad altre nanoparticelle di ossido metallico, relativamente poco costose, il che supporta il loro uso per la somministrazione di farmaci. Inoltre sono caratterizzate da varie applicazioni biomediche non solo per la somministrazione di farmaci, ma anche per attività antidiabetiche, antibatteriche, antimicotiche e antinfiammatorie (Figura 2.4). L'attività antitumorale delle nanoparticelle di ossido di zinco, oggetto di questa Tesi di Laurea Magistrale, è esplicata dalla produzione di ROS, che induce successivamente l'apoptosi. Inoltre, sperimentalmente sono state attuate alcune modifiche superficiali per migliorare ulteriormente la loro stabilità e aumentarne la selettività verso le cellule bersaglio. Infine, le loro proprietà di fotoluminescenza sono utili per applicazioni di imaging "in vitro" e "in vivo", fornendo informazioni complete per la diagnosi del tumore.

Sintesi

Il controllo delle dimensioni e della superficie delle nanoparticelle è l'obiettivo principale di ciascuno dei diversi metodi, che sono riportati per la preparazione delle nanoparticelle di ZnO . Tra questi metodi, quello di precipitazione e il metodo sol-gel sono i più interessanti. Il primo prevede una reazione tra un precursore di zinco e un reagente precipitante: una soluzione di agente precipitante (idrossido di sodio, urea, ecc.) viene aggiunta goccia a goccia alla soluzione acquosa di un precursore di zinco (nitrato di zinco, solfato di zinco, ecc.). Miscelando queste soluzioni, si forma un prodotto intermedio (tipicamente idrossido) e dopo la calcinazione ad alta temperatura viene convertito in ZnO . La seconda è una modifica del metodo di precipitazione, eccetto per il fatto che un additivo può essere utilizzato per stabilizzare le nanoparticelle formate. I metodi sol-gel, già utilizzati per sintetizzare gli ossidi metallici su scala macroscopica, sono stati adattati per la produzione di nanoparticelle: bassi costi, basse temperature, sicurezza dei reagenti utilizzati, controllo della morfologia e dimensioni del prodotto finale sono i principali vantaggi di questa sintesi. Sali inorganici e composti di zinco organico (nitrato di zinco e acetato di zinco) sono i precursori utilizzati per

la sintesi di nanomateriali di ossido di zinco: essi sono in grado di idrolizzare e formare intermedi ($Zn(OH)_4^{2-}$) che portano alla formazione di zinco cristallino ossido. Da questo meccanismo generale possono essere generate diverse strutture: nano- e microparticelle, nanowires, nanorods e multipods, modulando i parametri di sintesi. Non solo le dimensioni, ma anche la geometria e la forma influiscono sul rilascio di farmaci: il trasporto all'interno dell'organismo, l'interazione con il sistema immunitario e l'accumulo nei tessuti e nelle cellule sono alcuni esempi.

Biocompatibilità

Dopo il ferro, lo zinco è il secondo metallo più abbondante nel corpo umano ed è coinvolto in molti processi biologici, anche se è presente solo in tracce (~ 2 g in tutto il corpo). Nella sua forma massiva, l'ossido di zinco è stato classificato dalla FDA (Food and Drug Administration) come materiale GRAS (Generally Recognized as Safe), relativamente biocompatibile, biodegradabile e gli ioni prodotti dopo la biodegradazione possono diventare nutrienti per l'organismo.

Selettività e citotossicità

La possibilità di utilizzare le nanoparticelle di ZnO come agenti antitumorali si basa proprio sulla loro comprovata selettività contro le cellule tumorali, senza avere alcun impatto citotossico sulle cellule sane. Le proprietà elettrostatiche rendono le nanoparticelle di ZnO interessanti per l'attività antitumorale: presentano un diverso tipo di comportamento di carica superficiale, a causa dei gruppi idrossilici che si formano sulla superficie. A pH elevato, i protoni (H^+) migrano dalla superficie della particella verso il mezzo acquoso e la superficie rimane caricata negativamente con atomi di ossigeno parzialmente legati (ZnO^-). A pH inferiore, i protoni vengono trasferiti dall'ambiente acquoso sulla superficie della particella, risultando in una superficie caricata positivamente ($ZnOH_2^+$). Il punto isoelettrico delle nanoparticelle di ZnO è 9-10; per questo motivo in condizioni fisiologiche queste nanoparticelle hanno una forte carica superficiale positiva. Al contrario, le cellule tumorali presentano un'alta concentrazione di fosfolipidi anionici sulle loro membrane esterne e hanno ampi potenziali di membrana negativi. Pertanto, l'interazione delle cellule tumorali con le nanoparticelle di ossido di zinco caricate positivamente è favorita da aspetti elettrostatici, promuovendo l'internalizzazione cellulare, la fagocitosi e la citotossicità di queste nanoparticelle (Figura 2.5). Per quanto riguarda l'effetto citotossico dell'ossido di zinco, esso è collegato alla produzione di ROS, specie chimiche altamente reattive contenenti ossigeno (superossido, idrossile e perossido di idrogeno), che causano stress ossidativo alla cellula e in particolare al DNA, con conseguente arresto del ciclo cellulare e apoptosis. Alcune ricerche mostrano che la produzione di ROS sia una risposta citotossica data dalla cellula in presenza di ioni Zn^{2+} , generati dalla dissoluzione delle nanoparticelle a contatto con l'ambiente intracellulare: l'esposizione al pH acido di endosomi e lisosomi causa una rapida dissoluzione delle nanoparticelle, a cui seguono una risposta infiammatoria e danno ai mitocondri.

Biostabilità

Studi su soluzioni tampone evidenziano la forte tendenza delle nanoparticelle di ZnO ad aggregarsi.

Da questi esperimenti preliminari sono emersi diversi aspetti: ad esempio, la necessità di stabilizzare le nanoparticelle per proteggerle dalla biodegradazione e aumentarne la biocompatibilità.

In questo progetto di tesi è considerata la possibilità di creare una nanoparticella ibrida, che incapsula le nanoparticelle di ossido di zinco nei liposomi; inoltre, sulla membrana lipidica, è possibile aggiungere successivamente ligandi idonei, implementando metodi di targeting attivo. Le caratteristiche dell'ossido di zinco e dei liposomi li rendono candidati interessanti per lo sviluppo di un sistema efficace e innovativo per la terapia antitumorale.

Cellule Hela

Le cellule HeLa sono un tipo di cellule cancerose, appartenenti ad una linea cellulare comunemente usata nella ricerca scientifica. Questa linea cellulare deriva dalle cellule di cancro cervicale di Henrietta Lacks, una paziente morta di cancro il 4 ottobre 1951. L'importanza di questa ricerca si basa sul fatto che il cancro cervicale uterino è il secondo tumore maligno più comune nelle donne in tutto il mondo e presenta un alto tasso di mortalità, specialmente nei paesi in via di sviluppo: il cancro cervicale rappresenta il 4% dei casi di cancro diagnosticati nel mondo e circa l'84% dei casi di cancro cervicale si verificano in Africa, America Latina e Caraibi. Le cellule del cancro cervicale di Lacks sono state prese senza la sua conoscenza o il suo consenso. Il biologo cellulare George Otto Gey sviluppò una linea cellulare, isolando una cellula specifica e moltiplicandola, in modo che potessero essere mantenute in vita. Ciò rappresentò un notevole progresso perché prima le cellule coltivate sopravvivevano solo per pochi giorni e gli scienziati impiegavano più tempo a cercare di tenere in vita le cellule piuttosto che svolgere ricerche reali su di esse. Queste sono state le prime cellule umane cresciute in adesione: esse non morivano dopo un determinato numero di divisioni cellulari dovute alla senescenza cellulare (Figura 2.6). Ciò rappresentò una "nuova era" per la ricerca medica e biologica.

Materiali e Metodi

Sintesi e funzionalizzazione di nanoparticelle di ossido di zinco

Sintesi sol-gel

Acetato di zinco diidrato ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, 99.99%, Sigma Aldrich) e idrossido di sodio (NaOH , 98%, Sigma Aldrich) sono stati utilizzati come precursori per ottenere nanoparticelle sferiche di ossido di zinco con dimensioni intorno a 10-20 nm. Sono state preparate due diverse soluzioni: la prima con 818,2 mg (3,73 mmoli) di zinco acetato diidrato in 42 ml di metanolo (MeOH , Sigma Aldrich) e la seconda con 288,8 mg (7,22 mmoli) di idrossido di sodio in 23 ml di metanolo. La prima soluzione è stata agitata in continuo in un pallone di vetro, inserito in un bagno d'olio riscaldato a 60 ° C. Dopo che la temperatura ha raggiunto il set point, 318 μl di acqua bidistillata sono stati aggiunti alla soluzione di acetato di zinco e,

quindi, la soluzione di idrossido di sodio è stata fatta gocciolare per circa 15 minuti all'interno della prima soluzione (Figura 3.1). La reazione è avvenuta in 2 ore; un'altra sintesi è stata eseguita in 5 ore ed i risultati in termini di morfologia delle nanoparticelle non erano diversi, tuttavia la resa era più alta. È stato deciso di mantenere 2 ore per le successive sintesi per evitare lunghi tempi di processo. Alla fine della reazione, è stata ottenuta una soluzione opalescente contenente le nanoparticelle di ossido di zinco. Per rimuovere i residui di reazione, è stata eseguita una centrifugazione (3046 rcf per 5 min) seguita da lavaggio in etanolo (EtOH, Sigma Aldrich). La procedura è stata ripetuta due volte e poi le nanoparticelle di ossido di zinco sono state disperse in etanolo fresco. La concentrazione finale è stata misurata pesando un'eppendorf (tara) e quindi aggiungendo 500 µl di etanolo, in cui erano presenti le nanoparticelle di ZnO. L'etanolo è stato fatto evaporare per una notte in un forno a 60 ° C ed il giorno dopo l'eppendorf è stata pesata di nuovo (peso lordo). È stato possibile calcolare la concentrazione attraverso questo semplice calcolo: Concentrazione (mg / ml) = (peso lordo - tara) / (volume) (3.1). Le nanoparticelle di ossido di zinco ottenute mediante sintesi sol-gel sono state utilizzate soprattutto per i test di biodegradazione.

Sintesi sol-gel assistita a microonde

Acetato di zinco e idrossido di potassio (Sigma Aldrich) sono stati utilizzati come precursori in proporzioni 1: 2. La prima soluzione è stata preparata in un becher, dove l'idrossido di potassio è stato sciolto in metanolo, anche con l'aiuto del bagno ad ultrasuoni. La seconda soluzione è stata posta direttamente nel reattore: acetato di zinco e metanolo sono stati miscelati mediante agitazione e per favorire la nucleazione sono stati aggiunti 318 µl di acqua bidistillata. Quando l'idrossido di potassio è stato completamente dissolto in metanolo, la prima e la seconda soluzione sono state mescolate insieme nel reattore. La miscela finale è stata immessa nel forno a microonde sotto controllo della temperatura (60 ° C) e della pressione (P_{max} 10 bar) per 30 minuti. Quindi il prodotto è stato centrifugato per 10 minuti a 3500 rcf. Il precipitato è stato disperso in 15 ml di etanolo e centrifugato per 10 minuti a 3500 rcf (questo passaggio è stato eseguito due volte). Infine, le nanoparticelle di ossido di zinco stabili sono state disperse in etanolo fresco. L'ossido di zinco ottenuto tramite sintesi a microonde è stato utilizzato in particolare per i test di internalizzazione cellulare.

Funzionalizzazione

La funzionalizzazione è stata ottenuta combinando le nanoparticelle di ZnO con 3-amminopropiltrimetossilano (APTMS, 97% Sigma Aldrich) al fine di coprire la superficie delle nanoparticelle con gruppi amminici (-NH₂), che possono essere ulteriormente legati ai coloranti, essenziali per l'indagine microscopica in fluorescenza. Inoltre il gruppo amminico è in grado di protonare in un leggero substrato acido, che conferisce alle nanoparticelle una carica positiva netta, altamente desiderabile per l'ulteriore internalizzazione nelle cellule tumorali. APTMS è un alchilsilano, che è in grado di reagire e legarsi a gruppi -OH presenti sulla superficie delle nanoparticelle (Figura 3.2) e ottenere il campione funzionalizzato di amino-propile.

La reazione di funzionalizzazione è stata condotta in etanolo: in particolare per la

funzionalizzazione di 100 mg di nanoparticelle di ZnO (1,23 mmoli), è stato utilizzato un pallone di vetro da 100 ml sotto agitazione continua e un flusso di azoto per impedire la contaminazione di aria umida (e che APTMS polimerizzi su se stesso); la temperatura era di 78 ° C e alla soluzione sono stati aggiunti 21,4 µl di agente APTMS funzionalizzante, pari a 10 moli% della quantità totale di ZnO (0,123 mmoli, 22,05 g). La miscela ottenuta è stata mantenuta per 6 ore in condizioni di riflusso in atmosfera di azoto. Per rimuovere le molecole non legate, sono stati effettuati due lavaggi in etanolo per centrifugazione a 3046 rcf per 10 minuti e successiva ridispersione delle nanoparticelle di ZnO-NH₂ in solvente fresco. La concentrazione finale è stata fissata a 1 mg/ml.

Caratterizzazione delle nanoparticelle di ZnO

Sono state effettuate numerose analisi per verificare la riproducibilità della sintesi: i dati provenienti da diversi batch ottenuti dalla sintesi sol-gel e tramite la sintesi a microonde sono stati confrontati e caratterizzati. In primo luogo, la struttura cristallina e la morfologia (forma e dimensioni) sono state studiate rispettivamente con la diffrazione ai raggi X (XRD) e la microscopia elettronica a scansione di campo (FESEM). In secondo luogo, per avere maggiori informazioni sulla stabilità colloidale del sistema e lo stato di aggregazione delle nanoparticelle in sospensione, è stato eseguito il Dynamic Light Scattering (DLS) insieme alle misurazioni di Z-Potential, per ottenere informazioni sulla funzionalizzazione delle nanoparticelle e quindi del potenziale Z, collegato alla carica superficiale.

Test di biodegradazione

Sono stati eseguiti test di biodegradazione per studiare la stabilità e la reattività delle nanoparticelle di ossido di zinco nei seguenti fluidi:

- PBS (phosphate-buffered saline, Sigma Aldrich), una soluzione tampone, comunemente utilizzata nella ricerca biologica per il lavaggio delle cellule (pH 7,4);
- DPBS (Dulbecco's phosphate-buffered saline, Sigma Aldrich), che ha una concentrazione di fosfato inferiore a quella standard di PBS (pH 7-7,3);
- ALF (artificial lysosomal fluid), che simula le condizioni intercellulari nelle cellule che si verificano durante l'endocitosi (pH 4,5);

Tutti i prodotti chimici per produrre questa soluzione sono stati acquistati da Sigma Aldrich.

I test sono stati condotti sia su nanoparticelle di ossido di zinco tal quali (ZnO NPs) che su nanoparticelle rivestite con un doppio strato lipidico (ZNO-DOPC NPs).

In totale sono stati condotti sei esperimenti in parallelo e sono stati ripetuti due volte: questi test sono stati utili per studiare l'influenza delle caratteristiche della superficie sulla biodegradazione.

Composizione delle soluzioni

Per preparare il PBS, è stata disiolta una compressa in 200 ml di acqua deionizzata: ciò fornisce 0,01 M di tampone fosfato, 0,0027 M di cloruro di potassio e 0,137 M di cloruro di sodio, a pH 7,4 e 25 ° C. Alla fine il volume finale è stato portato ad 1 litro e nella tabella 3.1 la concentrazione è riportata in dettaglio. La soluzione tampone DPBS è stata acquistata da Sigma Aldrich già pronta per l'uso con la composizione descritta in dettaglio in tabella 3.2. Per quanto riguarda la preparazione di ALF, i reagenti sono stati aggiunti nello stesso ordine presentato nella tabella (Tabella 3.3) in 500 ml di acqua bidistillata in un becher di plastica sotto agitazione continua (400 rpm) e la temperatura è stata impostata a 37 ° C. Dopo che tutti i reagenti sono stati aggiunti e la soluzione è stata raffreddata a 25 ° C, sono stati aggiunti 500 ml di acqua bidistillata per dare un volume finale pari a 1 litro. Il pH è stato regolato a 4,5 aggiungendo HCl (1 M) goccia a goccia alla soluzione.

Test di biodegradazione

Sono state preparate sospensioni con una concentrazione di nanoparticelle pari a 2 mg/ml: in totale, 25 mg di nanoparticelle (ZnO NPs e ZnO-DOPC) sono state disperse in 12,5 ml di soluzione (PBS e DPBS). Le condizioni biologiche sono state simulate, mantenendo la temperatura costante a 37 ° C; i campioni sono stati tenuti sotto agitazione a 250 rpm per tutta la durata dell'intero esperimento, al fine di prevenire le precipitazioni. A tempi diversi (1h, 24h, 72h, 1 settimana) sono stati prelevati 2,5 ml da ciascuna soluzione, corrispondenti a 5 mg di nanoparticelle. Le nanoparticelle sono state dapprima centrifugate per bloccare la biodegradazione (3046 rcf per 5 min), il surnatante raccolto per analisi ICP-MS e il precipitato lavato due volte con acqua bidistillata (3046 rcf per 5 min) ed essiccato in un forno a 60 ° C. Sono stati eseguiti test DLS per un'ora, con lo scopo di studiare la biostabilità delle nanoparticelle di ZnO rivestite di lipidi in mezzi fisiologici: mantenendo costante la temperatura della camera di misurazione a 37 ° C, è stato possibile rilevare la dimensione ad intervalli di 5 minuti. A causa delle difficili condizioni fisiologiche di ALF (pH 4,5), i campioni sono stati analizzati a intervalli di tempo ridotti di biodegradazione (15 min, 30 min, 1h): dopo la prima centrifugazione per bloccare il processo (3046 rcf per 5 min), 1 ml di surnatante (top) è stato centrifugato a 10000 rcf per 5 minuti, disperso in acqua e quindi analizzato; anche il precipitato (bottom) è stato messo in 2 ml di acqua bidistillata, quindi lavato attraverso una fase di centrifugazione per 5 minuti a 3046 rcf e quindi analizzato.

Caratterizzazione

Tutti i campioni sono stati analizzati mediante diffrazione ai raggi X (XRD) e spettroscopia infrarossa (FT-IR) per verificare, rispettivamente, l'eventuale perdita di cristallinità e la presenza di nuovi elementi legati o precipitati sulla superficie. Per ottenere maggiori informazioni sulla morfologia e sulla composizione elementare, sono state eseguite analisi FESEM. Per quanto riguarda lo studio delle specie disiolte in soluzione, sono state sfruttate le analisi ICP-MS (Inductively Coupled Plasma Mass Spectroscopy); in particolare, sono state registrate le concentrazioni in soluzione di Zn²⁺, Ca²⁺ e P (legate alla presenza di ioni fosfato, PO₄³⁻). Le analisi sono state eseguite diluendo correttamente in acqua bidistillata il surnatante

scartato, che derivava dalla prima centrifugazione. Le diluizioni utilizzate, relative alle soglie di rilevamento dello strumento, sono le seguenti 1:50 per Ca^{2+} e PO_4^{3-} e 1: 250 per Zn^{2+} .

Accoppiamento con i lipidi

La possibilità di rivestire le nanoparticelle con lipidi (micelle costituite da una membrana con doppio strato fosfolipidico) è stata considerata al fine di migliorare la dispersione colloidale delle nanoparticelle in un ambiente biologico e prevenirne la degradazione e l'aggregazione. Le nanoparticelle di ossido di zinco sono state internalizzate nei liposomi costituiti da DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), fosfolipide a carica neutra, seguendo due protocolli diversi: solvent exchange e fusione. Il prodotto ottenuto è mostrato in Figura 3.6.

Metodo “Solvent exchange”

Le proprietà dei lipidi, stabili e dispersi come monomeri in soluzioni alcoliche con basso contenuto di acqua, sono utilizzate per formare micelle in seguito alla progressiva idratazione della soluzione. Sospendendo le nanoparticelle nella sospensione lipidica prima di aggiungere acqua, viene fornita una superficie di supporto su cui la formazione di liposomi è termodinamicamente favorita. La seguente procedura è stata utilizzata per accoppiare 1 mg di nanoparticelle di ZnO con 0,25 mg di DOPC. In primo luogo è stata preparata una miscela di lipidi dispersi: 1 mg di lipidi (DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids Inc.) sono stati posti in 1 ml di soluzione, aggiungendo prima 400 μl di etanolo (40% in volume) e in secondo luogo 600 μl di acqua bidistillata (60% in volume); quindi 250 μl di questa "soluzione madre" sono stati aggiunti a 1 mg di nanoparticelle, che sono state preventivamente separate dall'etanolo attraverso uno stadio di centrifugazione. A questo punto, le nanoparticelle e i lipidi sono stati omogeneizzati con l'aiuto di una pipetta e di un agitatore a vortice; successivamente, è stata ottenuta la formazione di micelle costituite da doppio strato lipidico che avvolge le nanoparticelle di ZnO, con l'aggiunta di 700 μl di acqua bidistillata.

Metodo di fusione

È stata sfruttata anche l'attrazione tra i liposomi DOPC già formati e le nanoparticelle di ZnO per l'internalizzazione. Grazie alle interazioni elettrostatiche, i liposomi si adsorbono sulla superficie, le forze di adesione aumentano l'area di contatto, determinando la rottura del liposoma, che si chiude intorno alla nanoparticella. È stata adottata la seguente procedura: 1 mg di DOPC in 1 ml di Hepes (Sigma Aldrich) è stato filtrato attraverso una membrana porosa undici volte in un estrusore per formare liposomi sferici. Da questa "soluzione madre" sono stati prelevati 200 μl e miscelati con 1 mg di nanoparticelle di ZnO; dopo aver disperso le nanoparticelle di ZnO tramite l'ausilio di una pipetta, sono stati aggiunti 200 μl di acqua bidistillata; quindi questa miscela è stata messa in uno shaker per 1.5 h (300 rpm) per consentire l'accoppiamento tra le nanoparticelle e i liposomi.

Test di co-localizzazione

Sono stati eseguiti esperimenti di co-localizzazione, depositando 20 µl della soluzione contenente le nanoparticelle accoppiate con i liposomi su un vetrino da microscopio per verificare il successo dei due protocolli di internalizzazione testati. Il campione è stato osservato mediante microscopia a fluorescenza ottica: è stato utilizzato un microscopio a fluorescenza a disco rotante (Zeiss Cell Observer Spinning Disk ,che utilizza un'unità di filatura Yokogawa CSU-X1). Sia le nanoparticelle funzionalizzate (ZnO-NH₂ NPs) che il doppio strato lipidico sono stati marcati con coloranti idonei ma con emissioni differenti tra loro. In dettaglio, prima di eseguire i due protocolli di accoppiamento, 2 µl di ATTO 488 NHS ester dye (Sigma Aldrich, 1 mg/ml) sono stati aggiunti alle nanoparticelle amino-funzionalizzate conservate in etanolo ad una concentrazione di 1 mg/ml. La soluzione contenente le nanoparticelle e il colorante è stata lasciata su uno shaker orbitale (700 rpm) durante la notte. Il giorno successivo, l'etanolo è stato separato mediante centrifugazione (10000 g per 5 minuti) e sono stati fatti alcuni lavaggi in acqua per rimuovere il colorante non legato ed in eccesso; in dettaglio, 2 lavaggi in acqua per 5 minuti a 10000 g e prima del terzo lavaggio la soluzione è stata lasciata per 15 minuti in un bagno ad ultrasuoni; dopo aver scartato il surnatante, sono stati eseguiti il solvent exchange o la fusione per accoppiare le nanoparticelle con i liposomi. Le misurazioni sono state effettuate in DLS per verificare la dimensione delle nanoparticelle accoppiate (500 µl/ml). Infine, per marcare i liposomi, sono stati aggiunti 0,1 µl di DID (1 µM), Indicatore lipofilo di Invitrogen. Atto488 (legato alle nanoparticelle ZnO-NH₂) è stato esaminato alla lunghezza d'onda di eccitazione del laser pari a 488 nm, mentre DID (legato allo strato lipidico) a 639 nm. Ottenendo l'emissione di fluorescenza nei due rispettivi canali (con una differenza temporale minima tra le due acquisizioni e confrontando le posizioni dei due segnali raccolti), è stato possibile determinare se le nanoparticelle e i liposomi fossero colocalizzati o meno.

Internalizzazione cellulare

Per gli esperimenti su cellule, le cellule tumorali HeLa ATCC® CCL-2™ derivate dal cancro cervicale (adenocarcinoma) sono state coltivate in DMEM supplementato con FBS 10% in volume e 1% in Streptococco. Tutti i reagenti sono stati acquistati da Thermofisherscientific. Il giorno precedente l'incubazione delle particelle, le cellule sono state poste su vetrini da 8 pozzetti ad una concentrazione di 5000 cellule per pozzetto contenente 300 µl di terreno. Le nanoparticelle di ossido di zinco sono state preparate come descritto sopra, sostituendo solo l'Atto 488 NHS ester con l'Atto 633 NHS ester. Il giorno dopo l'incubazione cellulare, 1 µl di Lysosomes-GFP (Thermo Fisher, 1 mg / ml) è stato aggiunto al terreno di coltura cellulare. Questo colorante è in grado di marcire i lisosomi nelle cellule, permettendo così di valutare la loro colocalizzazione con le nanoparticelle marcate. Infine le NPs tal quali e rivestite di lipidi, ottenute con il metodo solvent exchange, marcate con Atto 633, sono state aggiunte al terreno di coltura cellulare a diverse concentrazioni (10 µg / ml, 20 µg / ml, 30 µg / ml) e l'internalizzazione cellulare è stata valutata dopo 5 ore e 24 ore. Sono state eseguite quattro repliche per ciascuna concentrazione delle nanoparticelle e per i tempi di incubazione considerati per convalidare i dati ottenuti. Sono stati effettuati due lavaggi in DMEM prima di preparare il campione di coltura cellulare per il microscopio.

Cellule e particelle sono state esposte a 37 °C in atmosfera umidificata al 5% di CO₂ utilizzando il microscopio a fluorescenza a disco rotante (Zeiss Cell Observer SD che utilizza un'unità di filatura Yokogawa CSU-X1). L'obiettivo era un obiettivo di immersione in olio apocromatico da 1.40 NA 63 × Plan (Zeiss) (Figura 3.9). Atto633 è stato ripreso con una lunghezza d'onda di eccitazione pari a 639 nm, mentre Lysosomes-GFP con una lunghezza d'onda pari a 488 nm.

Risultati e discussione

Caratterizzazione di nanoparticelle di ossido di zinco tal quali e rivestite di lipidi

Caratterizzazione morfologica e strutturale

La morfologia e la dimensione delle nanoparticelle di ossido di zinco tal quali e rivestite di lipidi sono state caratterizzate dalla microscopia elettronica a scansione ad emissione di campo (FESEM) (Figura 4.1). Le immagini FESEM mostrano che queste nanoparticelle hanno una forma sferica con un diametro medio di 14 ± 2 nm (calcolato dal software Fiji, Open source, n = 50). L'analisi dei diffrazogrammi ai raggi X ha permesso di confermare la struttura cristallina delle nanoparticelle di ZnO. I picchi di diffrazione sono in accordo con i picchi caratteristici della struttura cristallina della wurtzite (Figura 4.2): lo spettro presenta picchi di diffrazione ampi per valori di 2θ pari a 31.9 °, 34.4 °, 36.4 °, 47.6 °, 56.7 ° e 62.9 °. Applicando l'equazione di Debye-Scherrer (Formula 3.3), è stata ottenuta una dimensione media di 15 nm dei nanocrystalliti, in accordo con i risultati ottenuti dal FESEM. Per le nanoparticelle rivestite da lipidi, preparate con un metodo di solvent exchange, usando il fosfolipide commerciale (1,2-dioleoyl-sn-glycero-3-phosphocholine), le analisi FESEM e XRD sono state utili per confermare che la morfologia e la struttura cristallina delle nanoparticelle non siano state modificate (Figura 4.1, Figura 4.2), mentre le dimensioni sono cambiate a causa del rivestimento (diametro medio di 21 ± 5 nm, misurato da immagini FESEM usando il software Fiji, n = 20).

Stabilità colloidale

Al fine di valutare il possibile uso di nanoparticelle di ossido di zinco nelle applicazioni cliniche, è stato importante studiare la stabilità della sospensione e lo stato di aggregazione, che influenza la bio-distribuzione all'interno dell'organismo e quindi l'efficacia terapeutica. Sono stati condotti esperimenti di Dynamic Light Scattering (DLS) per i due campioni per stimare i loro diametri idrodinamici e la stabilità in acqua (Figura 4.3). L'assenza di aggregati su scala micrometrica in tutte le misurazioni suggerisce una buona dispersione e un basso comportamento di aggregazione per entrambi i campioni. Inoltre, il rivestimento della superficie delle nanoparticelle di ZnO ha contribuito a un diametro idrodinamico medio più ampio (110 nm per ZNO-DOPC NP) rispetto a quello ottenuto per le nanoparticelle tal quali di ZnO (55 nm). Un altro parametro, importante per prevedere la stabilità delle sospensioni

colloidali, è il potenziale zeta. Corrisponde al valore del potenziale eletrostatico sul piano di scorrimento, in corrispondenza dello strato di liquido, formato da due o tre strati molecolari, che si muove con la particella. Questo valore consente di avere informazioni sulle forze repulsive che si creano tra le nanoparticelle e che sono in grado di stabilizzare la soluzione. Il potenziale Z delle micelle lipidiche e delle nanoparticelle di ZnO tal quali e rivestite di lipidi è stato valutato in acqua mantenendo un pH neutro mediante titolazione con NaOH e HCl 1 M. Come mostrato in Figura 4.3, le micelle DOPC presentano un potenziale Z negativo, pari a -15 mV, in accordo con gli studi di letteratura. Per quanto riguarda le nanoparticelle, sono stati ottenuti valori diversi a seconda delle proprietà superficiali delle nanoparticelle di ZnO. Per le nanoparticelle di ZnO tal quali, il potenziale Z misurato è positivo (26 mV) ed è dovuto alla protonazione dei gruppi idrossilici sulla superficie delle nanoparticelle, in accordo con i valori della letteratura. Una forte diminuzione del valore del potenziale Z è stata ottenuta per le nanoparticelle di ZnO rivestite di lipidi: il guscio fosfolipidico DOPC ha neutralizzato le cariche positive della superficie di ZnO, in modo che il potenziale Z scendesse a 1.3 mV. Questi diversi comportamenti in termini di diametri idrodinamici e misurazioni del potenziale Z suggeriscono che il processo di incapsulamento dell'ossido di zinco in un guscio lipidico protettivo è avvenuto con successo.

Test di biodegradazione

Morfologia e struttura

Le analisi FESEM sono state eseguite per i test di biodegradazione in PBS (phosphate-buffered saline) e DPBS (Dulbecco's phosphate-buffered saline) su nanoparticelle di ossido di zinco tal quali e rivestite di lipidi, al fine di ottenere ulteriori informazioni sulla morfologia delle nanoparticelle. Queste due soluzioni tampone hanno due diverse concentrazioni di fosfati: PBS a concentrazione più elevata (Tabella 3.1), mentre DPBS a concentrazione inferiore (Tabella 3.2). Durante questi test i prelievi sono stati effettuati a 1 ora, 24 ore, 72 ore e 1 settimana. Tutti i campioni erano già fortemente aggregati dopo la prima ora e l'aggregazione aumentava durante i test, come mostrato in Figura 4.4 e Figura 4.5: le nanoparticelle presentavano la tendenza a formare aggregati sferici, costituiti da ossido di zinco ricoperto da uno strato sottile di altro materiale inorganico, che deriva dalla precipitazione di sali presenti nelle soluzioni tampone (Tabella 4.1; Tabella 4.2). Tutti i campioni sottoposti a biodegradazione sono stati analizzati mediante diffrazione ai raggi X (Figura 4.6 e Figura 4.7), in modo che fosse possibile verificare la cristallinità delle nanoparticelle. In tutti i campioni sottoposti a biodegradazione, i picchi caratteristici della wurtzite, la struttura cristallina esagonale di ossido di zinco, sono stati trovati con valori di 2θ pari a 31.9° , 34.4° , 36.4° , 47.6° , 56.7° e 62.9° . Questo risultato supporta il fatto che l'ossido di zinco in PBS e DPBS abbia mantenuto la sua struttura cristallina. Inoltre, è stato possibile attribuire il picco di diffrazione a 2θ di 32° a sodio cloruro, un composto presente in PBS e DPBS. Inoltre, i picchi di diffrazione a 2θ pari a 21.36° , 27.2° , 34.61° , 52.14° , 53.17° e 56.09° erano dovuti ai fosfati che interagivano con la superficie di ossido di zinco. Per quanto riguarda i test di biodegradazione in ALF (artificial lysosomal fluid), le analisi

FESEM mostrano che il pH acido (4.5 pH) sia stato molto aggressivo nei confronti delle nanoparticelle di ossido di zinco tal quali e rivestite di lipidi: dopo pochi minuti la maggior parte delle nanoparticelle si sono sciolte in soluzione e sono rimasti pochi aggregati sferici (Figura 4.8). Inoltre, le nanoparticelle hanno perso la loro cristallinità, come mostrato nei diffrattogrammi delle nanoparticelle (Figura 4.9): l'assenza di picchi è legata al fatto che i campioni sono caratterizzati da uno stato amorfo.

Comportamento di dissoluzione

Per quanto riguarda le specie disciolte in soluzione, è stata eseguita l'analisi ICP-MS (inductively coupled plasma mass spectrometry): la concentrazione degli ioni PO_4^{2-} e Zn^{2+} è stata monitorata per verificare il comportamento di dissoluzione delle nanoparticelle in tutti i mezzi usati, PBS, DPBS e ALF. Per quanto riguarda le soluzioni di PBS e DPBS, gli studi di letteratura riportano un'elevata interazione di ioni fosfato con nanoparticelle di ossido di zinco: l'interazione degli ioni fosfato con la superficie delle nanoparticelle porta ad una diminuzione degli ioni fosfato in soluzione (Figura 4.10). Per quanto riguarda gli ioni Zn^{2+} in soluzione, è presente un aumento dopo 24 ore e una differenza tra le nanoparticelle tal quali e quelle rivestite di lipidi: il rivestimento lipidico sembra proteggere parzialmente le nanoparticelle dalla dissoluzione in PBS e DPBS, ovvero in tal caso sono presenti meno ioni Zn^{2+} rilasciati in soluzione (Figura 4.10). Si noti che non vi sono differenze nel comportamento di dissoluzione tra le soluzioni PBS e DPBS, nonostante il loro differente contenuto di ioni fosfato, maggiore nel PBS. Al pH acido del liquido lisosomiale artificiale, c'è un'alta produzione di ioni Zn^{2+} in soluzione (circa 1500 ppm) già dopo mezz'ora (Fig. 4.11). L'aggressività di questo mezzo determina un'elevata dissoluzione delle nanoparticelle sia tal quali sia rivestite di lipidi, così che il rivestimento non sembra proteggere le nanoparticelle. Quindi la presenza di ioni Zn^{2+} potrebbe generare una risposta citotossica sulle cellule: non appena le nanoparticelle sono presenti nei lisosomi, iniziano a dissolversi in breve tempo (30 min).

Composizione della superficie

Tutti i campioni dei test di biodegradazione sono stati analizzati mediante spettroscopia a infrarossi (FT-IR), al fine di verificare la presenza di nuove specie legate o di elementi precipitati sulla superficie delle nanoparticelle. Gli spettri risultanti sono mostrati in Figura 4.12 e Figura 4.13. Gli spettri delle nanoparticelle di ossido di zinco tal quali e rivestiti di lipidi prima dei test di biodegradazione (t_0) hanno picchi legati al legame Zn-O e ai gruppi idrossile e carbonile. Per quanto riguarda le nanoparticelle sottoposte a biodegradazione in PBS e DPBS, l'intensità dei picchi aumenta col tempo: tutti i campioni analizzati hanno un picco a 1045 cm^{-1} , caratteristico dello stiramento del legame P-O derivante dalla precipitazione sulla superficie dei gruppi fosfato (PO_4^{3-}), presente sia in PBS (a concentrazione più alta) che in DPBS (a concentrazione più bassa). I picchi a 1528 cm^{-1} e 1643 cm^{-1} sono legati agli ioni carbonato, mentre a 2955 cm^{-1} e 2915 cm^{-1} i picchi sono associati all'allungamento simmetrico e antisimmetrico del gruppo $-\text{CH}_3$, già presente nanoparticelle di ZnO tal quali, a causa dei residui di acetato nella sintesi. La banda di

stretching nella regione da 3600 a 3000 cm⁻¹ è attribuita ai gruppi -OH presenti sulla superficie.

Non è stato possibile effettuare questa analisi sui campioni biodegradati in ALF a causa dell'elevata dissoluzione.

Aggregazione e biostabilità

Come primo passo verso la comprensione del comportamento delle nanoparticelle di ZnO nei fluidi biologici, sono stati condotti esperimenti di Dynamic Light Scattering (DLS) in PBS ed è stato valutato l'effetto del rivestimento lipidico sulla stabilità colloidale delle nanoparticelle. Come mostrato in Figura 4.14a, quando le nanoparticelle sono state sospese in PBS, quelle tal quali mostrano un forte comportamento di aggregazione, formando aggregati di scala micrometrica (3200 nm), mentre le nanoparticelle rivestite di lipidi non mostrano alcuna aggregazione, confermando le dimensioni idrodinamiche ottenute in sospensione d'acqua (105 nm). Il raggio idrodinamico medio (media z) delle nanoparticelle è stato registrato in tempo reale per 1 ora (Figura 4.14b) per studiare ulteriormente questo comportamento. Non appena le nanoparticelle di ZnO tal quali sono state in contatto con la soluzione PBS (tempo zero), la media z misurata è relativamente alta (3200 nm), confermando che questo campione forma rapidamente enormi aggregati. Nel corso del tempo, la media z diminuisce moderatamente (fino a 1890 nm), suggerendo che le nanoparticelle mantengono la loro forma aggregata e parzialmente precipitano. La marcata diminuzione del derived count rate (Figura 4.14c) sostiene fortemente questa ipotesi. D'altra parte, le nanoparticelle di ZnO rivestite con lipidi non formano aggregati a scala micrometrica quando sospese in PBS, mantenendo nel tempo una dimensione media z compresa tra 100 e 250 nm, confermando così la loro maggiore stabilità colloidale rispetto a quelle tal quali.

Non è stato possibile fare misure DLS nel tempo per il campione delle nanoparticelle tal quali in ALF per via della degradazione immediata. Al contrario, è interessante osservare un comportamento stabile nel tempo delle nanoparticelle di ZnO rivestite di lipidi in ALF (Figura 4.15, Figura 4.16), dove il guscio lipidico impedisce ancora l'aggregazione delle nanoparticelle, sebbene la soluzione sia a pH acido (pH 4.5).

La dimensione media delle nanoparticelle è simile a quella osservata al FESEM in Fig. 4.8. Questi dati non sono tuttavia completamente confermati dalle misurazioni ICP-MS e XRD, che mostrano al contrario una forte degradazione del campione e l'assenza di qualsiasi picco di diffrazione dopo caratterizzazione XRD. Un'altra ipotesi potrebbe essere che il guscio lipidico rimanga intatto, ma le nanoparticelle di ossido di zinco si dissolvano e si diffondano fuori dal rivestimento lipidico. Pertanto si presume che il mezzo ALF sia molto ostile per entrambi i tipi di nanoparticelle, indipendentemente dal loro rivestimento e in particolare per tempi superiori a 30 min e 1 ora.

Test di co-localizzazione

È stata testata la possibilità di rivestire nanoparticelle di ossido di zinco con liposomi, micelle formate da un doppio strato di fosfolipide ampiamente utilizzato nella somministrazione di farmaci, al fine di prevenire l'aggregazione e migliorare la resistenza e la biocompatibilità del materiale. Come descritto nel capitolo Materiali e Metodi (capitolo 3), sono stati testati due

tipi di accoppiamento (fusione e solvent exchange) e il successo dell'internalizzazione è stato verificato grazie a esperimenti di co-localizzazione in microscopia a fluorescenza. Inizialmente il PBS è stato utilizzato per il protocollo di fusione: la vicinanza dei segnali fluorescenti relativi ai liposomi (canale rosso) e alle nanoparticelle (canale verde) ha permesso di confermare il successo dell'accoppiamento (Figura 4.17). Tuttavia, sono stati osservati così tanti aggregati, che il PBS è stato sostituito con HEPES (acido 4- (2-idrossietilico-1-piperazineetansolfonico) e questo ha portato a risultati migliori (Figura 4.18): le nanoparticelle sono risultate meno aggregate e caratterizzate da una migliore dispersione. Per quanto riguarda il metodo di solvent exchange, l'uso degli ultrasuoni è stato molto importante per facilitare la dispersione delle nanoparticelle: inizialmente i campioni non sono stati trattati con ultrasuoni nella procedura ed il campione è caratterizzato da molti aggregati (Figura 4.19), sebbene i segnali delle nanoparticelle (verde) siano molto vicini a quelli a doppio strato lipidico (rosso). Successivamente, sono stati eseguiti gli step a ultrasuoni descritti nel capitolo Materiali e Metodi: le nanoparticelle sono risultate meno aggregate e i segnali delle nanoparticelle in verde e del doppio strato lipidico in rosso risultano sempre molto vicini (Figura 4.20). I segnali non sono perfettamente sovrapposti a causa del rapido movimento delle nanoparticelle e dell'evaporazione del liquido durante la misurazione; tuttavia, la vicinanza dei segnali ha permesso di supporre che le nanoparticelle siano state ricoperte con successo da un doppio strato lipidico. Vale la pena ricordare che la barra della scala nelle immagini a fluorescenza ottica è di 10 µm. Pertanto, la dimensione risultante delle nanoparticelle può sembrare in disaccordo con le dimensioni misurate con la DLS e il FESEM. Tuttavia, l'ingrandimento utilizzato per l'imaging a fluorescenza (obiettivo 63×) non è sufficiente per risolvere dimensioni di 20 nm (che è la dimensione effettiva delle nanoparticelle) e sarà sempre limitato dal limite di Abbé, in questo caso circa 200 nm. Per questo motivo, non è possibile distinguere se vi sia una singola o più particelle all'interno di ciascun punto luminoso di 200 nm: si assume che siano state fotografate principalmente singole particelle a causa della elevata luminosità di emissione delle particelle e della loro forte diluizione.

Internalizzazione cellulare

Sono stati effettuati test di internalizzazione cellulare su cellule HeLa per confrontare il comportamento a diverse concentrazioni (10, 20 e 30 µg/ml) di nanoparticelle di ossido di zinco tal quale con quello delle nanoparticelle di ZnO rivestito con liposomi. I test a microscopia in fluorescenza sono stati eseguiti dopo 5 e 24 ore di incubazione con le cellule HeLa. Queste scale temporali sono state molto utili per studiare la cinetica di internalizzazione cellulare, essendo nel range necessario per avere le nanoparticelle internalizzate nelle cellule tumorali e co-localizzate nei lisosomi. La percentuale di internalizzazione cellulare, mediata su 4 test per ogni condizione studiata, è risultata maggiore aumentando la concentrazione ed è migliorata grazie al rivestimento lipidico, già dopo 5 ore di incubazione (Tabella 4.3). Per le nanoparticelle di ossido di zinco tal quali la presenza di aggregati impedisce alle nanoparticelle di entrare nelle cellule (Figura 4.21), rimanendo nel mezzo di coltura cellulare e all'esterno delle cellule. D'altro canto, le nanoparticelle di ossido di zinco con rivestimento lipidico sono più disperse e più stabili e il rivestimento favorisce la

loro interiorizzazione fino a quando sono visivamente co-localizzate con i lisosomi (Figura 4.22). Dopo 24 ore di incubazione questo comportamento è ancora più evidente: la percentuale di internalizzazione cellulare da parte delle cellule HeLa aumenta significativamente per le nanoparticelle rivestite di liposomi e soprattutto a concentrazioni più elevate (Tabella 4.4). Le immagini del microscopio a fluorescenza mostrano che ci sono molti aggregati micrometrici nel caso di nanoparticelle di ossido di zinco tal quale (Figura 4.23). Al contrario, nel caso delle nanoparticelle di ZnO rivestite di lipidi, il campione è più disperso e ciò favorisce l'internalizzazione cellulare, portando a risultati positivi (Figura 4.24). A questo punto, è importante capire l'effetto terapeutico delle nanoparticelle dopo che sono state internalizzate e co-localizzate nei lisosomi. In un precedente lavoro, la citotossicità e l'effetto fotodinamico delle nanoparticelle di ZnO tal quale e rivestito di lipidi sono stati studiati su cellule di Hela. Gli effetti di diverse concentrazioni di nanoparticelle ZnO e ZnO-DOPC sulle cellule HeLa dopo 24 ore di incubazione sono mostrati in Fig. 4.25. Da un punto di vista quantitativo, questi dati confermano il comportamento citotossico di entrambe le tipologie di nanoparticelle, anche in assenza di attivazione della luce UV, ma solo ad alte concentrazioni di nanoparticelle (vedere le barre nere e grigie nel grafico). I due tipi di nanoparticelle di ZnO non hanno mostrato effetti citotossici fino a una concentrazione di circa 18 µg/ml (molto vicina a quella utilizzata in questa Tesi Magistrale di 20 ug/mL), mentre entrambi hanno mostrato un significativo effetto citotossico per concentrazioni più elevate. In particolare, vale la pena notare la concentrazione a 36 ug/ml è vicina a quella utilizzata in questa Tesi Magistrale (30 ug/mL) in cui viene registrata la citotossicità più elevata per le nanoparticelle di ZnO rivestito di lipidi. Queste sono in grado di entrare efficientemente nelle cellule tumorali e poi vengono co-localizzate con i lisosomi, portando così ad un'alta concentrazione locale di cationi Zn²⁺; l'effetto tossico di questi cationi è qui particolarmente efficace. Al contrario, le nanoparticelle di ZnO tal quali sono meno internalizzate e quindi possono creare meno effetti citotossici nelle cellule HeLa. Ovviamente, per aumentare l'effetto citotossico dei nanomateriali di ZnO nelle cellule tumorali, è possibile sfruttare un comportamento di attivazione tramite stimolo esterno, quale la luce ultravioletta. Tale effetto non è stato studiato durante questa Tesi di Master, ma è riportato per completezza. Le colture cellulari HeLa irradiate con luce UV (a una lunghezza d'onda di 255 nm per 30 s) hanno mostrato una marcata diminuzione della vitalità cellulare anche a basse concentrazioni sia di nanoparticelle di ossido di zinco tal quali sia di quelle rivestite con i lipidi: questi dati preliminari suggeriscono che l'ossido di zinco sotto forma di nanoparticella, utilizzato a concentrazioni non tossiche, può indurre effetti citotossici se irradiato con luce UV.

Conclusioni

L'uso delle nanotecnologie nelle scienze della salute ha permesso lo sviluppo di nuove ed efficaci terapie per il trattamento di malattie altamente critiche, come il cancro. Recentemente, le nanostrutture di ossido di zinco hanno ricevuto grande attenzione nella ricerca grazie a diverse proprietà, che sono molto utili per scopi diagnostici e terapeutici. In particolare, le caratteristiche di biocompatibilità, biodegradabilità, citotossicità, facilità di sintesi e funzionalizzazione con leganti adatti ad aumentare la loro selettività, rendono le nanoparticelle di ossido di zinco buone candidate per diventare agenti antitumorali.

potenzialmente interessanti. Questo progetto di tesi si focalizza sullo studio della stabilità delle nanoparticelle di ossido di zinco tal quali e rivestite di lipidi in vari solventi, studiando il loro comportamento in termini di aggregazione e biodegradazione. In secondo luogo, è stato considerata l'internalizzazione di queste nanoparticelle da parte delle cellule tumorali HeLa. Per analizzare la reattività e la stabilità delle nanoparticelle, sono stati effettuati test di biodegradazione in PBS (phosphate-buffered saline) e DPBS (Dulbecco's phosphate-buffered saline), entrambe soluzioni tampone caratterizzate da forza ionica e percentuali di ioni disioliti molto elevate, con cui le cellule vengono lavate; inoltre, sono stati eseguiti test di biodegradazione in ALF (artificial lysosomal fluid), che simula il liquido lisosomiale. I test in PBS e DPBS mostrano la tendenza delle nanoparticelle ad aggregarsi in questi fluidi. Dalle immagini FESEM viene rilevata la presenza di aggregati: le nanoparticelle sono coperte da un sottile strato di materiale inorganico a base fosfato già dopo un'ora. L'analisi ai raggi X conferma la presenza di elementi estranei sulla superficie delle nanoparticelle, in particolare fosfati e carbonati e viene mantenuta la struttura cristallina dell'ossido di zinco. L'aggregazione è accompagnata da una modesta dissoluzione delle nanoparticelle di ossido di zinco, tuttavia il rivestimento lipidico fa diminuire le specie Zn^{2+} disiolte nella soluzione, che rimangono al di sotto di 100 ppm per la durata dei test. I test in ALF consentono di valutare l'interazione delle nanoparticelle con il fluido lisosomiale simulato: questo rappresenta la loro destinazione finale, dopo che sono entrate nelle cellule. L'analisi FESEM mostra che dopo la prima mezz'ora le nanoparticelle si dissolvono e si degradano e le analisi XRD confermano che perdono la loro struttura cristallina. Le analisi ICP-MS dimostrano che vengono generati molti Zn^{2+} (~ 1500 ppm), che potrebbero essere citotossici per le cellule. Ciò significa che le nanoparticelle potrebbero esercitare la loro attività citotossica già nella prima ora, dopo che sono state colocalizzate all'interno dei lisosomi. Attraverso l'uso della tecnica Dynamic Light Scattering (DLS), è stata valutata la stabilità colloidale delle nanoparticelle di ossido di zinco tal quali e rivestite di lipidi: il rivestimento impedisce alle nanoparticelle di entrare in contatto con il liquido e quindi migliora la stabilità delle nanoparticelle, evitando l'aggregazione. Inoltre, il rivestimento migliora l'internalizzazione cellulare, in quanto questo strato lipidico promuove l'ingresso delle nanoparticelle nelle cellule, formando un "Cavallo di Troia" nanometrico. Mentre le nanoparticelle di ossido di zinco tal quali formano molti aggregati, che rimangono nel terreno di coltura cellulare, così che il processo di internalizzazione non ha successo, le nanoparticelle ibride entrano nelle cellule già dopo 5 ore e dopo 24 ore la maggior parte di esse sono presenti nei lisosomi. Questi test sono importanti per avere informazioni sulla cinetica della terapia antitumorale: i test cellulari forniscono informazioni sulla cinetica di internalizzazione cellulare (dopo 5 ore le nanoparticelle con rivestimento lipidico si trovano nei lisosomi), mentre i test con il liquido lisosomiale simulato forniscono dettagli sulla cinetica di formazione di specie citotossiche, come gli ioni Zn^{2+} , che entro un'ora dalla colocalizzazione con i lisosomi sarebbero presenti e potrebbero esercitare la loro attività citotossica.

I risultati mostrano che un doppio strato lipidico migliora le prestazioni delle nanoparticelle e ciò rappresenta un inizio per ulteriori miglioramenti nell'efficacia della terapia.

Behaviour of biocompatible nanoparticles in biological fluids and in living cancer cells

1. Introduction and Objectives

Cancer remains one of the most important causes of mortality with over 10 million new cases every year worldwide [1]. Nowadays the most common cancer treatments are chemotherapy, radiation and surgery [2], but they come with several drawbacks: non-specific systemic distribution of antitumor agents, inadequate drug concentrations reaching the tumor site, intolerable cytotoxicity and limited ability to monitor therapeutic responses.

In recent years the medical application of Nanotechnology for cancer therapy has received considerable attention. As with any cancer therapy, the main aspects are efficacy, safety and biocompatibility in order to achieve the desired concentration of therapeutic agent in tumor sites, destroying cancerous cells while minimizing damage to normal cells.

The so called ‘Cancer Nanotechnology’ [1], an interdisciplinary area of research in engineering, medicine and science, provides an interesting and unique approach, that is expected to lead to major advantages through detection, diagnosis and treatment [3]. Scientists are interested in having an integrated therapeutic system that provides therapy and at the same time diagnosis and monitoring of therapy through imaging [4]: this approach called ‘theranostics’ helps to determine the correct line of treatment for an individual patient.

Developing ingenious biocompatible nanocomposites for drug delivery purposes is the key to revolutionize cancer therapeutics and diagnostics. These nanosystems have many properties that distinguish them from other cancer therapeutics: they can themselves have therapeutic properties or they can be designed to carry the active substance; besides, they can be attached to multivalent targeting ligands, which yield high affinity and specificity for target cells; they can be made to accommodate multiple drug molecules that simultaneously enable combinatorial cancer therapy and they can bypass traditional drug resistance mechanisms [1].

Due to their unique physical and chemical properties, metal oxide nanoparticles have been studied in recent years for drug delivery and targeting [5]. Their large surface area makes them useful for the cancer treatment and their inherent properties make them interesting for diagnostic and imaging purposes: for example the photoluminescence or paramagnetic properties of metal oxide nanoparticles are useful for imaging [6], whereas their ability to produce reactive oxygen species (ROS) can be used to kill cancer cells [7]. As ‘theranostic’ agents, metal oxide nanoparticles have advantages over other nanoparticles because of their inherent anticancer activity, which overcomes the requirement of other carriers for the delivery of therapeutic and diagnostic agents [8]. Besides, they are biocompatible in nature and can be easily excreted from the body. The surface of metal oxide nanoparticles can be modified to target cancer cells specifically by adding a specific targeting ligand to the surface of the metal nanoparticle; for imaging, fluorescent dye or optical imaging agents can be attached.

Among metal oxide nanoparticles, zinc oxide nanoparticles (ZnO NPs) have received much research attention: they are considered to be relatively biocompatible and test ‘*in vitro*’ presented their inherent preferential cytotoxicity against cancer cell [9].

In order to perform the transition from preclinical studies to ‘*in vivo*’ applications, it is important to understand the mechanisms of stability and toxicity in biological environment.

The basic scheme of this thesis can be summarized as follows: the first part focuses on the synthesis of zinc oxide nanoparticles, their functionalization with aminopropyl groups and their characterization from a morphological and chemical-physical point of view. Secondly, to test the stability of zinc oxide, biodegradation tests were performed in PBS (Phosphate-buffered saline), DPBS (Dulbecco’s phosphate-buffered saline) and ALF (Artificial lysosomal fluid). The analysis of the results obtained allowed to gain information on the aggregation and dissolution state of the ZnO NPs and on their interaction with the fluid components. The possibility of coupling ZnO NPs with commercial liposomes was evaluated to improve stability and biocompatibility. Finally, the uptake and intracellular behaviour of both bare and coated ZnO NPs by HeLa Cells were considered.

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2. Background theory

2.1 Smart drug delivery

The term drug delivery refers to systems and technologies, that transport a pharmaceutical compound in the body in order to achieve its desired therapeutic effect. In conventional drug delivery methods, the drug is distributed throughout the body through the systemic blood circulation. For most therapeutic agents, only a small portion of the drug reaches the organ to be affected, which implies non-specific bio-distribution, uncontrolled drug release, higher doses and less efficacy of the therapy [1]. To overcome these limitations, smart drug delivery systems have been developed in order to enable drug accumulation only in the target cells, tissues and organs of interest. In this way it is possible to increase the therapeutic index (defined as the ratio between the maximum tolerated dose and the lowest effective dose). Furthermore, it enables less fluctuations in circulating drug levels, more uniform effect of the drug, less frequency of the dosages taken by the patient and higher safety of the drug [2]. These systems are on a nanometric scale, ideally between 10 and 200 nm, at the same order of magnitude as the biomolecules with which they must interact, so that it is possible to reduce the non-specific toxicity and increase the effectiveness of the therapy; on the other hand, the disadvantage of these systems are high cost, potential accumulation into non-target vital organs, like liver, spleen, kidney, and potential immunogenicity, which makes their clinical use still an unsolved issue. Despite significant advances in the diagnosis and treatment, cancer remains the second most common cause of mortality worldwide after cardiovascular diseases, owing to the limited efficacy of chemotherapeutic agents [3]. Numerous studies are focusing on the possibility of carrying out anticancer therapies based on the use of nanoparticles made of a great variety of materials (polymers, dendrimers, liposomes, viruses, carbon nanotubes and metallic particles), which possess intrinsic anti-tumor properties or which can be used as carriers for traditional medicines [4]. In order to be effective in cancer treatment, nanoparticles should first reach the desired tumour tissues after administration through the penetration of barriers in the body with minimal loss of activity in the blood and minimal aggregation or clearance by Reticuloendothelial System (RES). Second, after reaching the tumor tissue, drugs should have the ability to selectively kill tumor cell without affecting normal cells with a controlled release mechanism of their active form from the nanoparticles. In principle, nanoparticle can reach the tumor tissues by either passive or active targeting [5]. Passive targeting refers to the accumulation of a drug or a drug carrier (nanoparticle) at a desired site owing to physico-chemical or pharmacological factors. It takes advantage of the inherent size of nanoparticles and the unique properties of tumor vasculature, such as the EPR effect (enhanced permeability and retention) and the tumor microenvironment. This approach can effectively enhance drug bioavailability and efficacy: it makes use of the anatomical and functional differences between normal and tumor vasculature to deliver the drug to a targeted site. The EPR effect is used to selectively deliver drug-loaded nanoparticles of size far below the micron (the ideal dimension should be below 200 nm). To enhance this effect, long circulation times of the nanoparticles should be provided, as well as the maintenance of nano-sized dimension in the blood system. In general, however, the EPR is a non-specific targeting mechanism, and the nanoparticles during blood circulation can also accumulate in other

healthy tissues, typically spleen, kidney, liver. Another contributor to passive targeting is the unique microenvironment surrounding tumor cells, which is different to that of normal cells. Fast-growing cancer cells have a high metabolic rate, and the supply of oxygen and nutrients is usually not sufficient for them to maintain this. Therefore, tumor cells use glycolysis to obtain extra energy, resulting in an acidic environment. To take chance of this microenvironment, for example pH-sensitive liposomes were designed to be stable at a physiologic pH of 7.4 but degraded to release active drug in target tissues in which the pH is less than physiologic values, such as in the acidic environment of tumour cells [6].

Active targeting enhances the accumulation of nanoparticles in the tumor sites and it involves the attachment of a specific targeting ligand able to recognize a specific receptor, in order thus to deliver a drug to pathological sites or to cross biological barriers based on molecular recognition processes. Firstly, the antigen or receptors should be expressed exclusively on tumor cells and not expressed on normal cells. Secondly, they should be expressed homogeneously on all targeted tumor cells. Finally, cell-surface antigens and receptors should not be shed into the blood circulation [7]. Due to the intrinsic mutagenesis of tumor tissues, the variability from patient to patient, the difficulties to identify specific antigens in several tumor diseases, it is clear the difficulties of developing a highly specific targeting ligand. Both passive and active targeted nanoparticles are taken up by tumor cells mainly through the endocytosis process (Fig.2.1). However, receptor-mediated endocytosis, as in the active targeting, is thermodynamically and kinetically favoured and lead to faster internalization process than the passive targeting mechanism [8]. There are multiple pathways for cellular entry of nanoparticles and in all cases the initial stage of endocytosis proceeds from the plasma membrane portals of cellular entry and involves engulfment of cargo into intracellular vesicles. For example, when a targeted conjugate binds with a receptor on the cell surface, the plasma membrane envelopes the complex of the receptor and ligand to form an endosome. Newly formed endosomes are transferred to target organelles. As the pH value in the interior of the endosome becomes acidic and lysozymes are activated, the drug is released from the conjugate and enters the cytoplasm, if the drug has the proper physico-chemical properties to cross the endosomal membrane. Nanoparticles made with this second strategy (active targeting) show very promising results, but they are still under preclinical stage or require further testing and optimization.

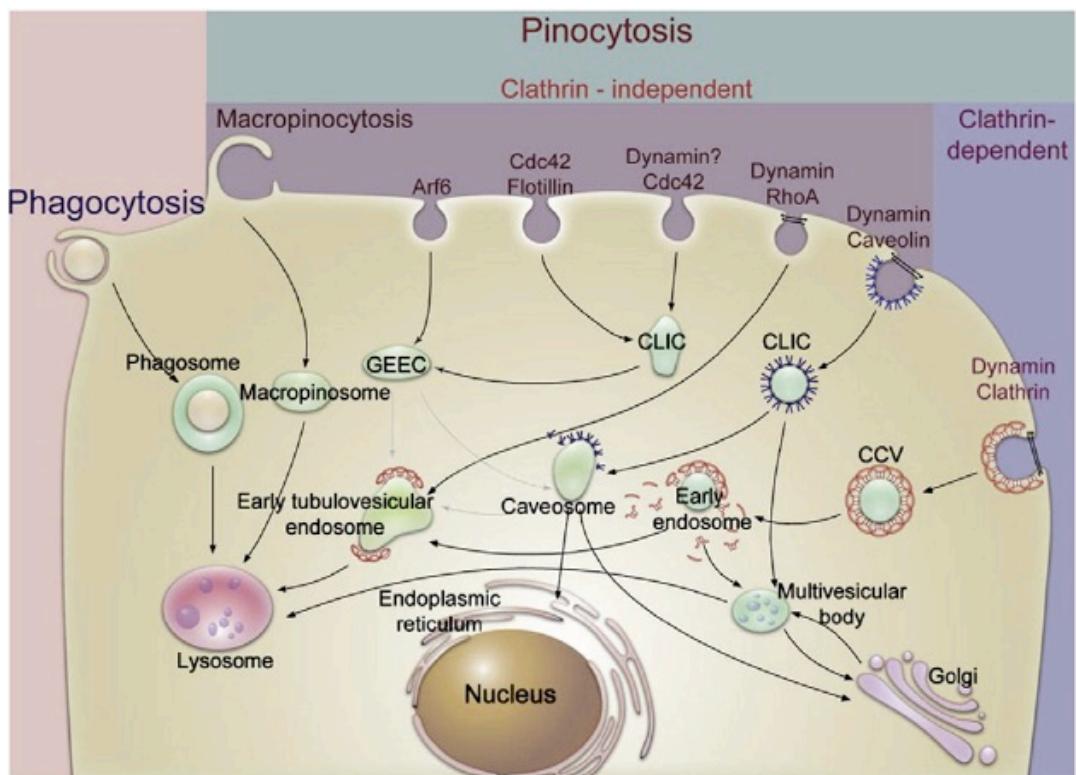


Fig. 2.1 Different mechanisms of endocytosis process (Modified from [5])

2.2 Nanoparticles for anticancer therapy

2.2.1 Nanocarriers

The term “nanoparticle” is defined as a nano-object with all three dimensions in the size range from approximately 1 nm to 100 nm [9]. The IUPAC also defines nanoparticles as a particle of any shape with dimensions in the 1-100 nm range [10] (Fig. 2.2). These nanomaterials have novel properties which bulk materials typically do not have and can be developed for biomedical applications: for example the high surface area and superficial reactivity allow to maximize the load capacity of the therapeutic agent and functionalization of the surface plays an important role.

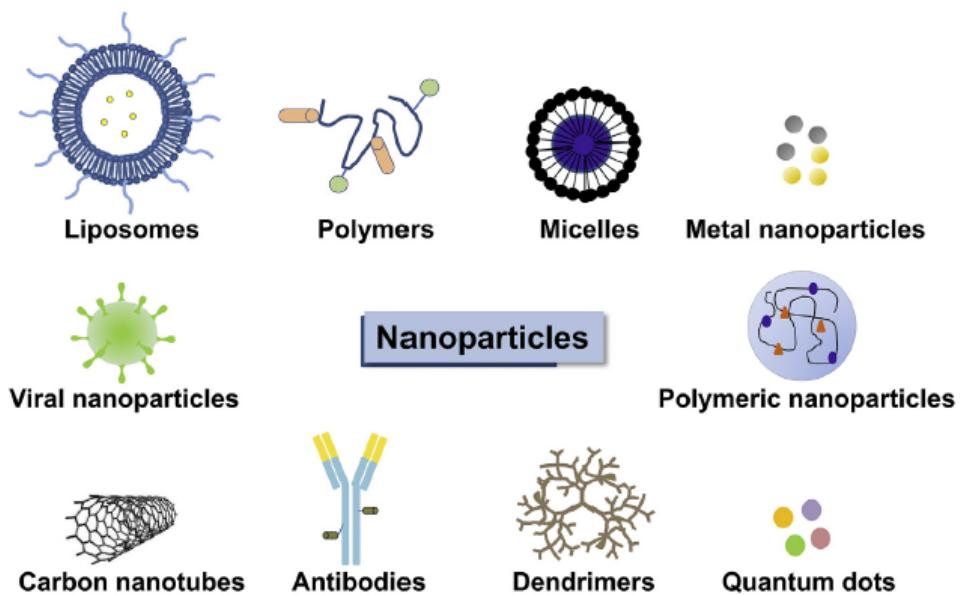


Fig. 2.2 Nanoparticles and antibodies used in antitumor therapy (Modified from [11])

These drug delivery nanosystems are essentially composed of two components: the therapeutic agent and the carrier, designed to resist the biological environment and protect the drug until the desired site is reached. However, there are several cases in which the therapeutic agent coincides with the nanocarrier, i.e. when nanoparticles can be activated to produce a therapeutic effect in the cancer cells. Some examples refer to metal, metal oxides and even magnetic nanoparticles to be activated under an external stimulus (light, magnetic field, ultrasound...) and develop high temperatures localized in the tumor tissue (hyperthermia) or toxic species (reactive oxygen species, ROS, or cytotoxic ions).

The polymeric nanoparticles are attractive drug carriers because of their great efficacy and reduced cytotoxicity on peripheral healthy tissues. The specific size and shape for tissue penetration and biodegradability allow them to be used notably for active and passive targeting. Some polymeric nanoparticles are prone to facile chemical modifications, which could enhance the binding specificity for active targeting purposes [12]. Polymeric micelles with a micellar core are also promising carriers to deliver chemotherapies to tumor cells due

to their various molecular interactions: it can help to the efficient incorporation of different therapeutic molecules as hydrophobic and they have an excellent stability [13]. Liposomes seem to be first carriers in drug delivery systems: examples of liposome-mediated drug delivery are doxorubicin (Doxil) and daunorubicin (Daunoxome), which are currently being marketed as liposome delivery systems [14]. They are composed of natural phospholipids that are biologically inert and feebly immunogenic, and they have low inherent toxicity. They can also modulate immune response and facilitate cell internalization, modifying the carrier surface [15]. They are extensively used as carriers for numerous molecules in cosmetic and pharmaceutical industries. Easy of synthesis, low batch-to-batch variability, biocompatibility, biodegradability, low toxicity, aptitude to trap both hydrophilic and lipophilic drugs and steric stabilization lead to the superiority of liposomes compared to other nanoparticle carriers [16]. However, they suffer for low stability versus time in the circulation system, thus the encapsulated drug can be lost and released unspecifically in the blood stream. Carbon nanotubes are another ideal carrier for target drug delivery systems: they are carbon cylinders composed of benzene rings with unique physicochemical properties as large surface areas that can be used as attractive carriers to deliver drug [17].

2.2.2 Inorganic Nanoparticles

Inorganic nanoparticles exhibit physical and chemical properties that depend on their size, shape and composition [18]. They have large surface-to-volume ratios and their behavior is strongly affected by their surface functionalization.

Recently researchers have been interested in the possibility of developing nanoparticles with their own antitumor characteristics (not loaded with the therapeutic and anticancer agent) or mediated by external stimuli: quantum dots and nanoparticles made of noble metals or metal oxides are part of this category. In recent years, semiconductor quantum dots have attracted the attention of many research groups because of their scientific and technological significance in microelectronics, optoelectronics and cellular imaging. They are tiny light-emitting particles on the nanometer scale and they are emerging as a new class of fluorescent probe for *in vivo* biomolecular and cellular imaging [19]: recent advances have led to the development of multifunctional quantum dots that are very bright and stable under complex *in vivo* conditions; which means they a high degree of selectivity and sensitivity for diagnostic purposes. Due to their magnetic properties, the use of iron oxide nanoparticles has received considerable interest: they serve as imaging probes for localizing and diagnosing cancerous lesions and at the same time as drug delivery vehicles that deliver therapeutic agents preferentially to those lesions [20]. In the presence of an external alternated magnetic field they become magnetized and their therapeutic properties are attributed to their energy absorption, which produces hyperthermia. Hyperthermia refers to an increase in the temperature of the microenvironment of a tissue up to 40-45°C, resulting in a series of subcellular events that cause apoptosis leading to cell death. When particles are exposed to an alternating external magnetic field, the magnetic dipole movements of nanosized particles reorient quickly and the power loss of nanoparticles in this process releases a considerable amount of heat, which kills the tumor cells.

Gold nanoparticles have unique optical properties that make them useful for cancer diagnosis and photothermal therapy (PTT). Photothermal therapy has attracted a great deal of attention in recent years, because this method is characterized by minimal invasiveness in cancer therapy [21]: the gold nanoparticles with maximum absorption in the visible or near IR region, receive light and generate heat, which causes the death of malignant tumors.

Also silver nanoparticles absorb and scatter a portion of light: after their selective uptake into cancer cells, the absorbed light can be used for thermal killing, while scattered light can be used for imaging. As far as the therapeutic activity of silver nanoparticles is concerned, they induce ROS accumulation, which initiates the inflammatory response and perturbation, resulting in programmed cell death [22].

These inorganic nanoparticles are still on the preclinical stage, even if they show interesting potentialities: for researchers it is important to understand in detail their biocompatibility, cytotoxicity and stability in biological environment.

2.3 Zinc oxide Nanoparticles

Zinc oxide (ZnO) is a semiconductor material with promising catalytic, electronic and optical properties: it possesses great potential because of its high electron mobility, high thermal conductivity, wide bandgap (~ 3.3 eV) and exhibits UV absorption in the range 200-350 nm and emission in the near UV [23]. Under ambient conditions the zinc oxide is thermodynamically stable in the form of wurtzite (Fig. 2.3), a hexagonal close-packed structure, where each zinc atom is surrounded by a tetrahedron of four oxygen atoms and vice versa; the resulting structure is composed of a combination of planes formed by oxygen and zinc atoms.

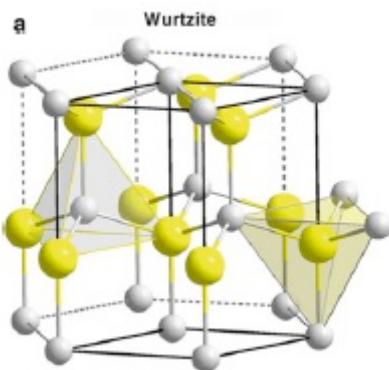


Fig. 2.3 Wurtzite crystal structure (Modified from [23])

Over the past few decades, metal oxide nanoparticles have been explored as standalone biomedical agents as well as novel carriers for the delivery of therapeutic agents for a variety of disorders. Among the metal oxide nanoparticle family, zinc oxide nanoparticles (ZnO NPs)

have received several attention: for example they have excellent ultraviolet absorbing properties and transparency for visible light, making these nanoparticles excellent sunscreen agents [24]. Moreover, they are also excellent drug carrier systems: they do not interact with the majority of pharmaceutically active molecules available and zinc oxide nanoparticles larger than 100 nm are considered to be relatively biocompatible, less toxic compared with other metal oxide nanoparticles, relatively inexpensive, which supports their use for drug delivery [25]. Therefore they are characterized by various biomedical applications not only for drug delivery applications, such as anticancer, but also for antidiabetic, antibacterial, antifungal and anti-inflammatory activities [26] (Fig. 2.4).

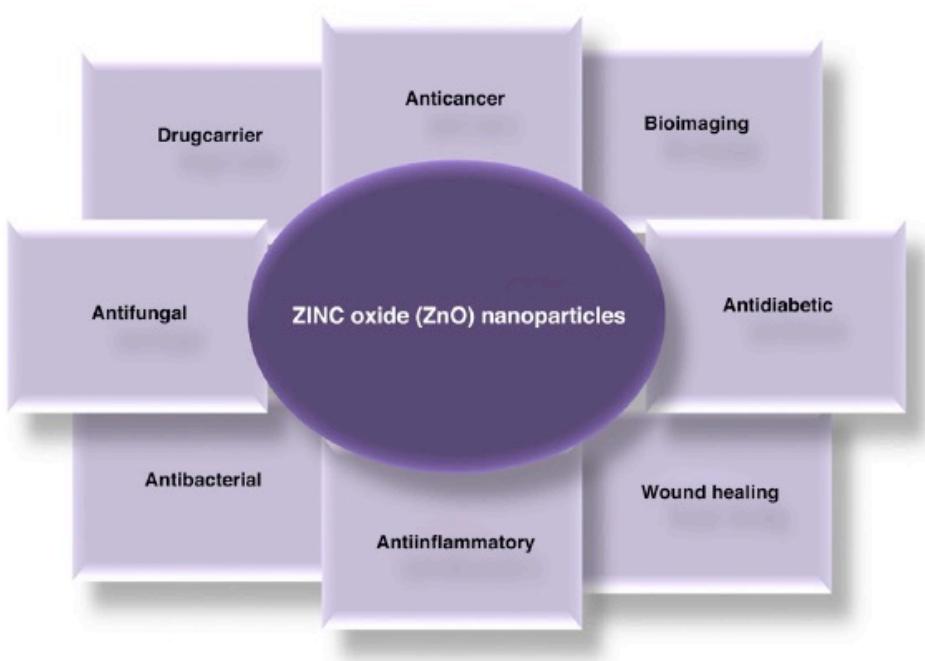


Fig. 2.4 Biomedical applications of zinc oxide (ZnO) nanoparticles (NPs) (Modified from [26])

Among several biomedical applications, the use of ZnO NPs in anticancer therapy has received several attention: ZnO NPs demonstrate their anticancer activity by inducing ROS generation and also by inducing apoptosis. Moreover, surface modifications of ZnO NPs have been undertaken to further improve their stability and to increase their selectivity of specific cells. Where the wide band-gap semiconductor properties of ZnO are useful for killing cells by producing ROS, their inherent photoluminescence properties are applicable for biosensing applications, imaging in vitro and in vivo, providing comprehensive information for tumor diagnosis [27]. The antidiabetic activity of ZnO NPs has been explored based on the fact that zinc has important role in insulin synthesis, storage and secretion: in a recent study, the antidiabetic activity of ZnO NPs resulted in improved insulin levels in diabetic rats [28]. The antibacterial activity of ZnO NPs lies in their ability to induce oxidative stress: when bacterial cells come into contact with ZnO NPs, they absorb Zn⁺, which then inhibits the action of respiratory enzymes, generates ROS and produces free radicals, causing oxidative stress. ROS

irreversibly damage bacterial membranes, DNA and mitochondria, resulting in the death of bacterial cells [29]. Due to their strong antimicrobial properties, ZnO NPs have also been successfully used in wound dressings. In addition to their antibacterial activity, ZnO NPs are also effective antifungal agents, so that they could be used in food safety and the agricultural industries [30].

2.3.1 Synthesis

The development of stable and uniform nanosized particles and their surface chemistry affects the interaction with the immune system, the accumulation of nanoparticles inside the tumor tissues and in general the efficacy of the therapy. The control of the nanoparticle size and surface is the primary focus of each of the several methods, which are reported for the preparation of ZnO NPs. Among these methods the most interesting ones resides in the wet chemical synthesis, such as: the precipitation method and the sol-gel method. The precipitation method involves a reaction between a zinc precursor and a precipitating reagent: a solution of precipitating agent (sodium hydroxide, urea, etc.) is added dropwise to the aqueous solution of a zinc precursor (zinc nitrate, zinc sulfate, etc.). Mixing these solutions, an intermediate product is formed (typically hydroxide species) and after calcination at high temperature it is converted to ZnO [31]. The wet chemical synthesis of ZnO NPs is a modification of the precipitation method except from the fact that an additive can be used to stabilize the NPs formed [26]. The sol-gel methods, already used to synthesize metal oxides at macroscopic scale, have been adapted for the production of NPs: low costs, low temperatures, safety of the reagents used, control on the morphology and the dimensions of the final product are the main advantages of this synthesis. The precursors used for the synthesis of zinc oxide nanomaterials include inorganic salts and organic zinc compounds (zinc nitrate and zinc acetate) which are able to hydrolyse and form intermediates ($Zn(OH)_4^{2-}$) which lead to the formation of crystalline zinc oxide [23]. From this general mechanism, different structures can be generated: nano and microparticles, multipods, nanowires and nanorods, modulating the synthesis parameters. Not only the size, but also the geometry and the shape have influence on the drug delivery [32]: the transport within the organism, the interaction with the immune system and the accumulation in the tissues and cells are some examples.

2.3.2 Biocompatibility

After iron, zinc is the second most abundant metal in the human body and it is involved in many biological processes, even if it is present only in traces (~ 2 g throughout the whole body). The US Food and Drug Administration (FDA) has accepted bulk ZnO as a “generally recognized as safe” (GRAS) substance, relatively biocompatible, biodegradable and the ions produced after biodegradation can become nutrients for the organism [33]. Moreover, zinc is a cofactor necessary for the functioning of more than 300 enzymes: it is involved in metabolic processes in the functioning of the immune system and contributes to the maintenance of the structural integrity of cells and organs [34]. Controlled zinc-ionic values in the cellular

environment are essential for good cellular functioning, while too high zinc values lead to malfunctions in the cell cycle, which can cause cell death for apoptosis and at higher concentrations for necrosis [34].

2.3.3 Selectivity & Cytotoxicity

The possibility of using ZnO NPs as antitumoral agents is based precisely on their demonstrated selectivity against cancer cells, without having any cytotoxic impact on normal cells [35]. The electrostatic properties make ZnO NPs interesting for anticancer activity: they exhibit a different type of surface charge behaviour, due to the hydroxyl groups chemisorbed on the surface. Protons (H^+) move out from the particle surface in aqueous medium at high pH, leaving a negatively charged surface with partially bonded oxygen atoms (ZnO^-). At lower pH, protons from the environment are transferred to the particle surface, resulting in a positively charged surface ($ZnOH_2^+$). The isoelectric point of ZnO NPs is 9-10; for this reason under physiological conditions these NPs carry a strong positive surface charge. By contrast, cancer cells comprise a high concentration of anionic phospholipids on their outer membranes and have large negative membrane potentials [36]. Thus, the interaction of cancer cells with positively charged ZnO NPs is driven by electrostatic interactions, promoting cellular uptake, phagocytosis and cytotoxicity of these NPs (Fig. 2.5).

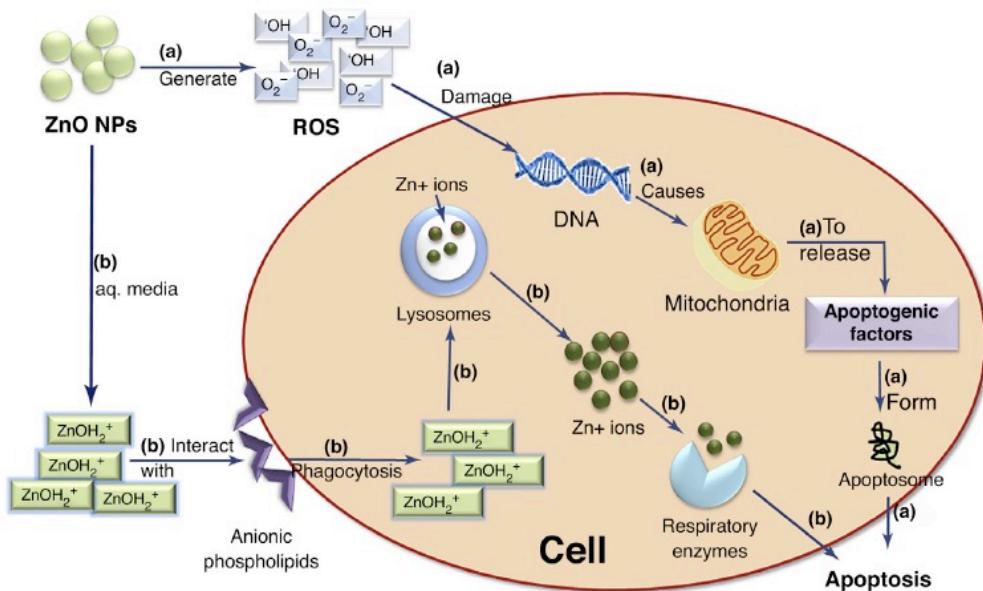


Fig. 2.5 Mechanisms of cytotoxic action of zinc oxide nanoparticles (ZnO NPs) (Modified from [26])

Regarding the cytotoxic effect of zinc oxide, it is connected with the production of ROS, highly reactive chemical species containing oxygen (free radicals superoxide, hydroxyl and hydrogen peroxide), which cause oxidative stress to the cell and in particular to the DNA, resulting in a cell cycle arrest and apoptosis. The semiconductor properties of ZnO affect their ability to generate ROS: the existence of a gap between the valence and the conduction band implies the fact that electrons endowed with an appropriate energy create highly reactive electron-hole pairs able to react with the oxygen and water present in the cell environment, generating ROS. The band gap measures approximately 3.3 eV for crystalline ZnO: the promotion of the electron can be obtained by irradiation with UV [26] or by ultrasonic application [37]. Furthermore, the crystal defects of nanosized materials make the formation of electron-hole pairs possible even without the presence of external stimuli [26].

Other research shows that ROS production is a cytotoxic response, linked to the presence of Zn^{2+} ions, generated by the dissolution of NPs in contact with the cellular environment: the acid pH exposure of endosomes and lysosomes causes a rapid dissolution of the nanoparticles, which causes an inflammatory response and damage to the mitochondria [38].

2.3.4 Biostability

Studies in buffer solutions highlight the strong tendency of ZnO NPs to aggregate, with a dependence on the ionic strength of the solution [36]. The state of aggregation also results in influencing the ability of the material to bind with proteins, which could strongly influence the in vivo behaviour of ZnO NPs. From these preliminary experiments different aspects are clear: for example the necessity to stabilize ZnO NPs to protect them from biodegradation and the increase in biocompatibility [39].

In this thesis project the possibility of creating a hybrid nanoparticle is considered, encapsulating ZnO NPs in liposomes; in addition, on the lipid membrane, suitable ligands can be added subsequently, by implementing active targeting methods.

The characteristics of ZnO and liposomes make them interesting candidates for the development of an effective and innovative system for anticancer therapy.

2.4 Hela Cells

HeLa is a cancer cell type in a cell line commonly used in scientific research.

This cell line was derived from cervical cancer cells taken from Henrietta Lacks, a patient who died of cancer on October 4, 1951. It was found to be remarkably durable and prolific and for this reason it is estimated 50 million tons of HeLa cells have been used in research since they were first taken [40]. The importance of this research is based on the fact that uterine cervical cancer is the second most common malignant tumor in women worldwide and presents a high mortality rate, especially in developing countries: cervical cancer accounts for 4% of the cases of cancer diagnosed in the world and about 84% of cervical cancer cases occur in Africa, Latin America and the Caribbean [41]. The cells from Lacks' cervical cancer were taken without her knowledge or consent. Cell biologist George Otto Gey developed a cell line, isolating one specific cell and multiplying it, so that they could be kept alive. This represented a considerable progress because before cells cultured from other human cells would only survive for a few days and the scientists spent more time trying to keep the cells alive than performing actual research on them. These were the first human cells grown in adhesion: they do not dye after a set number of cell divisions due to cellular senescence (Fig. 2.6).

In practice, these cells could be used for carrying out a multitude of biomedical experiments: in case the cells died, they could simply be discarded and the experiment performed again on fresh cells from the culture. This represented a 'new era' for medical and biological research.

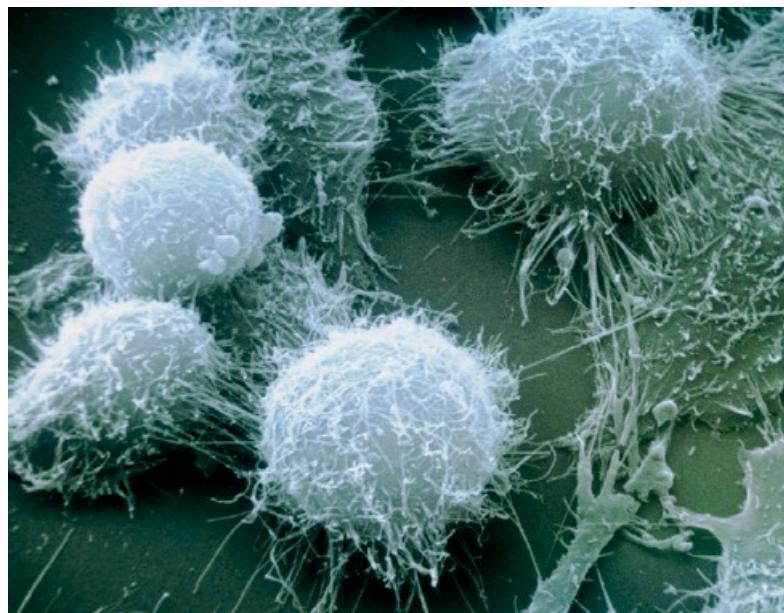


Fig. 2.6 Scanning electron micrograph of just-divided HeLa cells (Modified from [40])

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3. Materials and methods

3.1 Synthesis and functionalization of Zinc Oxide nanoparticles

3.1.1 Sol-Gel Synthesis

Zinc acetate dihydrate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, 99.99%, Sigma Aldrich) and sodium hydroxide (NaOH, 98%, Sigma Aldrich) were used as precursors to obtain spherical nanoparticles of zinc oxide with dimensions around 10-20 nm.

Two different solutions were prepared: the first one with 818.2 mg (3.73 mmol) of zinc acetate dihydrate in 42 ml methanol (MeOH, Sigma Aldrich) and the second one with 288.8 mg (7.22 mmol) of sodium hydroxide in 23 ml methanol [1]. The first solution was stirred continuously in a glass round flask, inserted in an oil bath heated at 60 °C. After the temperature reached the set point, 318 µl bidistilled water was added to the zinc acetate solution and then the sodium hydroxide solution was dropped for about 15 min inside the first solution (Fig 3.1). The reaction took place in 2 h; another synthesis was performed in 5 h and the results in terms of nanoparticles morphology were not different, however the yield was higher. It was however decided to keep 2 h as the final synthesis in order to avoid long processing times.

At the end of the reaction, an opalescent solution was obtained, containing the zinc oxide nanoparticles.

To remove reaction residues, centrifugation was performed (3046 rcf for 5 min) followed by washing in ethanol (EtOH, Sigma Aldrich). The procedure was repeated twice and then the zinc oxide nanoparticles were dispersed in fresh ethanol. The final concentration was measured by weighing an eppendorf (tare) and then adding into it 500 µl of ethanol, in which there were the ZnO nanoparticles. The ethanol was evaporated overnight in an oven at 60°C and the day after the eppendorf was weighed again (gross weight). It was possible to calculate the concentration through this calculation:

$$\text{Concentration } \left(\frac{\text{mg}}{\text{ml}} \right) = \frac{\text{gross weight} - \text{tare}}{\text{volume}} \quad (3.1)$$

The zinc oxide nanoparticles obtained through sol-gel synthesis were used especially for the biodegradation tests (Paragraph 3.3).



Fig. 3.1 Experimental apparatus for the sol-gel synthesis

3.1.2 Microwave-assisted sol-gel synthesis

Zinc acetate and potassium hydroxide (Sigma Aldrich) were used as precursors in 1:2 proportions.

The first solution was prepared in a beaker, where potassium hydroxide was dissolved in methanol, also with the help of ultrasonic bath. The second solution was put directly in the reactor vessel: zinc acetate and methanol were mixed through stirring and to help nucleation 318 µl of double distilled water was added. When potassium hydroxide was completely dissolved in methanol, the first and the second solution were mixed together in the reactor vessel.

The final mixture was put into microwave oven under control of temperature (60°C) and pressure (P_{\max} 10 bar) for 30 min. Then the product was centrifuged for 10 min at 3500 rcf. The precipitate was dispersed in 15 ml of ethanol and centrifuged for 10 min at 3500 rcf (this step was performed twice). Finally, zinc oxide nanoparticles were stable dispersed in fresh ethanol.

This synthesis procedure presents several advantages: short time of reaction and simultaneous nucleation [2,3].

The zinc oxide obtained via microwave synthesis were used in particular for cell uptake tests (Paragraph 3.5).

3.1.3 Functionalization

Functionalization was obtained combining ZnO NPs with 3-amminopropyltrimethoxysilane (APTMS, 97% Sigma Aldrich) in order to cover the nanoparticles' surface with amino groups (-NH₂), that can be then further linked to dyes, essential for fluorescence microscopy investigation [1,4]. Furthermore the amine group are able to protonate in slight acidic media,

which confers to the nanoparticles a net positive charge, highly desirable for the further internalization into cancer cells.

APTMS is an alkylsilane, which is able to react and bind to $-\text{OH}$ groups present on the surface of ZnO NPs (figure 3.2) and obtain the amino-propyl functionalized sample.

The functionalization reaction was carried out in ethanol: in particular to functionalize 100 mg of ZnO NPs (1.23 mmol), it was used a 100 ml round glass flask under continuous stirring and a nitrogen gas flow to prevent contamination of humid air (and thus to prevent the APTMS from polymerizing on itself); the temperature was 78 °C and 21.4 μl of functionalizing agent APTMS was added to the solution, equal to 10 mol% of total ZnO amount (0.123 mmol, 22.05 g).

The obtained mixture was maintained for 6 hours under reflux conditions in a nitrogen atmosphere . In order to remove the unbound molecules, two washes were carried out with ethanol alternated by centrifugation steps at 3046 rcf for 10 min and subsequent redispersion of ZnO-NH₂ NPs in fresh solvent.

The final concentration was set to 1 mg/ml.

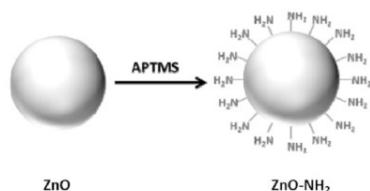


Fig. 3.2 Functionalization with amino groups of zinc oxide nanoparticles' surface (modified from [5])

3.2 ZnO NPs Characterization

Many analyses were carried out in order to verify the synthesis' reproducibility: data from different batches obtained from sol-gel synthesis and via microwave synthesis were compared and characterized.

Firstly, the crystal structure and morphology (shape and dimensions) were studied with X-Ray Diffraction (XRD) and Field-Emission Scanning Electron Microscopy (FESEM), respectively. Secondly, in order to have more information about the colloidal stability of the system and the aggregation status of the nanoparticles in suspension, Dynamic Light Scattering (DLS) was performed together with Z-Potential measurements, to get insight on the nanoparticle's functionalization and thus Z-Potential, linked to the surface charge.

3.2.1 X-Ray Diffraction –XRD

X-Ray Diffraction is essential to obtain accurate information regarding the crystallographic phase of solid samples. Within this technique the sample is irradiated with a beam of monochromatic x-rays, varying the incident angle range; diffracted x-rays are put in correlation with the interaction between atoms in the sample, when the Bragg equation is

satisfied. This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a crystalline sample according to the following Bragg Equation:

$$n\lambda = 2dsin\theta \quad (3.2)$$

where n is a positive integer, λ wavelength of incident wave, d lattice spacing, θ diffraction angles.

A Panalytical X' Pert diffractometer in θ -2 θ Bragg-Brentano configuration was exploited in order to perform X-Ray diffraction (Fig. 3.3). This instrument uses a source of Cu-K α radiation ($\lambda=1.54$ Å, 40 kV and 30 mA). By depositing drop to drop the sample on a flat silicon wafer, it was possible to have a sufficiently thick layer of nanoparticles, which was analyzed with step size of 0.02° (2 θ), 2 θ range of 20°-70° and acquisition time of 100 s. The crystallographic indices corresponding to well-defined peaks were found comparing the obtained spectra with those of zinc oxide nanoparticles present in literature and databases. Estimation of the particle size of each sample from the peak width at half height was obtained, exploiting the Debye-Scherrer Equation:

$$D = \frac{K\lambda}{\beta cos\theta} \quad (3.3)$$

where θ is the Bragg's angle, β the width at half height of peak (FWHM), λ wavelength of incident radiation, D dimension of crystallite and K is a factor that depends on particles' shape (K=0.9 for spherical particles).

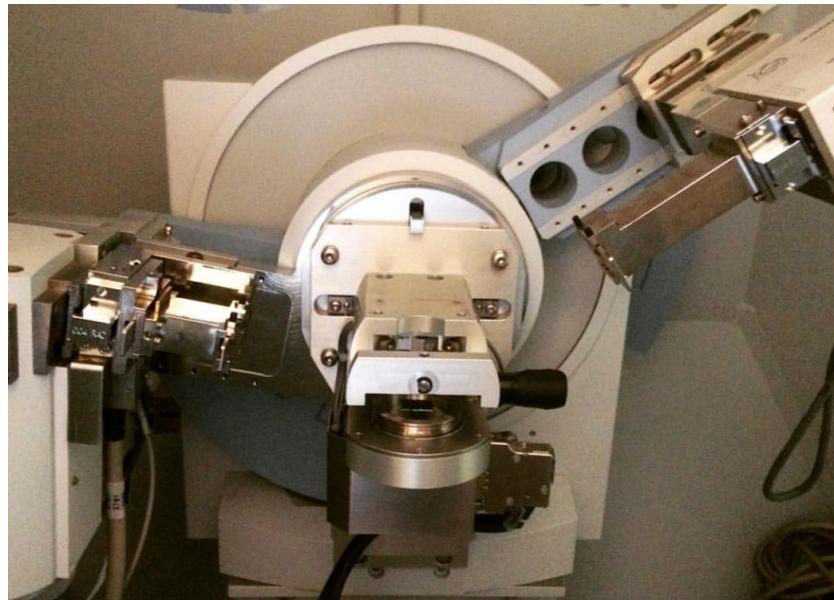


Fig. 3.3 XRD Experimental Apparatus

3.2.2 Field-Emission Scanning Electron Microscopy –FESEM

Field-Emission Scanning Electron Microscopy (FESEM) is a powerful and useful technique, that gives information about the morphology and elemental composition of a material's surface, with magnification ranging from 10x to 300000x, and with a virtually unlimited depth-of-field. Comparing the conventional scanning electron microscopy, FESEM is able to produce clearer and less electrostatically-distorted images with a better resolution. A field emission source frees the electrons, which are collected by a detector and an electronic signal is produced. This signal is amplified and converted into an image which can be seen on a monitor and saved.

The samples for FESEM (Carl Zeiss) were prepared by diluting in ethanol (1: 100) an aliquot of the nanoparticle solution and depositing 10 µl of this dilution on silicon wafers.

3.3.3 Dynamic Light Scattering-DLS

The DLS technique exploits the random thermal motion known as Brownian motion, typical for submicron particles. This random motion is governed by the Stokes-Einstein equation:

$$d_H = \frac{KT}{3\pi\eta D} \quad (3.4)$$

where d_H is hydrodynamic diameter, k is Boltzmann constant, T temperature, η viscosity and D diffusivity. This equation can be applied to spherical and non-interacting nanoparticles, which present Brownian motion in a quiescent fluid (laminar regime) and at a constant temperature.

In detail, the sample is irradiated by a laser and at very small time intervals the light scattered by the nanoparticles is detected by a fast photon detector at a known scattering angle θ ; that allows to calculate the diffusion coefficient of the nanoparticles (D), analyzing the variation of the intensity of this radiation spread over time and through a correlation function (Fig. 3.4). At this point, solving the Stokes-Einstein equation, it is possible to obtain the average hydrodynamic diameter (d_H), corresponding to the average size of the system formed by the particle and the layer of liquid which moves with it.

The measurements were performed with a Zetasizer Nano ZS90 from Malvern (equipped with a laser He-Ne of 633 nm) (Fig.3.5): the Software (Zetasizer Software) is able to provide a dimensional distribution of the analyzed sample, inserting the properties of the solvent (index of refraction and viscosity) and of the dispersed material (index of refraction and absorption coefficient).

It was possible to evaluate the aggregation status of the suspension and the interaction between the nanoparticles and the solvents in which they were tested.

In order to have good measurements quality, pristine nanoparticles (ZnO NPs), functionalized nanoparticles (ZnO-NH₂ NPs) and nanoparticles coated with liposomes (ZnO-DOPC NPs) were dispersed in bidistilled water and in different solvents at concentration of 500 µg/ml; this concentration value allows obtaining acceptable values of DCR (Derived Count Rate) and PDI (Polidispersity Index).

Each sample was analyzed three times and the results obtained were averaged.

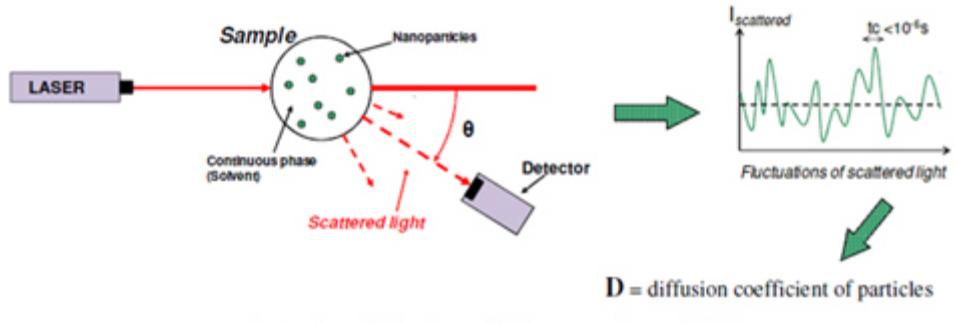


Fig. 3.4 Illustration of the dynamic light scattering principle [6]

Using the same instrument, zeta potential measurements were carried out: the value of this parameter is used to predict the stability of the colloidal system, linked to the surface charge of the nanoparticles and therefore to the repulsive electrostatic forces that are established between the nanoparticles in solution.

In this case, the technique used is the Laser Doppler Electrophoresis, which involves the application of an electric field between the electrodes of the measuring cell and illuminating the sample with a laser beam.

The displacement of the charged particles towards the electrode of opposite sign creates a variation of the frequency of the light diffused by the sample directly proportional to the electrophoretic mobility (U_E).

Solving the Henry equation, it is possible to derive the value of the zeta potential (ζ):

$$U_E = \frac{2\pi\zeta F(\kappa\alpha)}{3\eta} \quad (3.4)$$

where ϵ and η are respectively the dielectric constant and the viscosity of the medium and the value

$F(\kappa\alpha)$ is assumed equal to 1.5, according to the Smoluchowski approximation, valid in the case of polar solvents.

Zeta potential measurements were carried out on nanoparticles dispersed in bidistilled water: ZnO NPs, ZnO-NH₂ and ZnO-DOPC were considered. Also, titration measurements (zeta potential vs pH) were performed in order to investigate the stability of the system and to evaluate the isoelectric point (IEP).



Fig. 3.5 DLS Experimental Apparatus with the automatic titration set-up [7]

3.3 Biodegradation tests

Biodegradation tests were performed in order to study the stability and reactivity of zinc oxide nanoparticles in the following fluids:

- PBS (Phosphate-buffered saline, Sigma Aldrich), a water-based salt solution, commonly used in biological research to wash the cells (pH 7.4);
- DPBS (Dulbecco's phosphate-buffered saline, Sigma Aldrich), which has a lower phosphate concentration than standard PBS (pH 7-7.3);
- ALF (artificial lysosomal fluid), that simulates intercellular conditions in cells occurring in conjunction with phagocytosis, therefore representing relatively harsh condition (pH 4.5);

All chemicals to produce this solution were purchased by Sigma Aldrich.

The tests were carried out both on pristine zinc oxide nanoparticles (ZnO NPs) and on nanoparticles coated with liposomes (ZnO-DOPC NPs).

In total six experiments were conducted in parallel and repeated twice: these tests were useful to study the influence of surface characteristics on biodegradation.

3.3.1 Composition of solutions

In order to prepare PBS, one tablet was dissolved in 200 mL of deionized water: this yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25 °C [8].

At the end the final volume was 1 L and in the following table the concentration are reported in detail (Table 3.1):

Table 3.1 PBS composition (modified from [9])

Component	g/l
Inorganic salts	
KCl	0.2
KH ₂ PO ₄	0.24
NaCl	8.0
Na ₂ HPO ₄ (anhydrous)	1.44

DPBS was purchased by Sigma Aldrich already ready for use with the following composition (Table 3.2):

Table 3.2 DPBS composition (modified from [10])

Component	g/l
Inorganic salts	
KCl	0.2
KH ₂ PO ₄	0.2
NaCl	8.0
Na ₂ HPO ₄ (anhydrous)	1.15

Regarding the preparation of ALF, the reagents were added in the same order presented in the table (Table 3.3) in 500 ml of bidistilled water in a plastic beaker under continuous stirring (400 rpm) and the temperature was set at 37 °C.

After all the reagents were added and the solution cooled to 25 °C, 500 ml of bidistilled water was added to give a final volume equal to 1 liter.

pH was adjusted to 4.5 adding HCl (1M) drop by drop to the solution.

Table 3.3 ALF composition (modified from [11])

Component	ALF (g/l)
magnesium chloride	0.050
sodium chloride	3.21
disodium hydrogen phosphate	0.071
sodium sulfate	0.039
calcium chloride dihydrate	0.128
sodium citrate dihydrate	0.077
sodium hydroxide	6.00
citric acid	20.8
glycine	0.059
sodium tartrate dihydrate	0.090
Sodium lactate	0.085
sodium pyruvate	0.086
pH	4.5

3.3.2 Biodegradation tests

Suspensions with a concentration of NPs of 2 mg/ml were prepared: in total, 25 mg of nanoparticles (ZnO NPs and ZnO-DOPC) were dispersed in 12.5 ml of solution (PBS and DPBS).

Biological conditions were mimicked, keeping the temperature constant at 37 °C; the samples were kept under stirring at 250 rpm for the duration of the whole experiment, in order to prevent precipitation. At different timing (1h, 24h, 72h, 1week) 2.5 ml were taken out from each solution, corresponding to 5 mg of nanoparticles. The NPs were firstly centrifuged to block the biodegradation (3046 rcf for 5 min), the supernatant collected for ICP-MS analyses and the precipitate washed twice with bidistilled water (3046 rcf for 5 min) and dried in an oven at 60 °C.

DLS test over one hour were performed to study the biostability of lipid-coated ZnO nanoparticles in physiological media [11]: keeping the temperature of the measuring chamber constant at 37 °C, it was possible to detect the size at intervals of 5 minutes.

Due to the harsh physiological conditions of ALF (pH 4.5), the samples were analyzed at a reduced time steps of biodegradation (15 min, 30 min, 1h): after 1 step of centrifugation to block the process (3046 rcf for 5 min), 1 ml of supernatant (top) was centrifuged at 10000 rcf for 5 minutes, dispersed in water and then analyzed; also the precipitate was put in 2 ml of bidistilled water, then washed through a step of centrifugation for 5 min at 3046 rcf and then analyzed.

3.3.3 Characterization

All the samples were analyzed through X-ray diffraction (XRD) and infrared spectroscopy (FT-IR) to verify, respectively, the loss of crystallinity and the presence of new elements bound or precipitated on the surface.

In order to obtain more information on the morphology and the elemental composition, FESEM analyses were performed.

Regarding the study of the dissolved species in solution, ICP-MS (Inductively Coupled Plasma Mass Spectroscopy) analyses were exploited; in particular, the concentrations in solution of Zn^{2+} , Ca^{2+} and P (linked to the presence of phosphate ions, PO_4^{3-}) were recorded.

The analyses were carried out by diluting properly in double-distilled water the discarded supernatant, which derived from the first centrifugation.

The dilutions used, related to the detection thresholds of the instrument, are the following 1:50 for Ca^{2+} and PO_4^{3-} and 1: 250 for Zn^{2+} .

3.4 Coupling with lipids

The possibility of coating the nanoparticles with liposomes (micelles constituted by a membrane with a double phospholipidic layer) was considered in order to improve the colloidal dispersion of the nanoparticles in a biological environment and prevent their degradation and aggregation.

Zinc oxide nanoparticles were internalized in liposomes constituted of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), phospholipid with neutral charge, following two different protocols: solvent exchange and fusion. The obtained product is shown in Fig. 3.6.

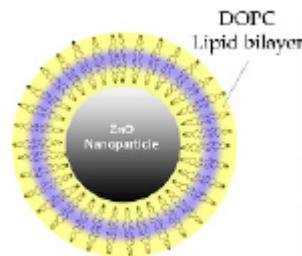


Fig. 3.6 ZnO nanoparticle internalized in a liposome, formed by a double layer of DOPC lipids [4]

3.4.1 Solvent Exchange

The properties of lipids, stable and dispersed as monomers in alcoholic solutions with low water content, are used to form micelles following the progressive hydration of the solution. By suspending the nanoparticles in the lipid suspension before adding water, a support surface is provided on which the formation of liposomes is thermodynamically favored [4].

The following procedure was used to couple 1 mg of ZnO NPs with 0.25 mg of DOPC. Firstly it was prepared a mixture of dispersed lipids: 1 mg of lipids (DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids Inc.) were dissolved in 1 ml of solution, adding firstly 400 µl of ethanol (40 vol%) and secondly 600 µl of bidistilled water (60 vol%); then 250 µl of this ‘mother solution’ were added to 1 mg of nanoparticles, which were previously separated from ethanol through a centrifugation step.

At this point, the Np sample and the lipids were homogenized with the help of a pipette and vortex stirrer; after that, the formation of micelles consisting of double lipidic layer enveloping the ZnO Nps was obtained adding 700 µl of bidistilled water.

3.4.2 Fusion

The attraction between already formed DOPC liposomes and the ZnO nanoparticles for internalization was also exploited. Thanks to the electrostatic interactions, the liposomes adsorb on the surface, the adhesion forces increase the contact area, determining the breakage of the liposome, which closes around the nanoparticle.

The following procedure was adopted [12]: 1 mg DOPC in 1 ml of Hepes (Sigma Aldrich) was filtered through a porous membrane eleven times in an extruder to form spherical liposomes.

From this ‘mother solution’, 200 µl were taken and mixed with 1 mg of ZnO NPs; after pipetting this solution to disperse the ZnO NPs, 200 µl of bidistilled water were added; then this mixture was put in a shaker for 1.5h (300 rpm) to enable the coupling between the nanoparticles and the liposomes.

3.4.3 Colocalization tests

Colocalization experiments were performed, depositing 20 µl of the solution containing the nanoparticles coupled to the liposomes on a microscope glass slide in order to verify the success of the two internalization protocols tested. The sample was observed by optical fluorescence microscopy: it was used a spinning disc fluorescence microscope (Zeiss Cell Observer SD utilizing a Yokogawa spinning disk unit CSU-X1).

The explosive growth in biomedical research has been characterized by a combination of events that include advances in confocal microscopy instrumentation coupled with the introduction of novel ultra-sensitive detectors [14]. In order to assure high image acquisition speeds of fluorescent proteins and synthetic dyes with reasonable contrast, microscopes must be able to quickly scan the field of view and record data using detectors with high quantum efficiency. Most laser scanning confocal microscopes are inadequate for capturing the millisecond dynamic events that are often essential to unravel the intricate molecular processes occurring in living cells. Spinning disk confocal microscopes are emerging as a powerful tool for rapid spatial and temporal imaging. Although the technique was originally introduced over 40 years ago, recent improvements in microscope optical design and camera technology have significantly expanded the versatility and potential of this approach [14].

The most advanced design in spinning disk instruments was engineered by Yokogawa Electric Corporation of Japan [15] (Fig. 3.7) and implemented in a series of complex disk scanning units termed the Confocal Scanning Unit (CSU). These scanners are equipped with a unique architecture that consists of two coaxially aligned disks featuring a dichromatic mirror positioned between the disks (Fig. 3.8). Each disk contains approximately 20.000 pinholes (with a 250-micrometer spacing). The upper disk is actually a glass plate containing microlenses on the top surface that direct and focus light onto perfectly aligned 50-micrometer pinholes in the lower disk for transmission to the objective and specimen. Because significantly more input illumination is gathered by the microlens disk than a regular Nipkow disk, even living cells expressing very low levels of fluorescent proteins are readily imaged in microscopes equipped with the Yokogawa scanning unit.

Fluorescence emission from the specimen is captured by the objective and focused back onto the pinhole Nipkow disk, where each conjugate pinhole now serves as a confocal exit pinhole and blocks emission from regions of the specimen not residing in the focal plane. The emission light reflected is passed through a barrier filter to remove any remaining stray light and out-of-band fluorescence before being focused on to the surface of the CCD array of the camera system to generate an image.

Among the most important aspects to understand about spinning disk confocal microscopy is that these instruments are capable of acquiring thin optical sections from specimens in a manner similar to laser scanning confocal microscopes, only much faster. Therefore, spinning disk microscopes are capable of imaging both thin and thick specimens in two (x-y) or three (x-y-z) dimensions in high spatial resolution as a function of time. Thus, spinning disk confocal microscopes can readily acquire two dimensional images at relatively slow time-lapse speeds: the real power of these instruments is manifested by the fact that they can rapidly acquire images in real time (approximately 30 frames per second), as well as in the

ultrafast timescale that ranges up to 1.000 frames per second and also possibly on living cell systems, leading to live-cell imaging.

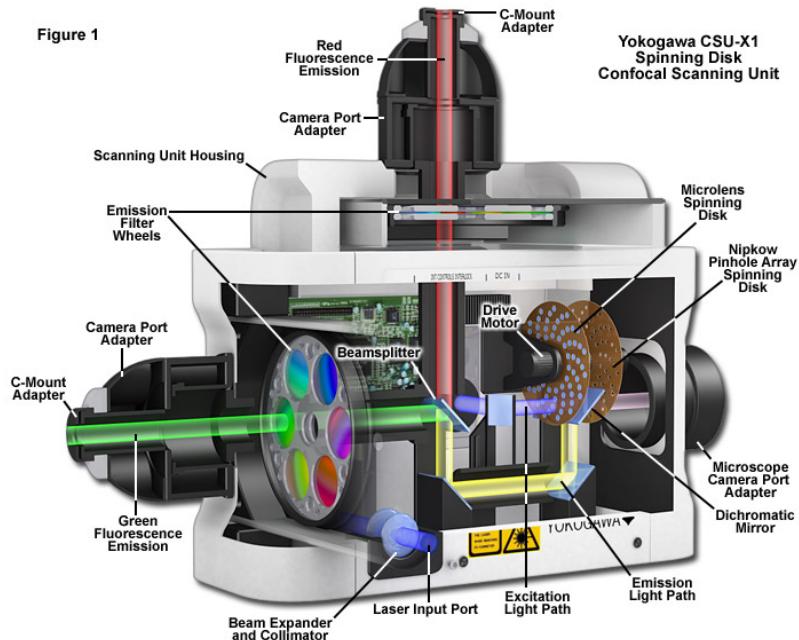


Fig. 3.7 Yokogawa Electric Corporation CSU-X1 spinning disk [13]

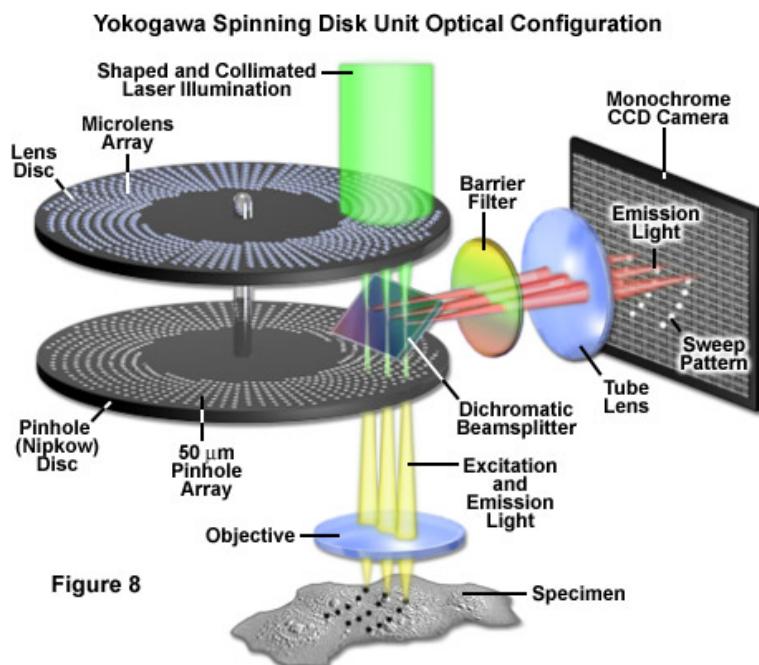


Fig. 3.8 Schematic diagram of the basic optical configuration in Yokogawa scanning units [13]

Both the functionalized nanoparticles (ZnO-NH₂ NPs) and the lipid shell were labeled with suitable dyes. In detail, before carrying out the two coupling protocols, 2 µl of ATTO 488 NHS ester dye (Sigma Aldrich, 1 mg/ml) were added to the amino functionalized nanoparticles stored in ethanol at a concentration of 1 mg/ml.

The solution containing the nanoparticles and the dye was left on an orbital shaker (700 rpm) overnight. The day after, the ethanol was separated by centrifugation (10000 g for 5 min) and some washes in water were made to remove the unbound and excess dye; in detail, 2 washes in water for 5 min at 10000 g and before the third wash the solution was left for 15 min in an ultrasonic bath; after discarding the supernatant, the solvent exchange or fusion procedure were performed to couple the nanoparticles with the liposomes.

Measurements were made in DLS to verify the size of the coupled nanoparticles (500 µl/ml). Finally, to mark the lipid shell, 0.1 µl of DID (1 µM), Lipophilic Indicator by Invitrogen was added.

Atto488 (linked to the ZnO-NH₂ nanoparticles) was imaged at the excitation wavelength of 488 nm and DID (linked to the lipid shell) at 639 nm laser excitation.

By obtaining the fluorescence emission in the two respective channels (with a minimum time difference between the two acquisitions and comparing the positions of the two collected signals), it was possible to determine whether the nanoparticles and the lipid shell were colocalized or not.

3.5 Cellular Uptake

For cell experiments, HeLa cancer cells HeLa ATCC® CCL-2™ derived from cervical cancer (adenocarcinoma) were cultivated in DMEM supplemented with 10 vol% FBS and 1 % Pen-strep [1]. All reagents were purchased from Thermo fisherscientific.

The day before particle incubation the cells were seeded onto 8-well slides at a concentration of 5000 cells per well containing 300 µl of medium. The zinc oxide nanoparticles were prepared and labelled as described above, only replacing Atto 488 by Atto 633 NHS ester dye.

The day after cell incubation, 1 µl of Lysosomes-GFP (Thermo Fisher, 1 mg/ml) was added to the cell culture medium. This dye is able to label the lysosomes in the cells, thus allowing to evaluate their colocalization with the labelled nanoparticles. Finally ZnO NPs and ZnO-DOPC NPs (obtained with Solvent Exchange method) and both labeled by Atto 633 were added to the cell culture medium at different concentrations (10 µg/ml, 20 µg/ml, 30 µg/ml) and the cell uptake was evaluated after 5 hours and 24 hours.

Four replicates were done for each NPs concentration and for the used time steps to validate the obtained data.

Two washes in DMEM were performed before preparing the cell culture sample for the microscope.

Cells and particles were imaged at 37 °C under a 5% CO₂ humidified atmosphere using the spinning disc fluorescence microscope, described above (Zeiss Cell Observer SD utilizing a Yokogawa spinning disk unit CSU-X1). The objective was a 1.40 NA 63× Plan apochromat oil immersion objective (Zeiss) (Fig. 3.9).

Atto633 was imaged with an excitation wavelength of 639 nm and Lysosomes-GFP with the 488 nm laser excitation.



Fig. 3.9 Fluorescence Microscope with 8-well slides for live-cell imaging experiments [16]

Bibliography Chapter 3

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4. Results and discussion

4.1 Characterization of bare and lipid-coated zinc oxide nanoparticles

4.1.1 Morphological and structural characterization

The morphology and particle size of bare and lipid-coated zinc oxide nanoparticles were characterized by Field Emission Scanning Electron Microscopy (FESEM) (Fig. 4.1). FESEM images show that these nanoparticles have a spherical shape with an average diameter of 14 ± 2 nm (calculated by Fiji software, Open source, n=50).

Analysis of the X-Ray diffraction pattern allowed to confirm the crystalline structure of the ZnO NPs. The diffraction peaks were in fair agreement with the characteristic peaks of wurtzite crystalline structure (Fig. 4.2): the spectrum presents broad diffraction peaks for values of 2θ equal to 31.9° , 34.4° , 36.4° , 47.6° , 56.7° and 62.9° . Applying the Debye-Scherrer equation (Formula 3.3), an average size of 15 nm of the nanocrystallites was obtained, in accordance with the electron imaging results.

For the lipid-coated nanoparticles, prepared by a solvent-exchange method using the commercial phospholipid DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), FESEM and XRD analyses were useful to confirm that the morphology and the crystalline structure of the NPs were not modified (Fig. 4.1, Fig. 4.2), while the size changed due to the coating (average diameter of 21 ± 5 nm, measured from FESEM images using Fiji software, n=20).

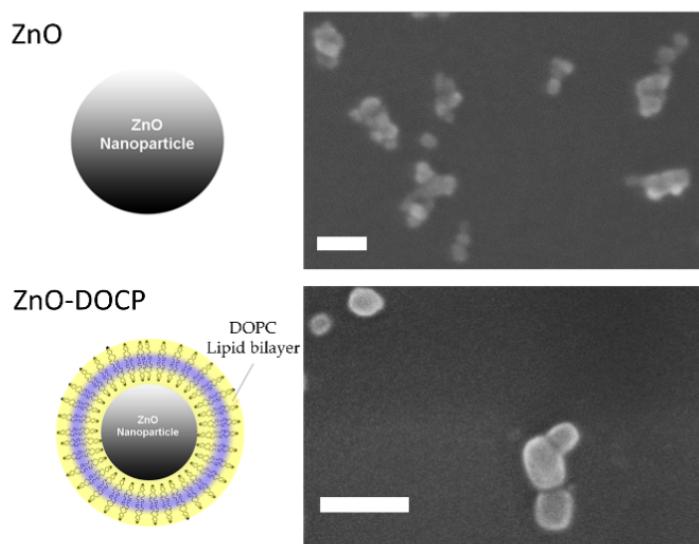


Fig. 4.1 FESEM images of bare and lipid-coated zinc oxide NPs (Modified from [1]); scale bar is 30 nm in both images

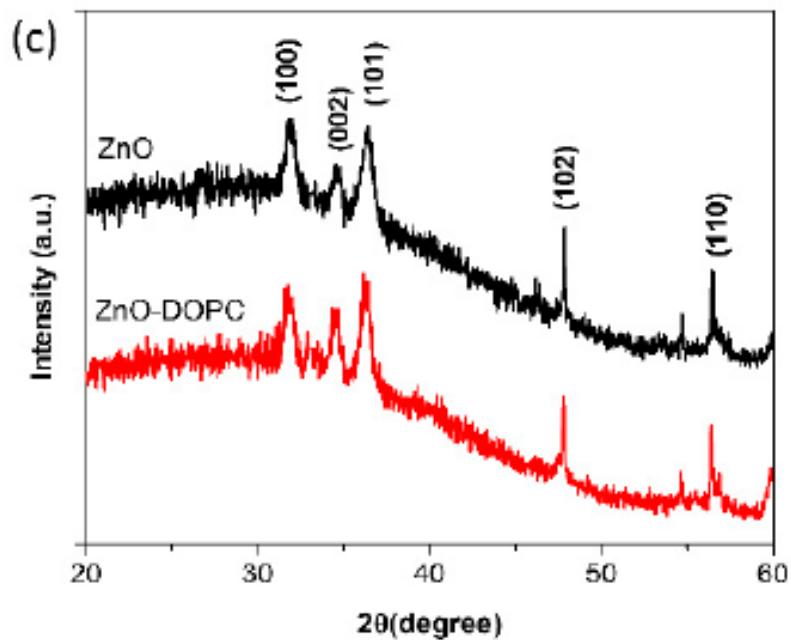


Fig. 4.2 Representative X-ray diffractograms of the pristine (black spectrum) and lipid-coated ZnO NPs (red spectrum) with Miller indices (Modified from [1])

4.1.2 Colloidal stability

In order to evaluate the possible use of zinc oxide nanoparticles in clinical applications, it was important to study the suspension stability and the state of aggregation, which influence the bio-distribution within the organism and therefore the therapeutic efficacy.

Dynamic Light Scattering (DLS) experiments were performed for the two samples to estimate their hydrodynamic diameters and stability in water (Fig. 4.3a). The absence of micrometer-scale aggregates in all measurements suggests good dispersion and low aggregation behaviour for both samples. Moreover, the coating of the ZnO nanoparticle surface contributed to a larger mean hydrodynamic diameter (110 nm for ZnO-DOPC NPs) than the one obtained for the pristine ZnO NPs (55 nm).

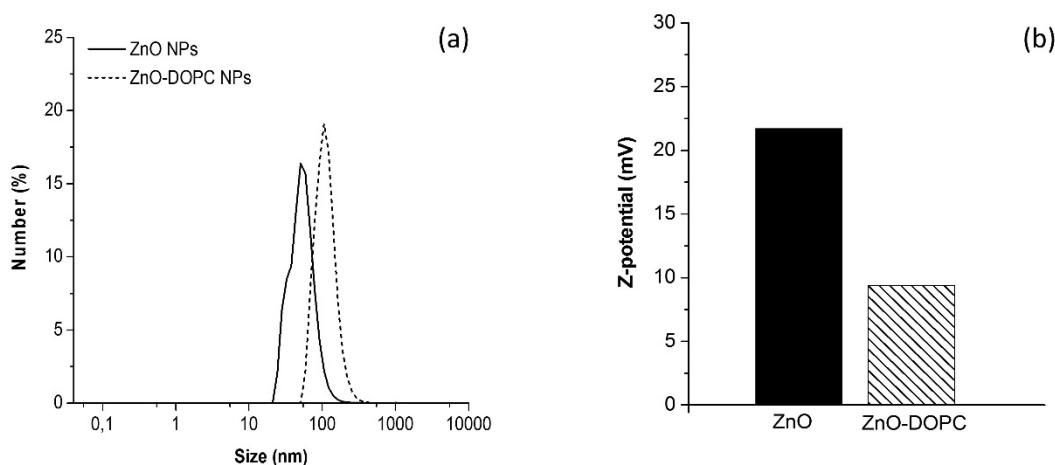


Fig. 4.3 Hydrodynamic diameters and Z potentials of bare and lipid-coated ZnO NPs. Dynamic Light Scattering (a) and Z-potential (b) measurements of the two samples [1]

Another parameter, important to predict the stability of colloidal suspensions, is the zeta potential. It corresponds to the value of the electrostatic potential at the sliding plane, in correspondence of the layer of liquid, formed by two or three molecular layers, which moves with the particle. This value allows to have information on the repulsive forces that are formed between the nanoparticles and that are able to stabilize the solution.

The Z-potential of lipids micelles and of bare and lipid-coated ZnO NPs was evaluated in water maintaining a neutral pH by titration with NaOH and HCl 1 M. As shown in Fig. 4.3b, the DOPC micelles present a negative Z potential, equal to -15 mV, in fair agreement with literature studies [2].

As far as the nanoparticles samples are concerned, different values were obtained depending on the surface properties of the ZnO NPs. For pristine ZnO NPs, the measured positive Z-potential (26 mV) is due to the protonation of the hydroxyl groups at the nanoparticle surface and it is in good agreement with the literature values [3]. A strong decrease of the Z-potential value was obtained for the lipid-coated ZnO nanoparticles: the DOPC phospholipidic shell

neutralized the positive charges of the ZnO surface, so that the Z-potential lowered down to 1.3 mV. These different behaviours for pristine and lipid-coated ZnO nanoparticles in terms of hydrodynamic diameters and Z-potential measurements clearly suggest that the process of encapsulating the ZnO NP in a protective lipid shell worked successfully.

4.2 Biodegradation tests

4.2.1 Morphology and structure

FESEM analyses were performed for the biodegradation tests in PBS (phosphate-buffered saline) and DPBS (Dulbecco's phosphate-buffered saline) on bare and lipid-coated zinc oxide nanoparticles, in order to obtain further information on the morphology of the nanoparticles. These two buffer solutions have two different phosphate concentrations: PBS at higher concentration (Table 3.1), while DPBS at lower concentration (Table 3.2).

During these tests, the withdrawals were made at 1 hour, 24 hours, 72 hours and 1 week.

All the samples were already strongly aggregated after the first hour and the aggregation increased during the tests, as shown in Fig. 4.4 and Fig. 4.5: the nanoparticles presented the tendency to form spherical aggregates consisting of zinc oxide covered by a thin layer of other inorganic material, which derives from the precipitation of salts present in the buffer solutions (Table 4.1; Table 4.2).

Table 4.1 EDS analysis of bare and lipid-coated zinc oxide nanoparticles in PBS biodegradation tests; results expressed in atomic%.

Element	1h PBS		1 week PBS	
	ZnO NPs	ZnO-DOPC NPs	ZnO NPs	ZnO-DOPC NPs
Zn	33.08	32.55	38.83	24.91
O	47.56	48.12	49.97	42.12
C	10.31	8.83	-	22.85
Na	6.52	6.43	9.16	7.15
P	2.28	3.80	2.03	2.37
Cl	0.25	0.26	-	0.59

Table 4.2 EDS analysis of bare and lipid-coated zinc oxide nanoparticles in DPBS biodegradation tests; results expressed in atomic%.

Element	1h DPBS		1 week DPBS	
	ZnO NPs	ZnO-DOPC NPs	ZnO NPs	ZnO-DOPC NPs
Zn	26.00	32.13	25.09	13.63
O	47.18	47.32	53.00	47.86
C	14.59	10.22	14.16	20.80
Na	7.87	6.41	-	9.62
P	3.77	3.92	7.75	7.47
Cl	0.58	-	-	0.33
K	-	-	-	0.30

All the samples subjected to biodegradation were analyzed by X-ray diffraction (Fig. 4.6 and Fig. 4.7), so that it was possible to verify the crystallinity of the nanoparticles. In all samples subjected to biodegradation, the characteristic peaks of wurtzite, a hexagonal crystalline structure of zinc oxide, was found with values of 2θ equal to 31.9° , 34.4° , 36.4° , 47.6° , 56.7° and 62.9° . This result supports the fact that zinc oxide in PBS and DPBS maintained its crystalline structure. Moreover, it was possible to attribute the diffraction peak at 2θ of 32° to sodium chloride [4], a compound present in PBS and DPBS. The XRD pattern showing the diffraction peaks at 2θ of 21.36° , 27.2° , 34.61° , 52.14° , 53.17° and 56.09° were due to phosphates, which interacted with the zinc oxide surface [5].

Concerning ALF (artificial lysosomal fluid) biodegradation tests, FESEM analyses show that the acidic pH (4.5 pH) was very aggressive towards both bare and lipid-coated zinc oxide nanoparticles: after few minutes most of the nanoparticles were dissolved in solution and few spherical aggregates remained (Fig. 4.8). Furthermore, the nanoparticles lost their crystallinity, as shown in the diffractograms of pristine and lipid-coated zinc oxide nanoparticles (Fig. 4.9): the absence of peaks is related to the fact that the samples were characterized by an amorphous state.

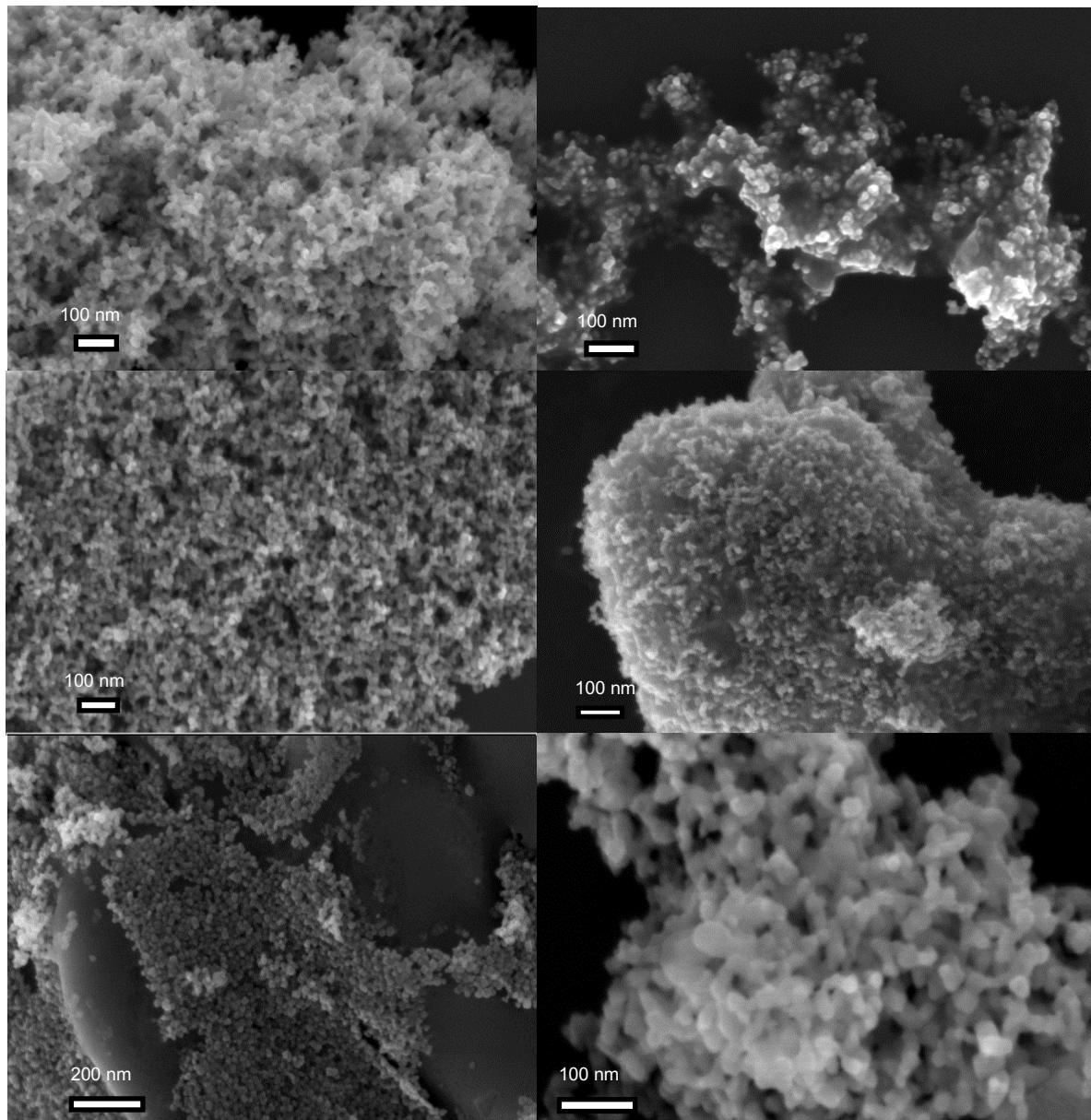


Fig. 4.4 FESEM images of pristine (left) and lipid-coated zinc oxide nanoparticles (right) in PBS biodegradation tests; from the top to the bottom sample images at 1 hour, 72 hours, 1 week.

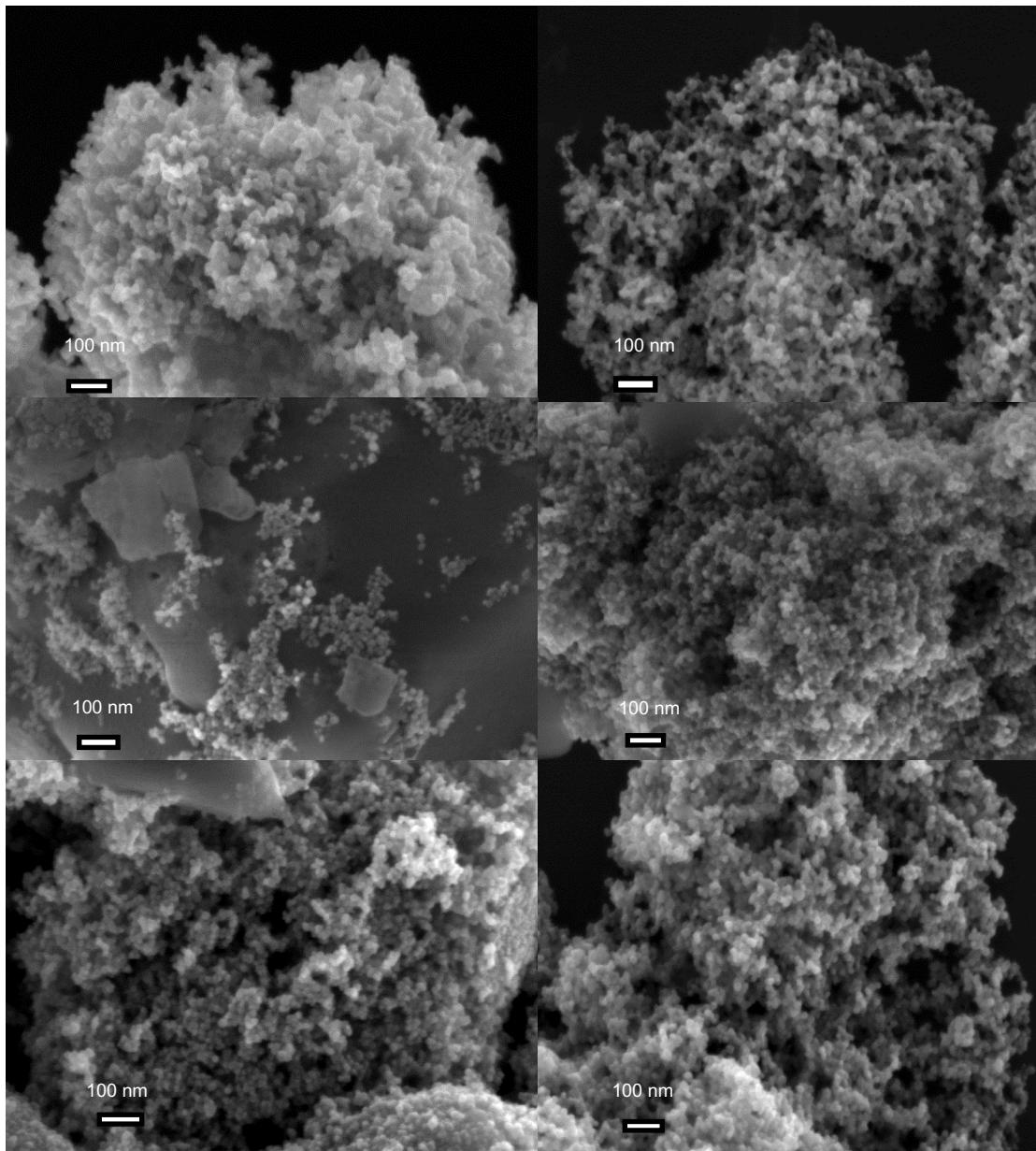


Fig. 4.5 FESEM images of pristine (left) and lipid-coated zinc oxide nanoparticles (right) in DPBS biodegradation tests; from the top to the bottom sample images at 1 hour, 72 hours, 1 week.

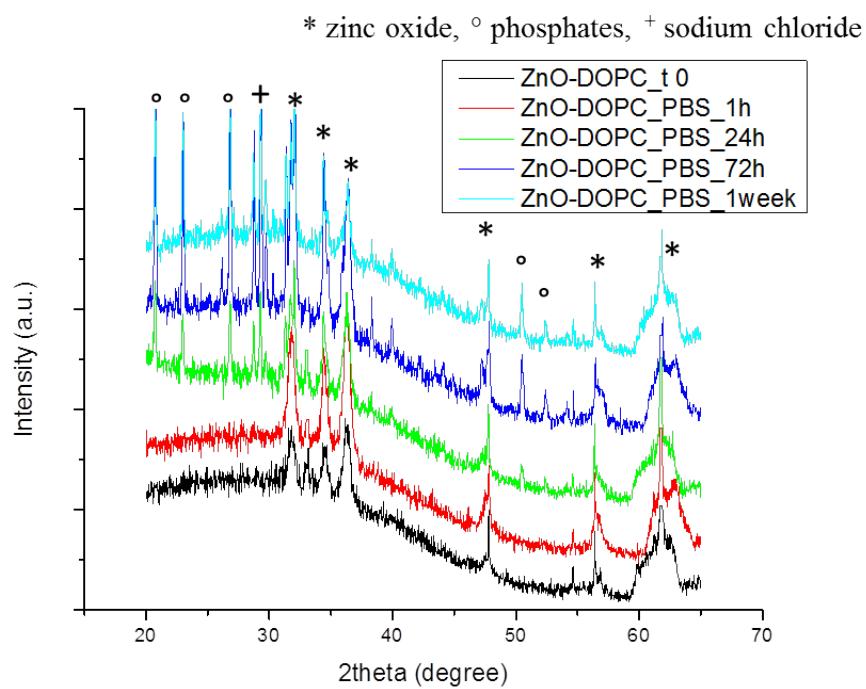
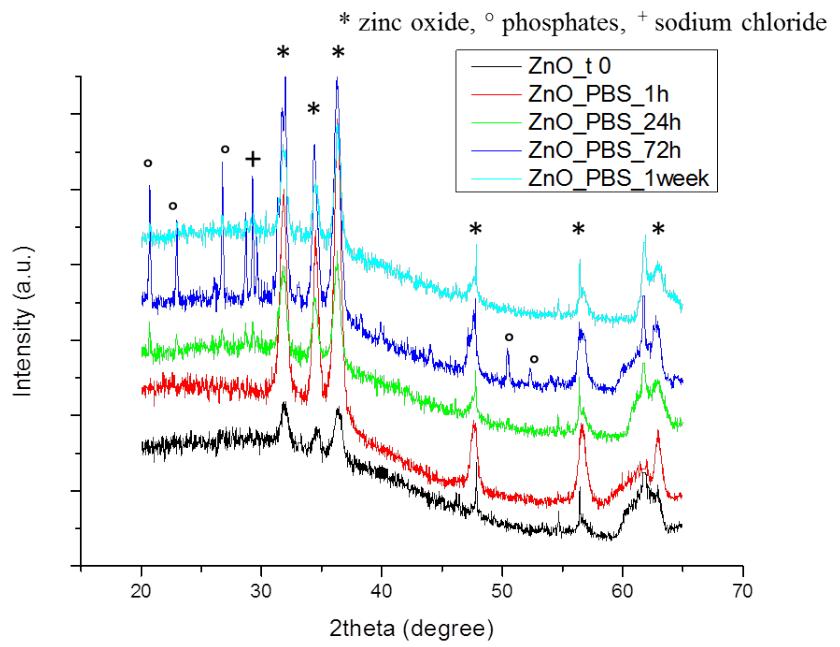


Fig. 4.6 Diffractograms of pristine (top graph) and lipid-coated (bottom graph) zinc oxide nanoparticles in PBS biodegradation tests.

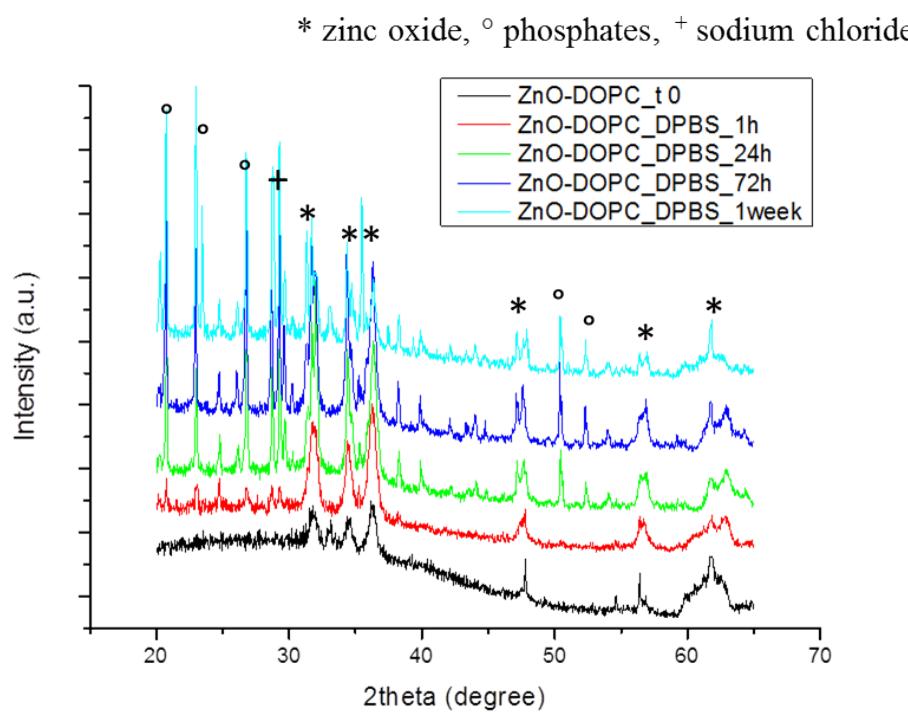
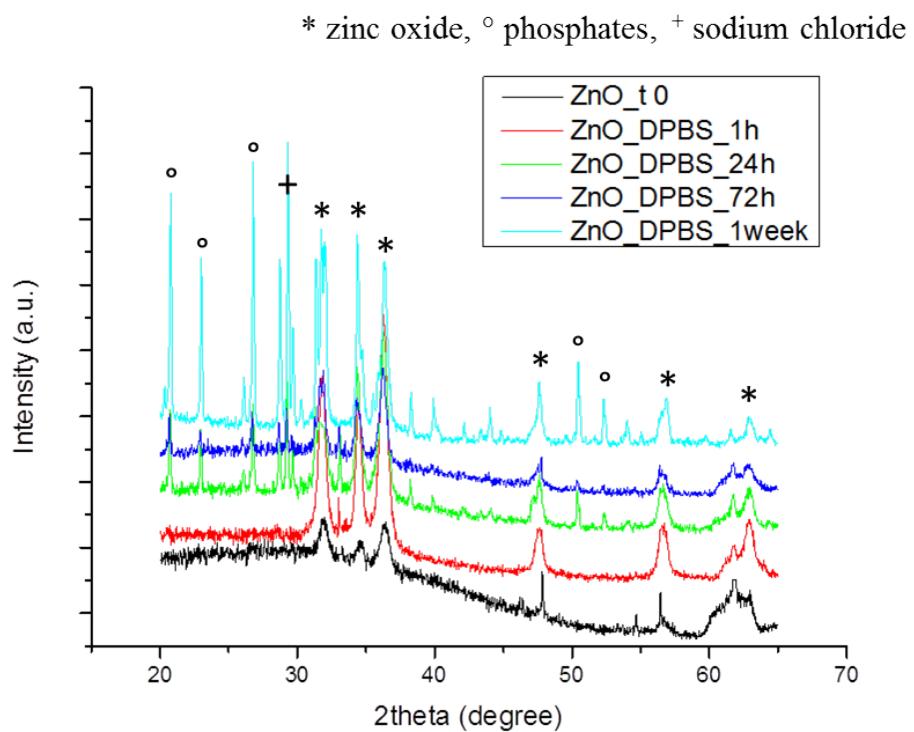


Fig. 4.7 Diffractograms of pristine (top graph) and lipid-coated (bottom graph) zinc oxide nanoparticles in DPBS biodegradation tests.

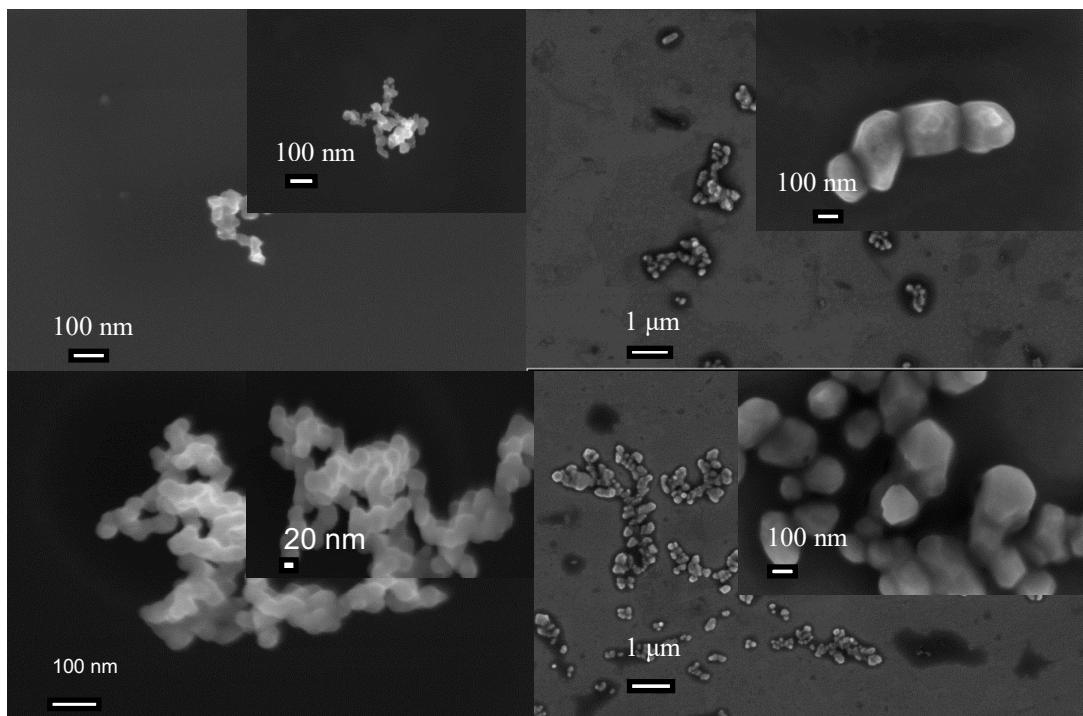


Fig. 4.8 FESEM images of pristine (left) and lipid-coated zinc oxide nanoparticles (right) in ALF biodegradation tests; from the top to the bottom sample images at 15 min, 30 min.

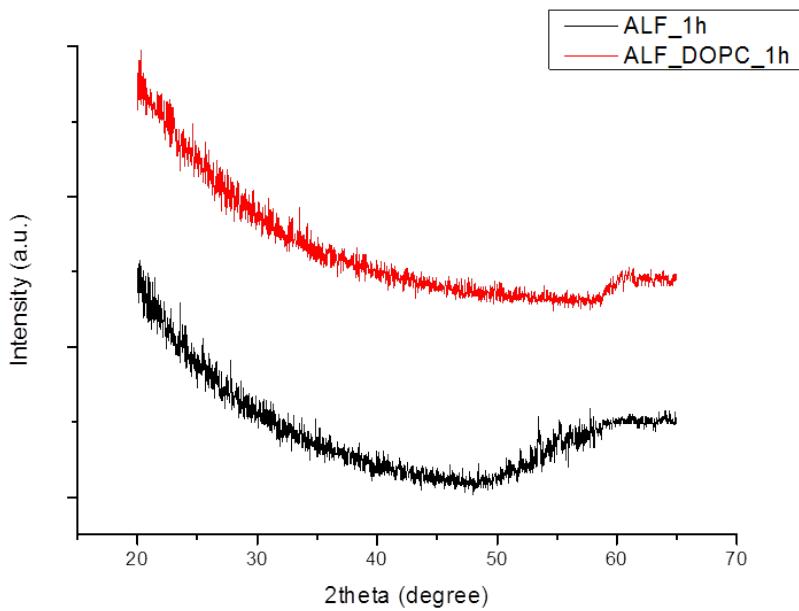


Fig 4.9 Diffractograms of pristine (black spectrum) and lipid-coated (red spectrum) zinc oxide nanoparticles in ALF biodegradation tests.

4.2.2 Dissolution behavior

Regarding the dissolved species in solution, ICP-MS (inductively coupled plasma mass spectrometry) analysis was performed: the concentration of the PO_4^{3-} and Zn^{2+} ions was monitored to verify the dissolution behavior of the nanoparticles.

Literature studies report a high interaction of phosphate ions with zinc oxide nanoparticles [6]: the interaction of the phosphate ions with the surface of the nanoparticles leads to a decrease of the phosphate ions in solution (Fig. 4.10).

As far as Zn^{2+} ions in solution are concerned, there is an increase after 24 h and a difference between the ZnO NPs and the ZnO-DOPC NPs: the lipid coating seems to partially protect the nanoparticles from dissolution in PBS and DPBS, which means that for lipid-coated ZnO NPs there are fewer Zn^{2+} ions released in solution (Fig. 4.10). It is to note that there are no differences of the dissolution behavior between the PBS and DPBS solutions.

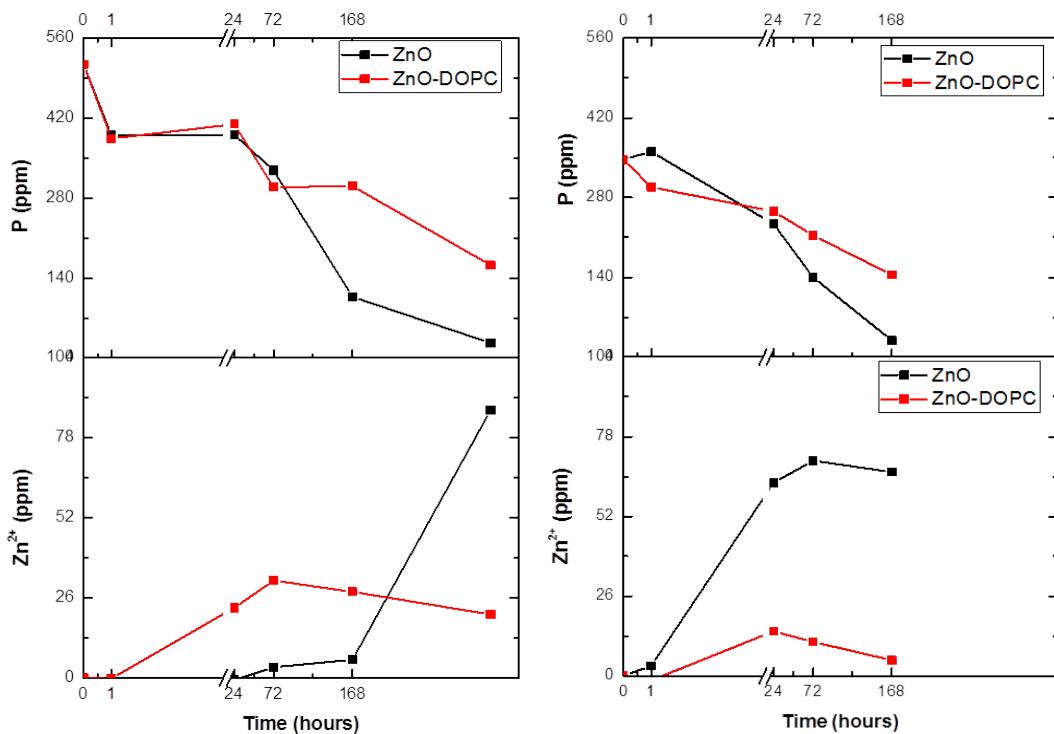


Fig. 4.10 Concentration of Zn^{2+} and PO_4^{3-} ions in PBS (left) and in DPBS (right)

In contrast, at the acidic pH of the artificial lysosomal liquid, there is a high production of Zn^{2+} ions in solution (about 1500 ppm) already after half an hour (Fig. 4.11).

The aggressiveness of this medium determines a high dissolution of both bare and lipid-coated ZnO NPs, so that the coating does not seem to protect the nanoparticles. Thus the presence of Zn^{2+} ions could generate a cytotoxic response on the cells: as soon as the nanoparticles are present in the lysosomes, they start to be dissolved in a short time (30 min).

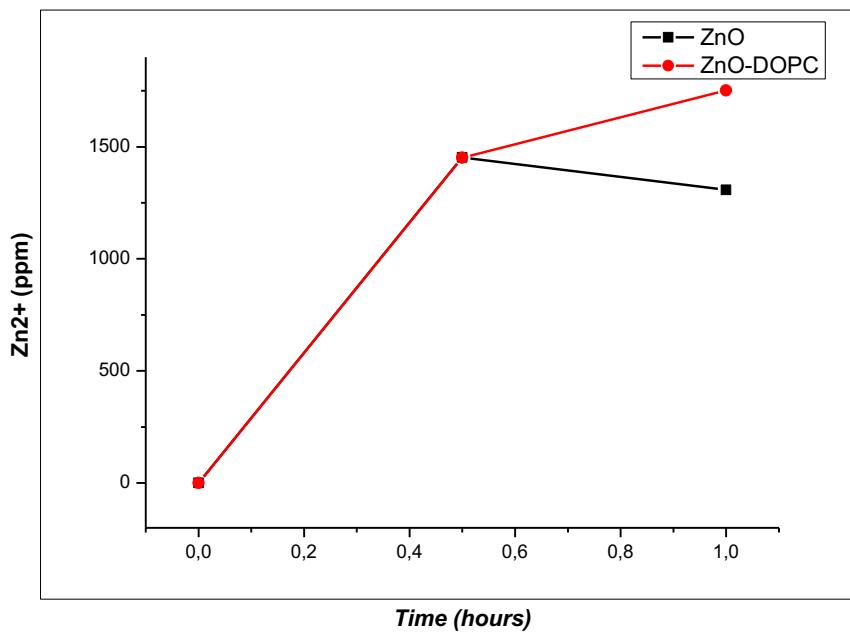


Fig. 4.11 Concentration of Zn^{2+} ions in ALF

4.2.3 Surface composition

All the samples of the biodegradation tests were analyzed by infrared spectroscopy (FT-IR), in order to verify the presence of new bound species or precipitated elements on the surface of the nanoparticles. The resulting spectra are shown in Fig. 4.12 and Fig. 4.13.

The spectra of both bare and lipid-coated zinc oxide nanoparticles before the biodegradation tests (t_0) have peaks related to the Zn-O bond and to the hydroxyl and carbonyl groups. In detail, both spectra show an intense peak at 440 cm^{-1} , characteristic of the Zn-O bond. At 1570 cm^{-1} and 1420 cm^{-1} the peaks are associated with the stretching of the C = O and C-O bonds, while the bands at 2860 cm^{-1} and 2925 cm^{-1} are related to the symmetrical and antisymmetric stretching of the $-\text{CH}_3$ group, which derive from the zinc acetate, used as a precursor in the synthesis not completely hydrolyzed and reacted.

Regarding the nanoparticles subjected to biodegradation, the intensity of the peaks increases with time: all the samples analyzed have a peak at 1045 cm^{-1} , characteristic of the P-O bond stretching deriving from the precipitation on the surface of phosphate groups (PO_4^{3-}), present in both PBS (at higher concentration) and DPBS (at lower concentration). The peaks at 1528 cm^{-1} and 1643 cm^{-1} are related to the carbonate ions, while at 2955 cm^{-1} and 2915 cm^{-1} the peaks are associated to the symmetric and antisymmetric stretching of the $-\text{CH}_3$ group, already present in pristine ZnO nanoparticles due to the acetate residues in the synthesis. The stretching band in the region from 3600 to 3000 cm^{-1} are attributed to the -OH groups present on the surface.

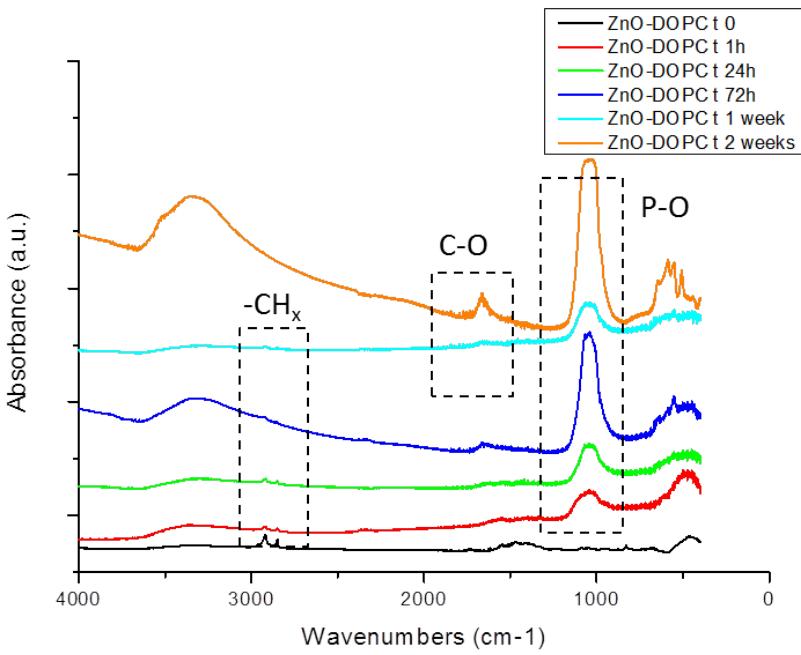
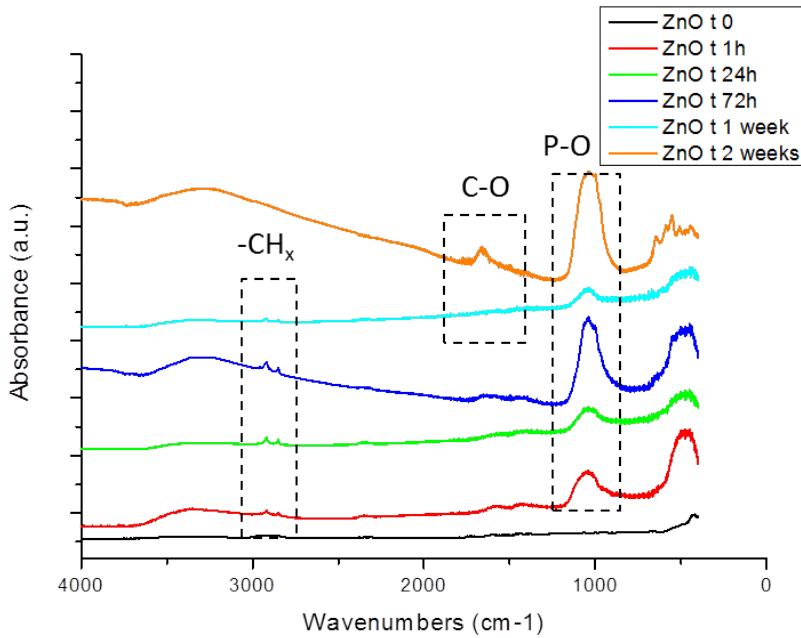


Fig. 4.12 FT-IR spectra of pristine (top graph) and lipid-coated (bottom graph) ZnO nanoparticles in PBS biodegradation tests.

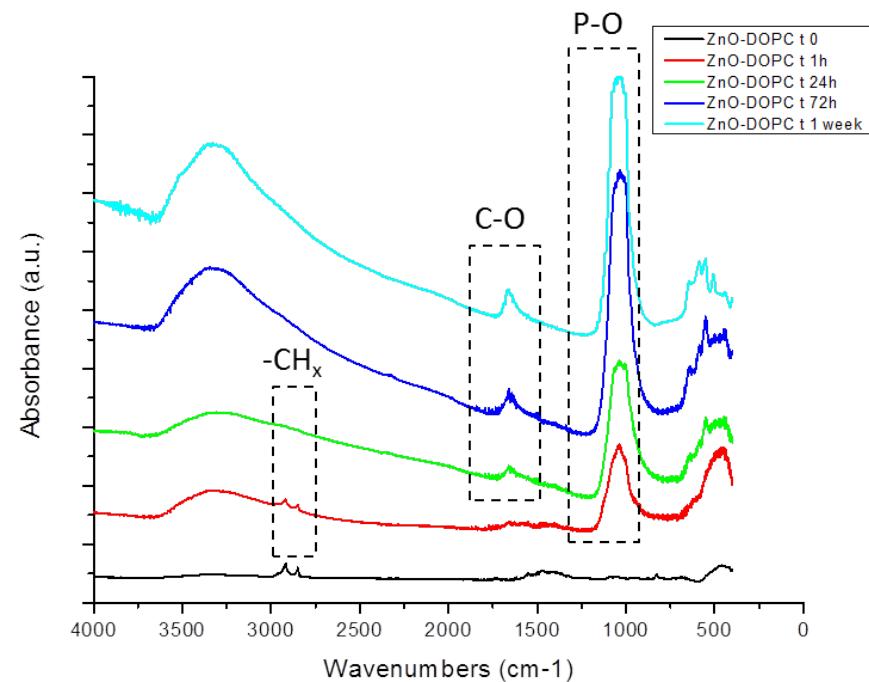
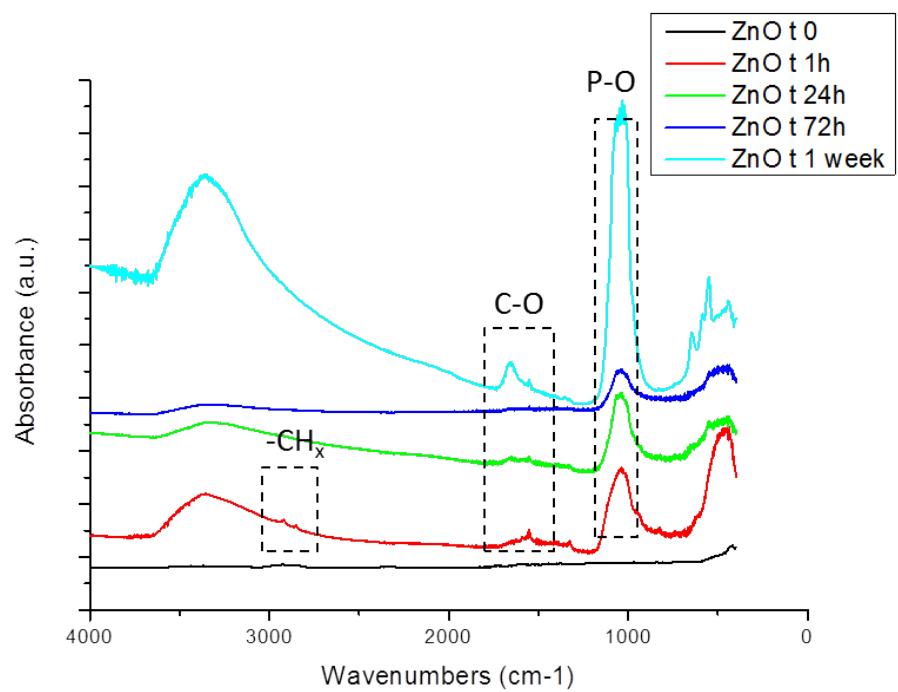


Fig. 4.13 FT-IR spectra of pristine (top graph) and lipid-coated (bottom graph) ZnO nanoparticles in DPBS biodegradation tests.

4.2.4 Aggregation and biostability

The efficient delivery of the nanoparticles to the pathological site of interest is a crucial step to achieve an effective therapeutic effect. In the case of injection into living systems, this could be hindered by the aggregation of the ZnO NPs in contact with plasma fluid: several studies have indeed shown that both circulation time in the blood stream and cellular uptake are strongly influenced by the nanoparticles' colloidal stability in these media [7].

As a first step towards the comprehension of the behaviour of ZnO NPs in biological media, Dynamic Light Scattering (DLS) experiments were performed in PBS and the effect of the lipid-coating on the colloidal stability of ZnO NPs was evaluated.

As shown in Fig. 4.14a, when the nanoparticles were suspended in PBS, bare ZnO NPs showed a strong aggregation behaviour, forming micrometer-scale aggregates (3200 nm), while lipid-coated ZnO NPs did not show any aggregation, confirming the hydrodynamic size obtained in water suspension (105 nm).

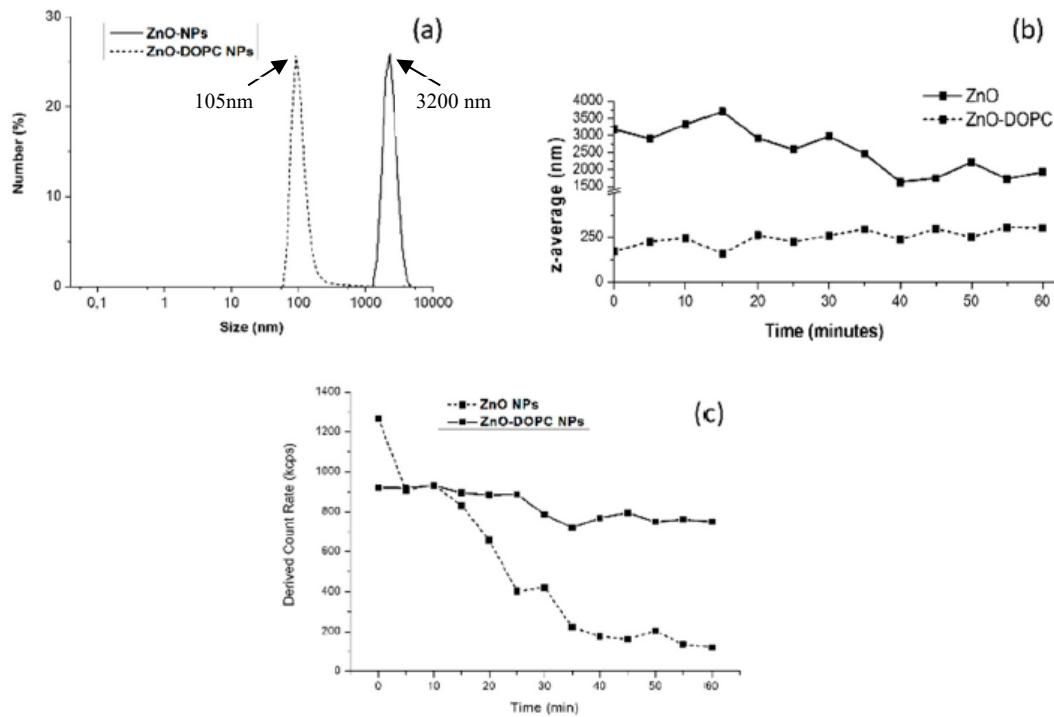


Fig. 4.14 The lipid-shell increases colloidal stability of ZnO NPs in PBS: (a) Dynamic Light Scattering (DLS) measurements in number percent of the bare (ZnO) and lipid-coated (ZnO-DOPC) nanoparticles in phosphate buffered saline (PBS); (b) mean hydrodynamic size (Z-average) of bare NPs (solid curve) and lipid-coated ZnO NPs (dotted curve) in PBS over time; and (c) derived count rate of the two samples over time (Modified from [1])

The mean hydrodynamic radius (z-average) of ZnO and ZnO-DOPC NPs was recorded in real-time for 1 h (Fig., 4.14b) to further study this behaviour. As soon as the bare ZnO NPs were in contact with PBS solution (time 0), the measured z-average was relatively high (3200 nm), confirming that this sample quickly formed huge aggregates. Over time, the z-average

moderately decreases (down to 1890 nm), suggesting that the NPs maintain their aggregated form and partially precipitate. The marked decrease of the derived count rate over time (Fig. 4.14c) strongly supports this hypothesis. On the other hand, lipid-coated ZnO NPs did not form micrometer-scale aggregates when suspended in PBS, maintaining over time a Z-average size between 100 and 250 nm, thus confirming their higher colloidal stability compared to the bare ZnO NPs. Similarly, the derived count rate remains stable over time.

In a previous work it was observed the same improvement of colloidal and chemical stability of lipid-coated ZnO nanoparticles in cell culture media (EMEM) and simulated human plasma (Simulated Body Fluid, SBF) [8]. This colloidal behaviour is improved due to the lipid shell stabilization: in particular, for PBS, the phosphate ions contained in large quantity in the buffer solution are strongly reactive towards ZnO leading to the formation of poorly soluble zinc-phosphate precipitates [9] and the lipid coating can limit the contact of solution's components with the ZnO surface. DLS over time could not be recorded for the pristine ZnO NPs sample in ALF due to immediate degradation. In contrast, it is interesting to observe a stable behaviour over time of the ZnO-DOPC NPs in ALF (Fig. 4.15, Fig. 4.16), where the lipid shell still prevents the ZnO NPs aggregation, although the solution is at acidic pH (pH 4.5). The average particle size is similar to what observed at FESEM in Fig. 4.8. These data are however not fully confirmed by the ICP-MS measurements, showing a strong sample degradation, and the absence of any diffraction peak after XRD characterization. Another hypothesis might be that the lipid shell stays intact, but the ZnO is dissolved and diffusing out of the lipid, but also have more ZnO ions in the solution. Therefore it is assumed that the ALF medium is very unfriendly to both kind of nanoparticles, irrespective to their coating and in particular for times higher than 30min-1hour.

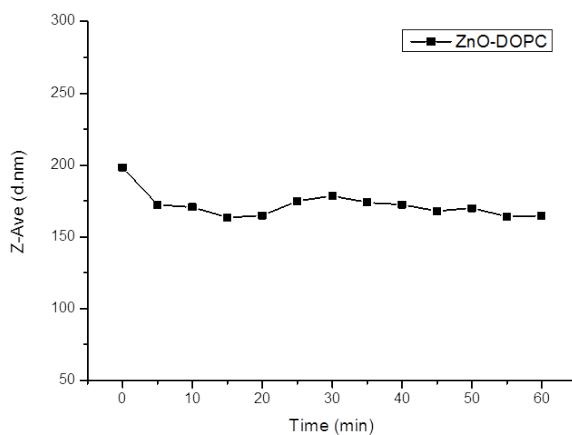


Fig. 4.15 Mean hydrodynamic size (Z-average) of lipid-coated ZnO NPs in ALF over time.

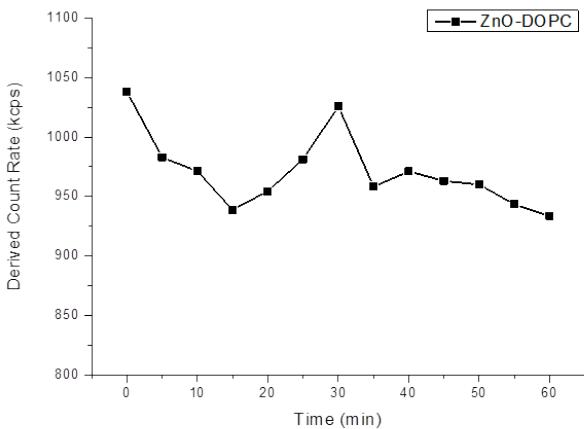


Fig. 4.16 Derived count rate of lipid-coated ZnO NPs in ALF over time

4.3 Colocalization tests

The possibility of coating zinc oxide nanoparticles with liposomes, micelles formed by a double phospholipid layer widely used in drug delivery, was tested in order to prevent aggregation and improve the resistance and biocompatibility of the material.

As described in the materials and methods chapter (chapter 3), two types of coupling (fusion and solvent exchange) were tested and the success of internalization was verified thanks to fluorescence microscopy colocalization experiments.

Initially PBS (Phosphate-buffered saline) was considered for the fusion protocol: the proximity of fluorescent signals related to the liposomes (red channel) and the nanoparticles (green channel) allowed to confirm the success of the coupling (Fig. 4.17). However, there were so many aggregates, that PBS was replaced with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in the fusion protocol and this led to better results (Fig. 4.18): the nanoparticles were less aggregated and they were characterized by better dispersion.

Concerning the solvent exchange method, the use of ultrasound was very important to facilitate the dispersion of the nanoparticles: initially they were not treated with ultrasound in the procedure and the sample was characterized by many aggregates (Fig. 4.19), although the nanoparticles' signals (green) were very close to the lipid bilayer ones (red).

Subsequently, ultrasound steps were carried out and they are described in the materials and methods chapter: the nanoparticles were less aggregated and the signals of the nanoparticles in green and of the lipid bilayer in red were always very close (Fig. 4.20).

The signals were not perfectly overlapped due to the fast movement of the nanoparticles and to the evaporation of the liquid during the measurement; nevertheless, the proximity of the signals allowed to suppose that the nanoparticles have been successfully covered by a double lipidic bilayer.

It is worth mentioning that the scale bar in the optical fluorescence images is 10 μm . Thus, the resulting size of the nanoparticles can seem in disagreement with the sizes measured by DLS and FESEM. However, the magnification used for fluorescence imaging (in this case, 63 \times objective) is not enough to resolve sizes of 20 nm (which is the actual size of the NPs) and will always be constrained by Abbé's limit, in this case about 200 nm. For this reason, it is not possible to distinguish whether there is one single or more particles within each bright spot of 200 nm: we assume that mainly single particles were imaged due to the high particles brightness and their strong dilution.

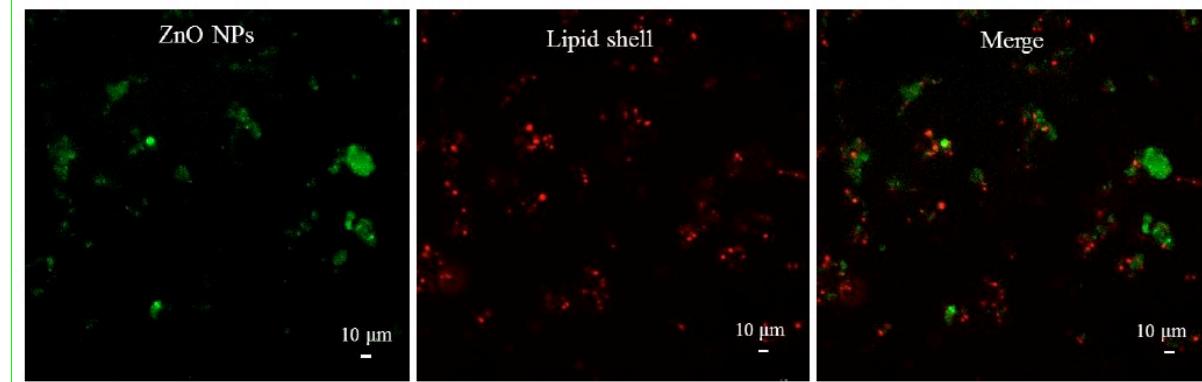


Fig. 4.17 Fluorescence microscopy images of zinc oxide nanoparticles ($\text{ZnO-NH}_2 + \text{ATTO} 488$) internalized in liposomes (DOPC + DID, Lipophilic Indicator) through fusion protocol in PBS; scale bar is 10 μm .

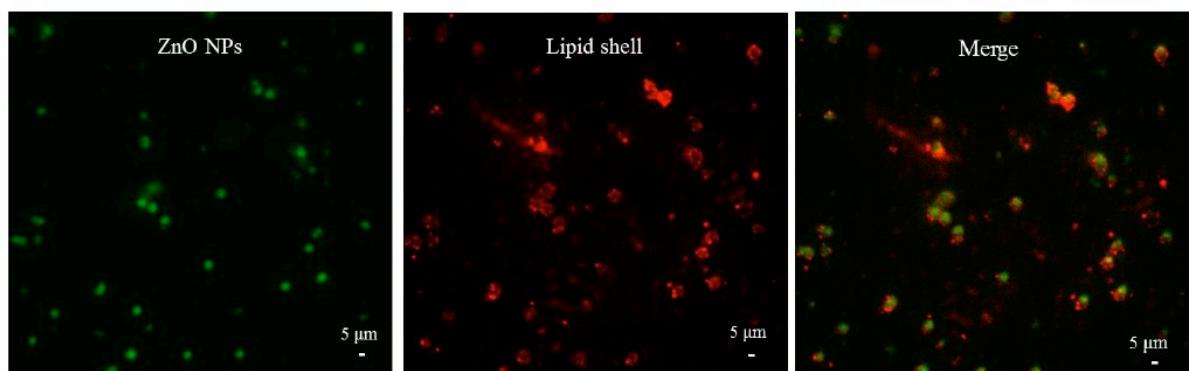


Fig. 4.18 Fluorescence microscopy images of zinc oxide nanoparticles ($\text{ZnO-NH}_2 + \text{ATTO} 488$) internalized in liposomes (DOPC + DID, Lipophilic Indicator) through fusion protocol in HEPES; scale bar is 5 μm .

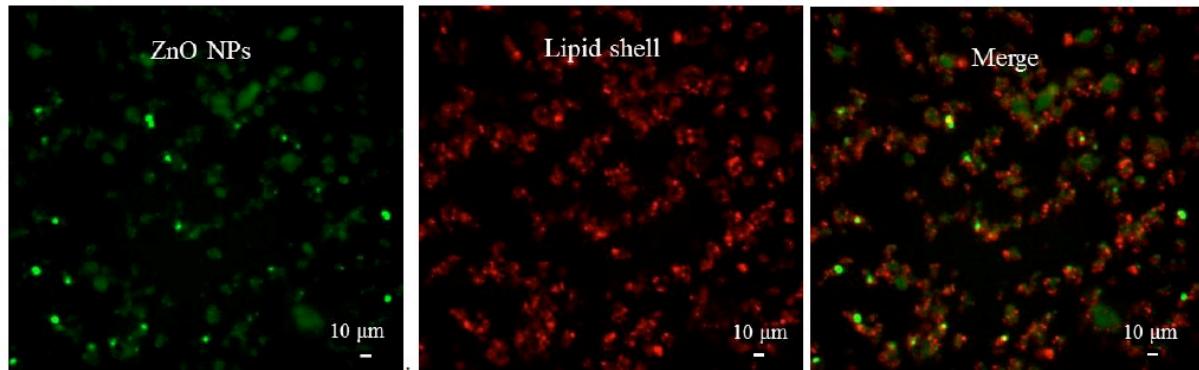


Fig. 4.19 Fluorescence microscopy images of zinc oxide nanoparticles ($\text{ZnO-NH}_2 + \text{ATTO} 488$) covered by a double lipidic bilayer (DOPC + DID, Lipophilic Indicator) through solvent exchange protocol; scale bar is $10 \mu\text{m}$.

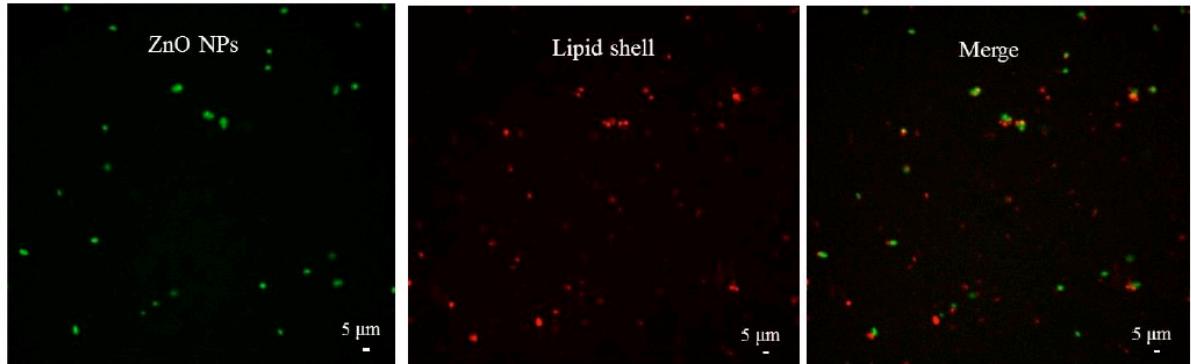


Fig. 4.20 Fluorescence microscopy images of zinc oxide nanoparticles ($\text{ZnO-NH}_2 + \text{ATTO} 488$) covered by a double lipidic bilayer (DOPC + DID, Lipophilic Indicator) through solvent exchange protocol, well dispersed with ultrasounds; scale bar is $5 \mu\text{m}$.

4.4 Cellular uptake

Cellular uptake tests were performed on HeLa Cells to compare the behaviour of pristine zinc oxide nanoparticles with those coated with liposomes and at different concentrations (10, 20 and 30 µg/ml).

Tests under spinning disk fluorescence microscopy were carried out after both 5 and 24 hours of NPs incubation with cells. These time scales were very useful to study cell uptake kinetics, being in the range necessary to have the nanoparticles internalized into the cancer cells and co-localized in the lysosomes.

The percentage of cell uptake, averaged over 4 tests per each condition studied, is higher increasing the concentration and it is improved thanks to the lipidic bilayer coating, already after 5-hour of incubation (Table 4.1). For the pristine zinc oxide nanoparticles the presence of aggregates prevents the nanoparticles from entering the cells (Fig. 4.21), remaining in the cell culture medium and outside the cells. On the other hand, lipid-coated zinc oxide nanoparticles are more dispersed and more stable and the coating favours their internalization until they are visibly co-localized with the lysosomes (Fig. 4.22).

After 24 hours of incubation this behaviour is even more evident: the percentage of NPs uptaken by the HeLa cells increases significantly for the liposome-coated nanoparticles and especially at higher concentrations (Table 4.2). Fluorescence microscope images show that there are many micrometric aggregates in the case of pristine zinc oxide nanoparticles (Fig. 4.23). In contrast, in the case of lipid-coated ZnO NPs, the sample is more dispersed and this favours the cell uptake, leading to positive results (Fig. 4.24).

Table 4.3 Percentage of cell uptake for bare and lipid-coated ZnO NPs after 5 hours of incubation

NPs Concentration	5h	
	ZnO	ZnO-DOPC
10 µg/ml	5%	38%
20 µg/ml	14%	41%
30 µg/ml	17%	47%

Table 4.4 Percentage of cell uptake for bare and lipid-coated ZnO NPs after 24 hours of incubation

Concentration	24h	
	ZnO	ZnO-DOPC
10 µg/ml	11%	61%
20 µg/ml	12.5%	63%
30 µg/ml	21.5%	66%

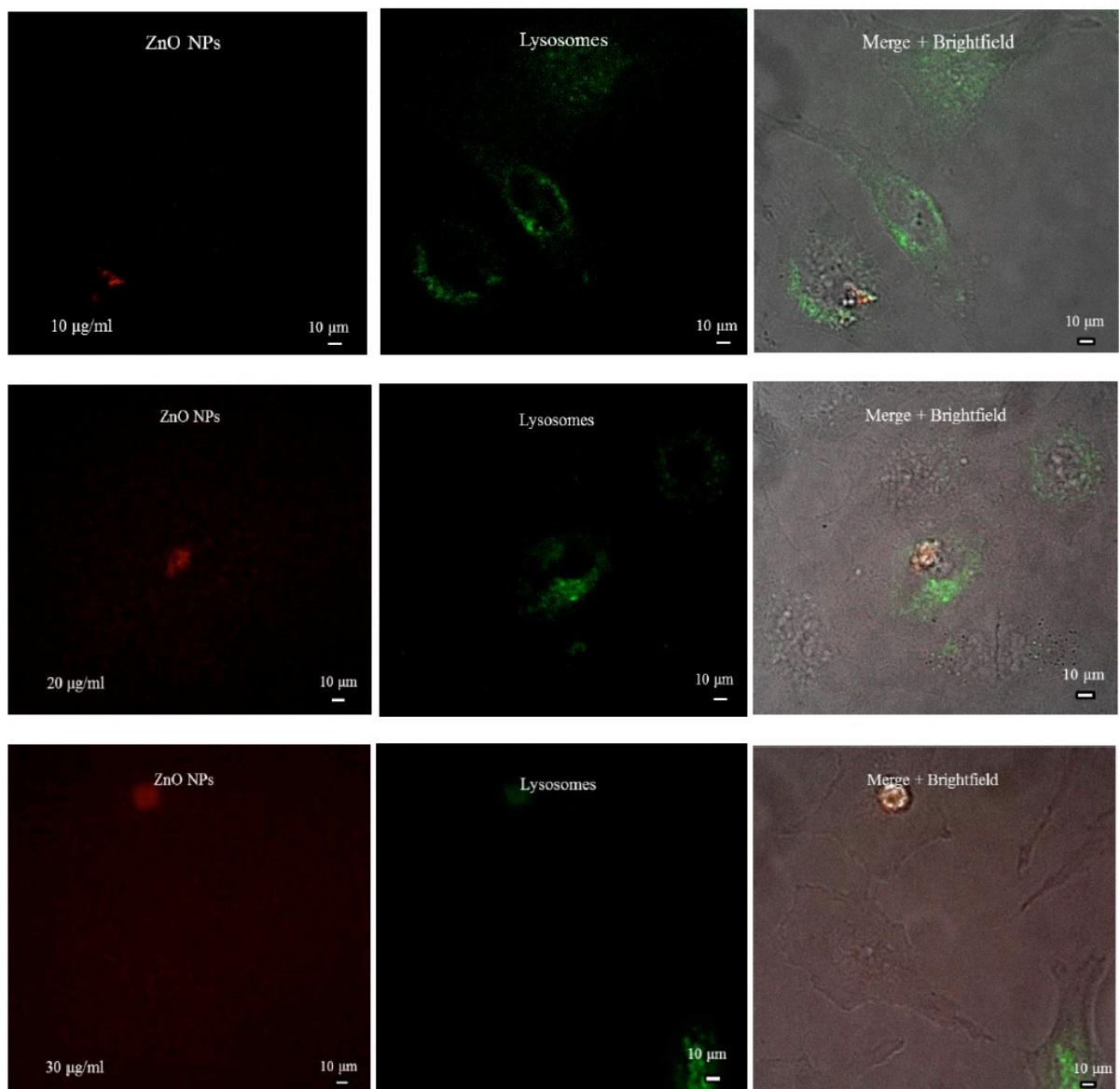


Fig. 4.21 Cell uptake of pristine ZnO nanoparticles (ZnO-NH_2 + ATTO 633, in the red channel) in lysosomes (Lysosomes-GFP, in the green channel) after 5 hours of incubation; scale bar is 10 μm .

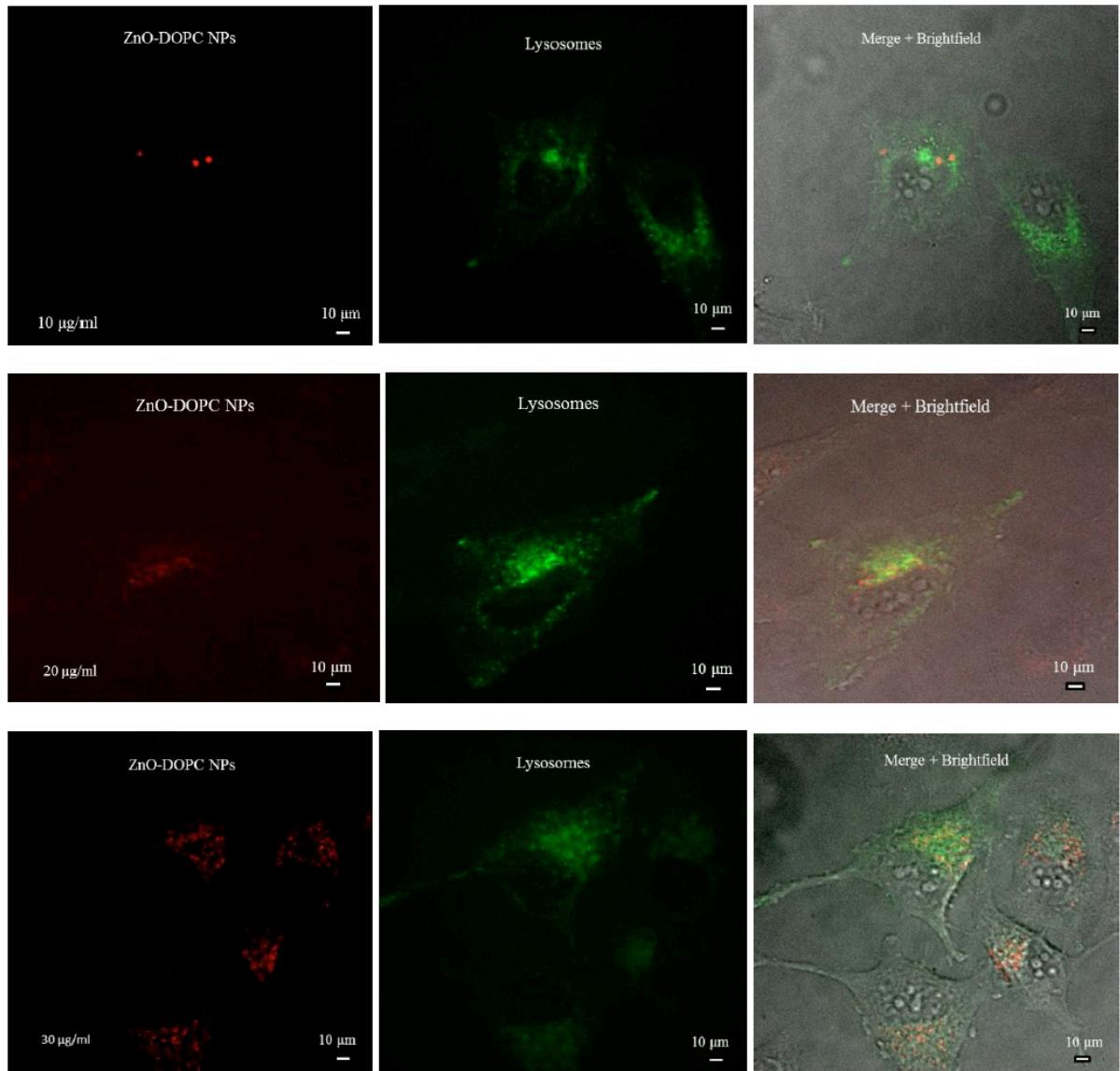


Fig. 4.22 Cell uptake of lipid-coated ZnO nanoparticles (ZnO-NH₂-DOPC + ATTO 633, red channel) in lysosomes (Lysosomes-GFP, green channel) after 5 hours of incubation; scale bar is 10 µm.

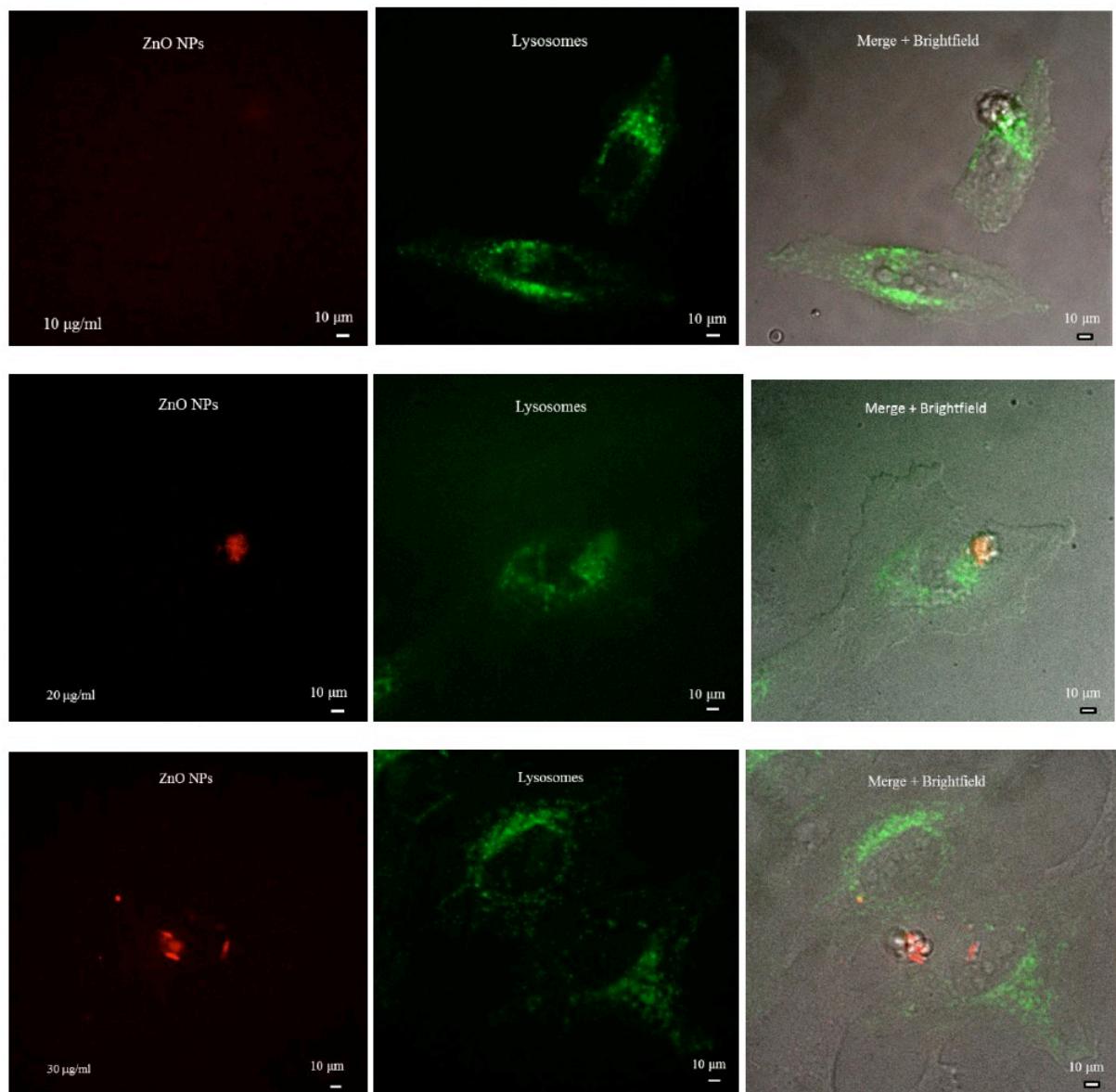


Fig. 4.23 Cell uptake of pristine ZnO nanoparticles (ZnO-NH₂ + ATTO 633, red channel) in lysosomes (Lysosomes-GFP, green channel) after 24 hours of incubation; scale bar is 10 µm.

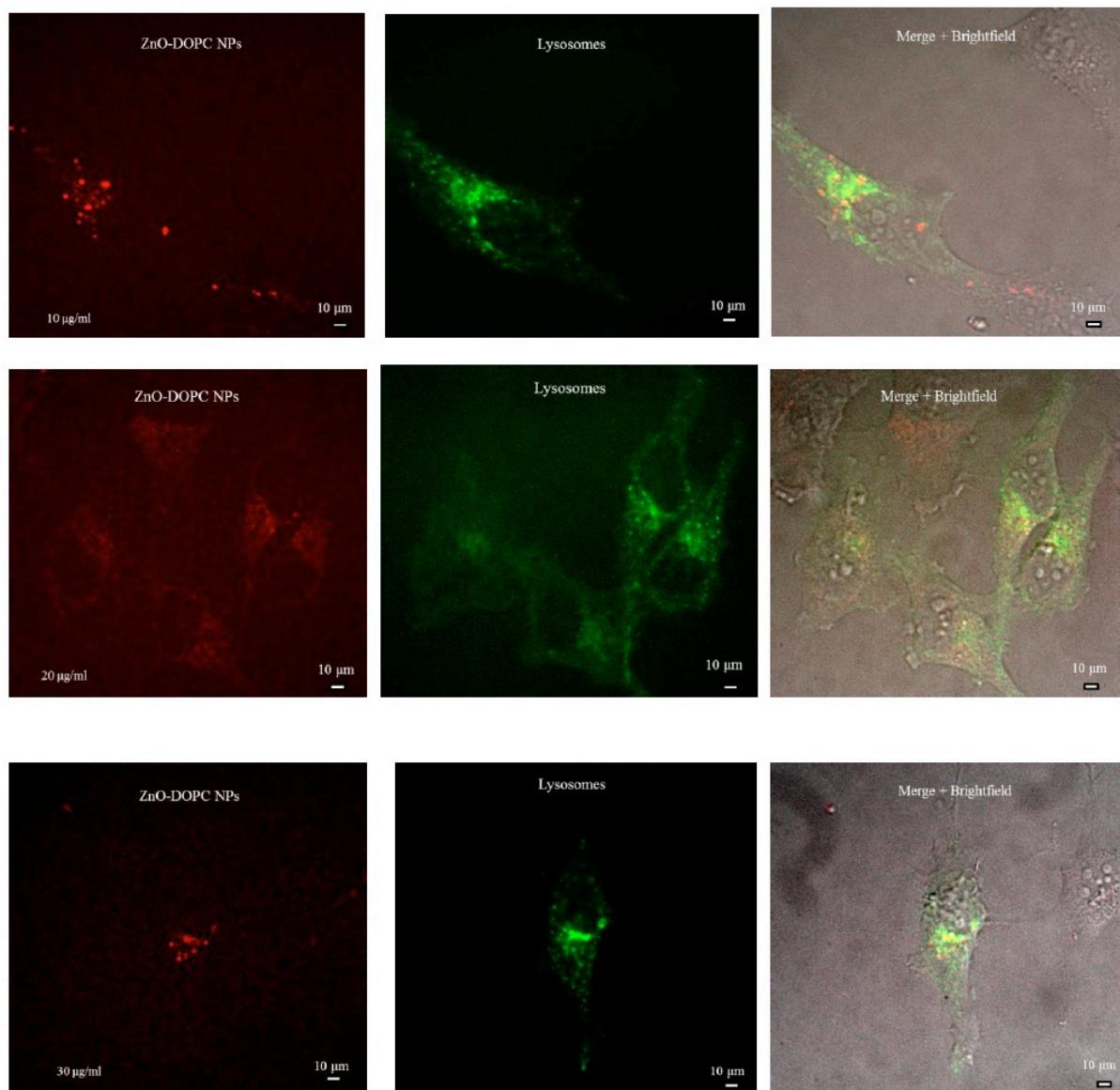


Fig. 4.24 Cell uptake of lipid-coated ZnO nanoparticles (ZnO-NH₂-DOPC + ATTO 633, red channel) in lysosomes (Lysosomes-GFP, green channel) after 24 hours of incubation; scale bar is 10 µm.

At this point, it is important to understand the therapeutic effect of the nanoparticles after they are internalized and co-localized in the lysosomes.

In a previous work, cytotoxicity and photodynamic effect of both bare ZnO and lipid-coated ZnO nanoparticles were investigated on HeLa cells [1]. The effects of different concentrations of ZnO and ZnO-DOPC nanoparticles on HeLa cell culture after 24 hours of incubation are shown in Fig. 4.25. From a quantitative point of view, these data confirm the cytotoxic behavior of both ZnO and ZnO-DOPC NPs even in the absence of UV light activation, but only at high NPs concentration (see the black and grey bars in the graph). The two kinds of ZnO NPs had no visible cytotoxic effect up to a concentration of about 18 µg/ml, while both showed a significant cytotoxic effect for higher concentrations. It is in particular worth of note the concentration at 36 µg/ml, close to the one used in this Master Thesis (30 µg/mL) where the higher citotoxicity is recorded for the ZnO-DOPC nanoparticles. As it was reported that the lipid coated NPs are able to efficiently enter into the cancer cells and are co-localized with lysosomes, thus leading to a high local concentration of Zn²⁺ cations, the toxic effect of these cations is here particularly observed. In contrast the pristine ZnO NPs are less internalized and thus can create less cytotoxic effects into the HeLa cells. Of course, to increase the citotoxic effect of ZnO nanomaterials in cancer cells, it is possible to exploit a stimuli-responsive behaviour (which was not however investigated during this Master Thesis, but it is reported for completeness). HeLa cell cultures irradiated with UV light (at a wavelength of 255 nm for 30 s) showed a marked decrease of cell viability even at lower concentrations of both pristine and lipid-coated ZnO NPs: these preliminary data suggest that ZnO, used at non-toxic concentrations, can induce cytotoxic effects only when irradiated with UV light.

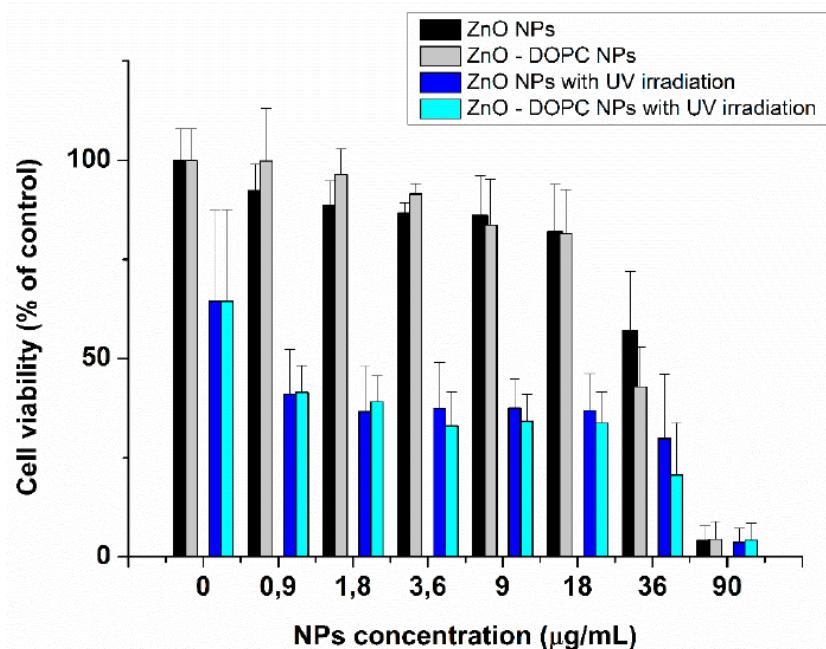


Fig. 4.25 Cytotoxicity and photodynamic effect of different concentrations of ZnO nanoparticles and lipid-coated ZnO nanoparticles with and without UV irradiation (30 s) (Modified from [1]).

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5. Conclusions

The use of nanotechnologies in the health science has allowed the development of new and effective therapeutic techniques for the treatment of highly critical diseases, such as cancer. In the anticancer therapy, nanoparticles are used for their size and chemical-physical properties, which make them able to exploit pathophysiological characteristics of the tumor areas to selectively target their action, obtaining important advantages based both on the efficacy and on the toxicity of the therapy [1].

Recently, zinc oxide nanostructures have received great attention in the biomedical field and in the nanomedicine thanks to different properties, which are very useful for the development of new diagnostic and therapeutic tools. In particular, the characteristics of biocompatibility, biodegradability, cytotoxicity, ease of synthesis and functionalization with suitable binders to increase their selectivity, make the zinc oxide nanoparticles good candidates to become potentially interesting anticancer agents [2].

This thesis project focuses on the study of the stability of bare and lipid-coated zinc oxide nanoparticles in various solvents, focusing the attention on the aggregation and biodegradation behaviour. Secondly, the uptake of these nanoparticles by Hela cells was considered.

In order to analyze the reactivity and stability of the nanoparticles, biodegradation tests were carried out in PBS (phosphate-buffered saline) and DPBS (Dulbecco's phosphate-buffered saline), both buffer solutions characterized by ionic strength and very high dissolved ion percentages, by which the cells are washed; then biodegradation tests were performed also in ALF (artificial lysosomal fluid), which simulates the lysosomal liquid.

The tests in PBS and DPBS show the tendency of the nanoparticles to aggregate in these fluids, since they strongly interact with both of them. From FESEM images the presence of aggregates is detected: the nanoparticles are covered by a thin layer of material already after one hour. X-ray analysis confirm the presence of foreign elements on the surface of the nanoparticles, particularly with phosphates and carbonates and the crystalline structure of zinc oxide is maintained. This aggregation behaviour is accompanied by a modest dissolution of the zinc oxide nanoparticles: the coating also decreases the Zn^{2+} species dissolved in the solution, which remain below 100 ppm for the duration of the tests.

The tests in ALF allow to evaluate the interaction of the nanoparticles with the simulated lysosomal fluid: this represents their final destination, after they have entered the cells. FESEM analysis show that after the first half hour the nanoparticles are dissolved and degraded and XRD analyses confirm that they lose their crystalline structure. ICP-MS analyses demonstrate that many Zn^{2+} (~ 1500 ppm) are delivered in simulated lysosomal fluid solution; moreover, they could be cytotoxic for the cells. This means that the nanoparticles could exert their cytotoxic activity already in the first hour, after they are colocalized within the lysosomes.

Through the use of the Dynamic Light Scattering (DLS) technique, the colloidal stability of the pristine and lipid-coated zinc oxide nanoparticles was evaluated: the coating prevents the nanoparticles from the contact with the liquid and therefore improves the stability of the nanoparticles, avoiding aggregation. In addition, the coating improves cell uptake, as this lipidic layer promotes the entry of nanoparticles into cells, forming a nanosized “Trojanhorse”.

While the pristine zinc oxide nanoparticles form many aggregates, that remain in the cell culture medium, so that the process of uptake is not successful, the hybrid nanoparticles enter the cells already after 5 hours and after 24 hours the majority of them are present in the lysosomes.

These tests are important to have information on the kinetics of the anticancer therapy: cell tests provide information on cellular uptake kinetics (after 5 hours the lipid-coated nanoparticles are found in the lysosomes), while the tests with the simulated lysosomal fluid give details on the kinetics of formation of cytotoxic species, such as Zn^{2+} ions, which within an hour after internalization in the lysosomes would be present and could exert their cytotoxic activity, leading to cell death.

The results show that a lipid bilayer improves the performance of the nanoparticles and it represent a beginning for further improvements in the effectiveness of the therapy.

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List of Figures

Figure 2.1 Different mechanisms of endocytosis process (Modified from [5]).....	6
Figure 2.2 Nanoparticles and antibodies used in antitumor therapy (Modified from [11])	7
Figure 2.3 Wurtzite crystal structure (Modified from [23]).....	9
Figure 2.4 Biomedical applications of zinc oxide (ZnO) nanoparticles (NPs) (Modified from [26]).....	10
Figure 2.5 Mechanisms of cytotoxic action of zinc oxide nanoparticles (ZnO NPs) (Modified from [26]).....	12
Figure 2.6 Scanning electron micrograph of just-divided HeLa cells (Modified from [40])... <td>14</td>	14
Figure 3.1 Experimental apparatus for the sol-gel synthesis.....	20
Figure 3.2 Functionalization with amino groups of zinc oxide nanoparticles' surface (modified from [5]).....	21
Figure 3.3 XRD Experimental Apparatus.....	22
Figure 3.4 Illustration of the dynamic light scattering principle [6].....	24
Figure 3.5 DLS Experimental Apparatus with the automatic titration set-up [7].....	25
Figure 3.6 ZnO nanoparticle internalized in a liposome, formed by a double layer of DOPC lipids [4].....	28
Figure 3.7 Yokogawa Electric Corporation CSU-X1 spinning disk [13].....	30
Figure 3.8 Schematic diagram of the basic optical configuration in Yokogawa scanning units [13].....	30
Figure 3.9 Fluorescence Microscope with 8-well slides for live-cell imaging experiments [16].....	32
Figure 4.1 FESEM images of bare and lipid-coated zinc oxide NPs (Modified from [1]); scale bar is 30 nm in both images.....	34
Figure 4.2 Representative X-ray diffractograms of the pristine (black spectrum) and lipid-coated ZnO NPs (red spectrum) with Miller indices (Modified from [1]).....	35
Figure 4.3 Hydrodynamic diameters and Z potentials of bare and lipid-coated ZnO NPs. Dynamic Light Scattering (a) and Z-potential (b) measurements of the two samples [1].....	36
Figure 4.4 FESEM images of pristine (left) and lipid-coated zinc oxide nanoparticles (right) in PBS biodegradation tests; from the top to the bottom sample images at 1 hour, 72 hours, 1 week.....	39

Figure 4.5 FESEM images of pristine (left) and lipid-coated zinc oxide nanoparticles (right) in DPBS biodegradation tests; from the top to the bottom sample images at 1 hour, 72 hours, 1 week.....	40
Figure 4.6 Diffractograms of pristine (top graph) and lipid-coated (bottom graph) zinc oxide nanoparticles in PBS biodegradation tests.....	41
Figure 4.7 Diffractograms of pristine (top graph) and lipid-coated (bottom graph) zinc oxide nanoparticles in DPBS biodegradation tests.....	42
Figure 4.8 FESEM images of pristine (left) and lipid-coated zinc oxide nanoparticles (right) in ALF biodegradation tests; from the top to the bottom sample images at 15 min, 30 min.....	43
Figure 4.9 Diffractograms of pristine (black spectrum) and lipid-coated (red spectrum) zinc oxide nanoparticles in ALF biodegradation tests.....	43
Figure 4.10 Concentration of Zn ²⁺ and PO ₄ ³⁻ ions in PBS (left) and in DPBS (right).....	44
Figure 4.11 Concentration of Zn ²⁺ ions in ALF	45
Figure 4.12 FT-IR spectra of pristine (top graph) and lipid-coated (bottom graph) ZnO nanoparticles in PBS biodegradation tests.....	46
Figure 4.13 FT-IR spectra of pristine (top graph) and lipid-coated (bottom graph) ZnO nanoparticles in DPBS biodegradation tests.....	47
Figure 4.14 The lipid-shell increases colloidal stability of ZnO NPs in PBS: (a) Dynamic Light Scattering (DLS) measurements in number percent of the bare (ZnO) and lipid-coated (ZnO-DOPC) nanoparticles in phosphate buffered saline (PBS); (b) mean hydrodynamic size (Z-average) of bare NPs (solid curve) and lipid-coated ZnO NPs (dotted curve) in PBS over time; and (c) derived count rate of the two samples over time.....	48
Figure 4.15 Mean hydrodynamic size (Z-average) of lipid-coated ZnO NPs in ALF over time.....	49
Figure 4.16 Derived count rate of lipid-coated ZnO NPs in ALF over time.....	50
Figure 4.17 Fluorescence microscopy images of zinc oxide nanoparticles (ZnO-NH ₂ + ATTO 488) internalized in liposomes (DOPC + DID, Lipophilic Indicator) through fusion protocol in PBS; scale bar is 10 μm.....	51
Figure 4.18 Fluorescence microscopy images of zinc oxide nanoparticles (ZnO-NH ₂ + ATTO 488) internalized in liposomes (DOPC + DID, Lipophilic Indicator) through fusion protocol in HEPES; scale bar is 5 μm.....	51
Figure 4.19 Fluorescence microscopy images of zinc oxide nanoparticles (ZnO-NH ₂ + ATTO 488) covered by a double lipidic bilayer (DOPC + DID, Lipophilic Indicator) through solvent exchange protocol; scale bar is 10 μm.....	52

Figure 4.20 Fluorescence microscopy images of zinc oxide nanoparticles (ZnO-NH ₂ + ATTO 488) covered by a double lipidic bilayer (DOPC + DID, Lipophilic Indicator) through solvent exchange protocol, well dispersed with ultrasounds; scale bar is 5 µm.....	52
Figure 4.21 Cell uptake of pristine ZnO nanoparticles (ZnO-NH ₂ + ATTO 633, in the red channel) in lysosomes (Lysosomes-GFP, in the green channel) after 5 hours of incubation; scale bar is 10 µm.....	54
Figure 4.22 Cell uptake of lipid-coated ZnO nanoparticles (ZnO-NH ₂ -DOPC + ATTO 633, red channel) in lysosomes (Lysosomes-GFP, green channel) after 5 hours of incubation; scale bar is 10 µm.....	55
Figure 4.23 Cell uptake of pristine ZnO nanoparticles (ZnO-NH ₂ + ATTO 633, red channel) in lysosomes (Lysosomes-GFP, green channel) after 24 hours of incubation; scale bar is 10 µm.....	56
Figure 4.24 Cell uptake of lipid-coated ZnO nanoparticles (ZnO-NH ₂ -DOPC + ATTO 633, red channel) in lysosomes (Lysosomes-GFP, green channel) after 24 hours of incubation; scale bar is 10 µm.....	57
Figure 4.25 Cytotoxicity and photodynamic effect of different concentrations of ZnO nanoparticles and lipid-coated ZnO nanoparticles with and without UV irradiation (30 s) (Modified from [1]).....	58

List of Tables

Table 3.1 PBS composition (modified from [9]).....	26
Table 3.2 DPBS composition (modied from [10]).....	26
Table 3.3 ALF composition (modified from [11]).....	26
Table 4.1 EDS analysis of bare and lipid-coated zinc oxide nanoparticles in PBS biodegradation tests; results expressed in atomic%.....	37
Table 4.2 EDS analysis of bare and lipid-coated zinc oxide nanoparticles in DPBS biodegradation tests; results expressed in atomic%.....	37
Table 4.3 Percentage of cell uptake for bare and lipid-coated ZnO NPs after 5 hours of incubation.....	53
Table 4.4 Table 4.2 Percentage of cell uptake for bare and lipid-coated ZnO NPs after 24 hours of incubation.....	53