UNIVERSITY OF NOVA GORICA
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IMPACT OF DISPERSION PREPARATION METHOD ON TOXICITY OF SELECTED NANOMATERIALS

DIPLOMA THESIS

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STATEMENT

This diploma thesis is a result of my own research work. Results which have been created/provided by other researchers either in collaboration or separately are properly cited in this thesis.

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Summary

Due to the unique physico-chemical properties of nanomaterials, development and production have dramatically increased recently, however, their safety aspect on the biological systems is still not clear. One of the main problems is how to define unified toxicity protocols to accurately interpret obtained results. Particle dispersion methods may potentially alter nanomaterials characteristic and thus, mechanism of action and behavior in biological systems. In this work, TiO_2 nanoparticles (P25 and PC500), inorganic nanotubes and nanowires (MoS_2 and MoO_3) were analyzed to investigate the impact of three dispersion protocols on the toxicity results. Cytotoxic and genotoxic effects were tested on cell culture model using MTT cell viability test and H2A.X histone phosphorylation test, while ecotoxicity measured bioluminescence inhibition in *Vibrio fischeri*. Our results show that in some cases the dispersion method influenced the results of toxicity studies. The presence of smaller agglomerates induced significantly higher cytotoxic effects. In genotoxicity testing, dispersion preparation dependent genotoxicity was observed for TiO_2 based nanomaterials, whereby protein serum coating decreased generation of γH2A.X. Molybdenum compounds did not activate DNA repair marker H2A.X phosphorylation in our experiments. Our data showed a certain ecotoxicity of the nanomaterials tested in bioluminescence inhibition assay, but further analyzes are necessary for better understanding of this complex work.

Key words: Ti- and Mo-based nanomaterials, secondary characterization, cytotoxicity, genotoxicity and ecotoxic evaluation, dispersion methods

Povzetek

Razvoj in proizvodnja nanomaterialov sta močno narasla v zadnjem času, predvsem zaradi njihovih posebnih fizikalno-kemijskih lastnosti, kljub temu pa je njihov škodljiv vpliv na biološke sisteme še vedno dokaj neznan. Eden glavnih problemov je določitev enotnih protokolov, ki bi omogočala pravilno interpretacijo rezultatov, pridobljenih s toksikološkimi raziskavami. Različne metode disperzije delcev lahko potencialno spremenijo lastnosti nanomaterialov in posledično tudi mehanizem delovanja in njihovega obnašanja v bioloških sistemih. Za določevanje vpliva treh protokolov priprave disperzije na rezultate toksikoloških študij smo testirali TiO_2 nanodelce (P25 in PC500) ter molibdenove anorganske nanocevke in nanožice (MoS_2 in MoO_3). Za testiranje citotoksičnosti in genotoksičnosti smo uporabili MTT test metabolne aktivnosti celic ter test fosforilacije histona H2A.X, medtem ko smo za določevanje ekotoksičnosti merili inhibicijo bioluminiscence v bakteriji *Vibrio fischeri*. Naši rezultati kažejo, da v nekaterih primerih metoda disperzije lahko vpliva na rezultat toksikoloških študij. Manjša aglomeracija v primeru citotoksičnosti je povzročila znatno višje učinke. Genotoksični učinki, odvisni od metode priprave disperzije, so bili dokazani za TiO_2 nanomateriale, pri čemer disperzije, ki so vsebovale proteinski serum, niso povzročile genotoksičnosti. Molibdenove spojine niso aktivirale fosforilacije H2A.X, kazalca DNK popravljalnega mehanizma. Podatki za inhibicijo bioluminiscence nakazujejo določeno ekotoksičnost nanomaterialov, pri čemer bo potrebna nadaljnja analiza za razumevanje tega kompleksnega delovanja.

Ključne besede: nanomateriali na osnovi Ti in Mo, sekundarna karakterizacija, citotoksičnost, genotoksičnost in ocena ekotoksičnosti, metode disperzije

Obsežen povzetek v slovenščini

Vpliv metode priprave disperzije na toksičnost izbranih nanomaterialov

Diplomsko delo

TEORETIČNE OSNOVE

V zadnjih nekaj letih so se razvoj, tehnologija in proizvodnja nanomaterialov drastično povečali. Po definiciji Znanstvenega odbora za nastajajoča in na novo ugotovljena zdravstvena tveganja (Scientific Committee on Emerging and Newly Identified Health Risks-SCENIHR, 2007a) so nanomateriali definirani kot vsaka oblika materiala, ki je sestavljena iz diskretnih funkcionalnih delov, kateri imajo eno ali več dimenzij v obsegu 100 nm ali manj. Na drugi strani so po tej isti definiciji nanodelci definirani kot vsaka oblika materiala, sestavljena iz diskretnih funkcionalnih delov, ki imajo vse tri dimenzije v obsegu 100 nm ali manj. Tovrstne definicije so za raziskave o ocenah tveganja in toksikološke raziskave primernejše od drugih, zato so kot take tudi uporabljene v tem diplomskem delu.

Buzea in sod. (2007) so izpostavili dva faktorja, ki povzročata, da se nanomateriali obnašajo signifikantno drugače kot ostali materiali večjih dimenzij. Ta dva faktorja sta:

- Površinski efekti (specifična površina in delež atomov na površini sta znatno večja pri nanodelcih kot pri večjih delcih, kar vpliva na različne lastnosti, kot so povišana reaktivnost, katalitična aktivnost, sprememba temperature vrelišča in sprememba topnosti ter druge).
- Kvantni efekti (opisujejo fizikalne lastnosti elektrona v trdninah, katerim se je močno zmanjšala velikost, kot posledica se spremenijo optične, magnetne, električne in mehanske lastnosti materiala).

Prav te lastnosti omogočajo široko uporabnost nanomaterialov, kar botruje k njihovem intenzivnem razvoju (Stone in sod., 2010). Nanotehnologije tako omogočajo manipulacijo snovi na ravni atomov in odkrivajo nove lastnosti in funkcije materialov, ki bi lahko povečale njihovo uporabnost (SCENIHR, 2007a). Te lastnosti omogočajo možnosti za ogromen napredek, tako v vsakdanji uporabi kot tudi v znanosti in informacijskih tehnologijah (Buzea in sod., 2007).

Nanotehnologija se dandanes uporablja na različnih področjih, kot so: elektronika in komunikacije, materiali in gradbeništvo, stroji in orodja, farmacevtsko in zdravstveno varstvo, okolje in voda ter energetika (OECD, 2009a).

Posebne fizikalno kemijske lastnosti nanomaterialov, ustvarjene z namenom uporabe v različnih aplikacijah, pa so največkrat tudi lastnosti, ki izzovejo toksične učinke (SCENIHR, 2009). Lewinsky in sod. (2010) so poudarili, da medtem ko število vrst nanomaterialov in njihovih aplikacij nezadržno narašča, pa je v primerjavi študij, ki bi opredelile njihove škodljive učinke po izpostavljenosti, zelo malo. Zaradi tega je izrednega pomena, da se standardizirano določi toksičnost nanomaterialov, kar pa zahteva podrobno primarno in sekundarno karakterizacijo njihovih lastnosti.

Trenutno še vedno ostajajo odprta vprašanja, katere lastnosti je potrebno karakterizirati, ko določamo toksične učinke nanomaterialov (Thomas in sod., 2006;

OECD, 2008; SCENIHR, 2007a; SCENIHR, 2007b; SCENIHR, 2009). Med fizikalne lastnosti, ki jih je potrebno določiti, se na podlagi predloga Organizacije za gospodarsko sodelovanje in razvoj (Organisation for Economic Co-operation and development-OECD, 2008) tako uvrščajo velikost, oblika, specifična površina, aglomeracijsko/agregacijsko stanje, porazdelitev velikosti, morfologija/topografija površja, kristalinična struktura in topnost. Za vedenje nanomaterialov in za učinke le-teh v bioloških in ekoloških sistemih pa so ključne lastnosti: dinamičnost disperzije, stopnja razpada, značilnosti agregatov, površina in zmožnost adsorpcije snovi na površino nanomaterialov (SCENIHR, 2007b). Kemijska karakterizacija naj bi se osredotočala na študijo kemičnih lastnosti površine (naboj, trenje, reaktivnost, fizična struktura, fotokatalitične lastnosti, zeta potencial) ter sestavo (stopnja čistosti, znani aditivi in nečistoče), strukturno/molekulsko formulo in hidrofilnost/lipofilnost (OECD, 2008).

Med najvidnejšimi in najpomembnejšimi fizikalno kemijskimi lastnostmi pri določevanju toksičnih učinkov nanomaterialov v bioloških sistemih je porazdelitev velikosti delcev v disperziji. Za določanje le-te obstaja veliko različnih metod, med katerimi pa je najpogosteje uporabljena metoda dinamičnega sipanja svetlobe (DLS) (Lamberty in sod., 2011; Couteau in sod., 2010).

Za določevanje toksičnih efektov nanomaterialov se uporablja mnogo različnih pristopov in metod, kar pa vodi v pridobitev neprimerljivih rezultatov. Ta neskladnost nakazuje potrebo po uvedbi standardiziranih testov, če želimo pridobiti primerljive rezultate pri določanju toksičnosti nanomaterialov (Oberdörster in sod., 2005). Kot priporočeno s strani OECD (2009b) obstaja torej velika potreba po razvoju novih ali spremembi obstoječih protokolov, ki bi bili primerni in ustrezni za *in vitro* testiranje toksičnosti nanomaterialov.

Primerljivost rezultatov različnih študij v preteklosti je bila močno ovirana predvsem zaradi dejavnikov, ki so vključevali različne metode priprave disperzij, različne vrste toksikoloških testov, koncentracije, čase izpostavljenosti in uporabo različnih celičnih linij. Velikokrat pa je bila primerljivost ovirana tudi zaradi pomanjkljivih informacij o primarni in sekundarni karakterizaciji kot tudi o podrobnostih metode priprave disperzije nanomaterialov.

Nanodelci v fizioloških raztopinah z določeno slanostjo in pH vrednostjo, ki je kompatibilna z biološkimi študijami, tvorijo aglomerate mikrometrskih velikosti, ki imajo različne lastnosti in lahko izzovejo drugačne biološke učinke od nanodelcev v optimalno disperzijskem stanju.

Ker lahko različni testni pogoji vplivajo direktno na lastnosti nanomaterialov in posledično tudi na izide toksikoloških študij, je izrednega pomena, da se metode priprave disperzije standardizirajo in da se upošteva vpliv različnih metod priprave disperzije na toksičnost nanomaterialov.

Pri testiranju toksičnosti v bioloških sistemih je potrebno nanomateriale raztopiti v ustreznem mediju z določeno stopnjo slanosti in pH vrednostjo, pri čemer pa so fizikalno kemijske lastnosti nanomaterialov, ki so vzrok toksičnosti, v veliki meri odvisne od lastnosti medija in načina disperzije. Interakcija takšnega medija in nanomateriala ima lahko signifikanten učinek na vedenje disperzije in posledično na toksičnost (SCENIHR, 2009).

Nanomateriali v raztopinah tvorijo aglomerate in/ali agregate, še posebej kadar so v obliki praškov v suhih pogojih (SCENIHR, 2009). Aglomeracija se odraža v učinkoviti odstranitvi nanofrakcije iz disperzije. Če želimo kvantificirati stabilnost nanodelcev v okolju, moramo najprej predvideti stabilnost v disperziji in njihovo težnjo po aglomeraciji, agregaciji ali po interakciji z drugimi molekulami (Nowack in Bucheli, 2007).

Kako dispergirati nanomateriale, še vedno ostaja odprta debata. Večina nanomaterialov je zelo slabo topnih, zato je potrebno uporabiti različne metode disperzije. Veliko znanstvenih raziskav je proučevalo, kako se izogniti tvorbi aglomeratov v fizioloških raztopinah, primernih za biološka testiranja. Poudarek je predvsem na uporabi primerne energije pri sonifikaciji in uporabi stabilizatorjev disperzije (Pohl in sod., 2004; Mandzy in sod., 2005; Bihari in sod., 2008; Buford in sod., 2007).

Stabilnost disperzije delcev je odvisna od ravnotežja med privlačnimi in odbojnimi silami med delci. V principu obstajata dva načina priprave stabilnih raztopin: elektrostatična in sterična stabilizacija. Pri elektrostatični stabilizaciji zeta potencial delcev zagotavlja odbojno silo. Zeta potencial je močno odvisen od pH-ja in koncentracije elektrolitov v disperziji. Pri pH-ju, ki ga imajo fiziološke raztopine, zeta potencial ni zadosten za stabilizacijo disperzije in nanodelci tvorijo aglomerate. Da to preprečimo, se lahko poslužujemo sterične stabilizacije, kjer dodamo stabilizator, ki se adsorbira na površje delcev in jim tako preprečuje, da bi se fizično približali eden drugemu (Bihari in sod., 2008).

Metoda sonifikacije je kritizirana s strani nekaterih raziskav, ki argumentirajo svoje nestrinjanje s tem, da sonifikacija vsekakor ni naraven pojav in da takšnemu načinu disperzije primanjkuje okoljske relevantnosti. Prav tako obstajajo kritike pri uporabi stabilizatorjev disperzije. Čeprav je uporaba naravnih stabilizatorjev vsekakor bolj okoljsko relevantna kot uporaba sintetičnih, pa ima kljub temu vsako sredstvo lahko potencialen vpliv na dinamiko obnašanja nanomaterialov v raztopini. Poleg tega lahko kakršenkoli ovoj okoli delcev spremeni interakcijo med delci in celičnim površjem in tako spremeni biološko razpoložljivost nanomaterialov in posledično njihove toksične učinke (Handy in sod., 2012).

Za ponazoritev scenarija, pri katerem pričakujemo najvišjo toksičnost, moramo opazovati delce v raztopinah s čim manjšo aglomeracijo, t.i. optimalnih disperzijah. Za pripravo optimalne disperzije v fiziološki raztopini so potrebni naslednji koraki v točno določenem zaporedju (Bihari in sod., 2008):

1. sonifikacija v deionizirani vodi, pri čemer mora biti energija ultrazvoka zadostna za deaglomeracijo (>4.2×10^5 kJ/m^3),
2. dodatek stabilizatorja disperzije (dodatek raztopine z visoko vsebnostjo proteinov, ki vsebuje albumin ali serum, z zadostno koncentracijo, da pokrije vso površino nanodelcev),
3. na koncu dodatek puferske solne raztopine.

Optimalna disperzija torej vključuje dodatek albumina, ki je sicer naravno prisoten protein v krvnem obtoku, zato ni verjetnosti interference zaradi nerealistične izpostavitve stabilizatorju (Bihari et al., 2008). Kakorkoli pa albumin po inkubaciji skupaj z nanodelci kot rezultat tvori t.i. proteinsko korono. Na takšen način je obnašanje nanodelcev določeno z obnašanjem in lastnostmi proteinske korone in njenih interakcij z biomolekulami in ne z obnašanjem samih nanodelcev (Lynch in Dawson, 2008).

Celična internalizacija nanodelcev je pomembna, ker lahko poveča toksičnost zaradi interakcij z normalno celično fiziologijo in funkcijo. V nekaterih primerih lahko pride do internalizacije delcev, ki pa ne povzroči nobenega učinka. Toksičnost delcev v celici je torej odvisna od lokalizacije v celici in količine vnosa. Možno je tudi delovanje nanodelcev na celico od zunaj navznoter, ki vključuje interakcije delcev s celičnim površjem in povzročanjem mehanske škode na celicah (Johnston in sod., 2009).

CILJI DIPLOMSKEGA DELA

- Izvesti sekundarno karakterizacijo (s tehniko dinamičnega sipanja svetlobe-DLS) izbranih nanomaterialov (TiO_2 P25 in TiO_2 PC500 nanodelcev, MoS_2 nanocevk in MoO_3 nanožic), pripravljenih z uporabo treh različnih metod disperzije, z namenom določitve njihove porazdelitve velikosti in potrditve razlik v aglomeraciji med tremi različnimi metodami priprave disperzije.

- Določiti citotoksičnost (z uporabo MTT testa metabolne aktivnosti celic), genotoksičnost (z uporabo testa fosforilacije histona H2A.X) ter ekotoksičnost (z uporabo testa inhibicije bioluminiscence v bakteriji *Vibrio fischeri*) izbranih nanomaterialov.

- Diskutirati, ali metoda disperzije vpliva na rezultat citotoksikoloških, genotoksikoloških in ekotoksikoloških testov s primerjavo toksikoloških rezultatov različnih protokolov disperzije.

- Demonstrirati povezavo med porazdelitvijo velikosti delcev, dobljeno s sekundarno karakterizacijo, in toksičnostjo, pri čemer se ne izključuje tudi možnih učinkov zaradi različnih časov izpostavljenosti, kristalinične velikosti in faze (za TiO_2 vzorce).

PRAKTIČNI DEL

Testirani nanomateriali

Kot referenčni material smo v naši raziskavi uporabili TiO_2 nanodelce Millennium PC500 in Degussa P25, saj so TiO_2 nanodelci najbolj raziskana in uporabljena skupina nanomaterialov (Johnston in sod., 2009). Njihova široka uporaba sega v področja okoljskih in energetskih aplikacij, ki vključujejo fotokatalitično čiščenje odpadne vode, razgradnjo pesticidov in sodelovanje v reakcijah pri proizvodnji vodika (Gupta in Tripathi, 2011).

PC500 je sestavljen iz anatasa (>99.5%) in ima manjšo kristalinično velikost, medtem ko P25 sestavlja 80% anatasa in 20% rutila, njegova kristalinična velikost pa je skoraj šestkrat večja od velikosti kristalov PC500 (Gumy in sod., 2006).

Iz skupine anorganskih molibdenovih spojin smo kot testni material uporabili MoS_2 nanocevke in MoO_3 nanožice. Te molibdenove anorganske spojine so bile proizvedene in za skupne namene testiranja toksičnosti donirane s strani Dr. Maje Remškar z Inštituta Jožef Stefan v Ljubljani.

MoS_2 nanocevke z votlimi strukturami, podobnimi fulerenom, kažejo odlične uporabne lastnosti v aplikacijah kot trdni lubrikanti, v električnih napravah, kot katalizatorji itd. (Remškar in sod., 2007). Te spojine so ekstremno anizotropične s plastovito strukturo. Šibke interakcije, ki ohranjajo plasti, so predvsem van der Waalsove privlačne sile. Molekulska plast S-Mo-S kaže trigonalno simetrijo. Atom prehodne kovine je koordiniran s šestimi žveplovimi atomi, ki so pozicionirani v kotih trigonalne prizme (Remškar in Mrzel, 2004). Nanocevke služijo dvema vlogama: kot nanoreaktorji in nanokontejnerji. Zaradi zelo tankih sten, ki se lahko podrejo ob vzbujanju z ultrazvokom, se strukture, podobne fulerenom, sprostijo na kontroliran način. Ta posebna morfologija odgovarja na mnoga vprašanja povezana z varno proizvodnjo, shranjevanjem in transportom teh nanomaterialov (Remškar in sod., 2007).

Stena nanocevke ima debelino okoli 13 nm, dolžino okoli 10 μm in premer okoli 100-500 nm, medtem ko velikosti struktur, podobnih fulerenom, znotraj nanocevk segajo od 40 nm pa tja do približno 300 nm (Remškar in sod., 2007).

MoO_3 je polprevodnik, ki ima energijsko vrzel okoli 3 eV. Molibdenovi oksidi so lahko dobri katalizatorji in visoko občutljivi plinski senzorji, njihova proizvodnja in sinteza pa je trenutno pod drobnogledom, predvsem ker bi z njihovo uporabo lahko pridobili mnoge nadomestke za drage in redke kovine (Suemitsu in Abe, 2010).

Premeri in dolžine MoO_3 nanožic so približno enaki kot pri MoS_2, saj je bil za sintezo obeh spojin uporabljen enak material.

Priprava disperzij

Referenčne in testne nanomateriale smo pripravili na tri načine s tremi različnimi metodami disperzije. Prva metoda je bila optimalna metoda disperzije, ki je vključevala sonificiranje ter dodatek proteinskega stabilizatorja disperzije. Druga metoda disperzije je vključevala le sonificiranje, medtem ko je bila tretja disperzija pripravljena z mešanjem. Vsem disperzijam smo na koncu dodali fosfatno-pufersko solno raztopino, da smo dosegli vsebnost soli in pH vrednost, ki je primerna za biološka testiranja.

Sekundarna karakterizacija

Po pripravi disperzij je sledila sekundarna karakterizacija le-teh, kjer smo za določevanje porazdelitve velikosti delcev uporabili metodo dinamičnega sipanja svetlobe (DLS). DLS meritev je bila opravljena pod nadzorom Prof. Dr. Nataše Novak Tušar s Kemijskega Inštituta v Ljubljani. Princip te metode je osnovan na študiji intenzitete fluktuacij svetlobe pri prehajanju skozi disperzijo z nanomateriali, ki so podleženi Brownovem gibanju. Fluktuacije so povezane z difuznim koeficientom in tako s premerom delcev (Couteau in sod., 2010).

Določevanje toksičnosti

Določevanje citotoksičnosti, genotoksičnosti in ekotoksičnosti posameznih nanomaterialov je bilo vezano na testiranje različnih metod priprave disperzij.

Citotoksičnost je bila določena z uporabo MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolijev bromid) kolorimetričnega testa celične metabolne aktivnosti, pri čemer smo

uporabili celično linijo človeških embrionalnih ledvičnih celic (Human Embryonic Kidney cells-HEK 293) za testiranje koncentracij 1, 10, 100 in 1000 mg/L ob različnih časih izpostavljenosti (24, 48 in 72 ur). Vsak vzorec je bil testiran v treh ponovitvah.

Princip metode je osnovan na absorpciji MTT soli v celice, kjer se v mitohondrijih reducira v netopen vijoličen formazan s pomočjo NADH reduktaze in flavin oksidaze. V procesu MTT metabolizma so samo celice z delujočim metabolizmom sposobne redukcije MTT soli v netopen produkt, ki ga lahko karakteriziramo z merjenjem absorbance pri valovni dolžini 570 nm. Izmerjena absorbanca nam tako služi kot ocena aktivnosti metabolizma in s tem povezane viabilnosti celic (Lü in sod., 2012).

Vrednosti z nižjim razmerjem absorbanc ($A_{test\ povprečje}/A_{kontrola\ povprečje}$) kot kontrola kažejo na zmanjšanje števila aktivnih mitohondrijev (ki korelira s celično smrtjo ali upočasnitvijo metabolne aktivnosti), medtem ko razmerja, višja od absorbance kontrole, nakazujejo pospešitev celičnega metabolizma (celično proliferacijo).

Za določevanje genotoksičnosti smo uporabili test fosforilacije histona H2A.X. Aktivacija fosforilacije histona H2A.X je eden od zgodnjih znakov odzivov popravljalnih mehanizmov na poškodbe DNK. Fosforilacija histona se zgodi v nekaj minutah po poškodbi, kar ga uvršča med uporabne in občutljive indikatorje DNK poškodb (Garcia-Canton in sod., 2012). Prednost testa je tudi v tem, da do fosforilacije lahko pride v kateremkoli delu celičnega cikla, tudi med mitozo (Nakamura in sod., 2010).

Za preverjanje sposobnosti generiranja fosforilirane oblike H2A.X smo uporabili celično linijo HEK 293, pri čemer smo testirali koncentracijo 1000 mg/L v dveh ponovitvah.

Za označevanje jeder je bila DNK obarvana s Hoechst 33342. Imunofluorescenčni signali jeder in fosforiliranih oblik H2A.X so bili analizirani z uporabo rešetanja visokih zmogljivosti (High Content Analysis-HCA) in uporabo Metamorph programske opreme. S pomočjo avtomatskega fluorescenčnega mikroskopa smo dobili 9 mikrofotografij obarvanih celic za posamezen testiran vzorec, pri čemer so bile uporabljene 20x leče.

S to metodo smo dobili informacije o številu celic, proliferaciji ter pa informacije o obsegu aktivacije DNK popravljalnih mehanizmov.

Za testiranje ekotoksičnosti smo uporabili test inhibicije bioluminiscence v bakteriji *Vibrio fischeri* (DIN EN ISO 11348-3), ki je eden izmed najbolj pogosto uporabljenih testov v ekotoksikologiji (Mortimer in sod., 2008). Test je osnovan na merjenju produkcije svetlobe, ki je direktno sorazmerna z metabolno aktivnostjo bakterijske populacije. Vsakršna inhibicija encimske aktivnosti po izpostavljenosti nanomaterialom v primerjavi s kontrolo povzroči ustrezno zmanjšanje v intenziteti bakterijske bioluminiscence. Test tako zagotavlja merjenje sub-letalnih odzivov (Parvez in sod., 2006).

Uporabljeni bakterijski test je izredno hiter, pri čemer dobimo rezultate, ki so primerljivi z drugimi metodami. Odlikujeta ga tehnična preprostost ter občutljivost, kar kaže na njegovo potencialno široko uporabnost pri testiranju različnih nanomaterialov (Zheng in sod., 2010).

Vsi pridobljeni rezultati so bili analizirani s pomočjo programa MS Excel. Najprej smo uporabili F-test za variance, na podlagi katerega smo potem določili vrsto Student t-testa. Odzivi so bili ovrednoteni kot signifikantno različni od kontrole v primerih, kjer je p vrednost znašala manj kot 0.05.

REZULTATI IN DISKUSIJA

Sekundarna karakterizacija (metoda dinamičnega sipanja svetlobe)

Naša raziskava je potrdila izsledke preteklih študij, kjer so opazili, da se sekundarne velikosti (hidrodinamični premeri) nanomaterialov v disperziji močno razlikujejo od primarnih velikosti (premerov), ki jih deklarirajo proizvajalci. Vsi nanomateriali v naši študiji so bili v disperziji prisotni v obliki aglomeratov, vendar pa je prišlo do razlik v velikosti aglomeratov med različnimi metodami priprave disperzije. Nanodelci niso bili prisotni v nobeni od disperzij, zato rezultati testov toksičnosti niso povezani s toksičnostjo samih nanodelcev, ampak s toksičnostjo njihovih aglomeratov.

Primerjava rezultatov številčnih porazdelitev velikosti med različnimi metodami disperzije je pokazala, da je najnižja stopnja aglomeracije dosežena pri pripravi optimalne disperzije (sonifikacija+stabilizator), medtem ko so bili glede na prostorninsko porazdelitev najmanjši aglomerati najdeni pri uporabi metode disperzije, ki je vključevala le sonifikacijo. Vzrok temu je verjetno proteinski ovoj, ki ga stabilizator tvori okoli delcev in s tem poveča njihovo prostornino. Ko primerjamo porazdelitve velikosti v disperziji, ki je bila pripravljena z mešanjem, lahko ugotovimo, da so prisotni aglomerati precej večji kot pri ostalih dveh metodah disperzije.

Glede na dobljene rezultate pričakujemo večjo toksičnost disperzij z manjšimi aglomerati, vendar pa moramo izpostaviti dejstvo, da proteinski ovoj lahko pripomore k zmanjšani reaktivnosti površine delcev in tako povzroči manjšo toksičnost.

Ugotavljanje citotoksičnosti

V naši raziskavi smo opazili, da je citotoksičnost TiO_2 nanodelcev odvisna od primarne (kristalinične) velikosti oz. relativne površine in kristalinične faze, saj so delci manjših primarnih velikosti (približno 8 nm) z večjo relativno površino (335 m^2/g) in večjim deležem anatazne faze (>99.5%) v njihovi strukturi (PC500) povzročili signifikantne citotoksične učinke, medtem ko jih večji delci (približno 25-40 nm) z manjšo relativno površino (50 m^2/g) in z manj anatazne faze (80%) v njihovi strukturi (P25) niso. Predhodne študije so prav tako potrdile večji toksični efekt anatazne faze (Dunford in sod., 1997; Nakagawa in sod., 1997; Wang in sod., 2007; Warheit in sod., 2007).

Pri PC500 citotoksičnost ni bila opažena pri vseh metodah priprave disperzije in različnih časih izpostavljenosti, kar nakazuje, da citotoksičnost TiO_2 nanodelcev ni odvisna le od primarnih velikosti delcev in kristalinične faze, ampak tudi od metode priprave disperzije (velikosti aglomeratov) in časa izpostavljenosti vzorcem.

MoS_2 nanocevke in MoO_3 nanožice so pokazale signifikantne citotoksične učinke na HEK 293 celično linijo. Za MoO_3 so bili signifikantni učinki na celično viabilnost opaženi pri koncentracijah nad 100 mg/L, razen v primeru 48 urne izpostavljenosti optimalni disperziji, kjer je bila signifikantna toksičnost zabeležena že pri koncentraciji 10 mg/L. Pri 24 urni izpostavljenosti spojinam MoS_2 ni bilo opaziti signifikantne toksičnosti. Signifikantno znižanje metabolne aktivnosti je bilo moč opaziti pri 48 urni (pri koncentracijah nad 10 mg/L) in 72 urni izpostavljenosti (pri koncentracijah nad 1 mg/L). Pridobljeni rezultati sovpadajo z redkimi študijami, ki so testirale toksičnost teh spojin (Braydich-Stolle et al., 2005; Hussain et al., 2005; Wu et al., 2011).

Ta toksičnost bi bila lahko povezana z njihovo močno anizotropično obliko in plastovito strukturo. Vendar pa je na tem mestu potrebno poudariti, da je pri testiranju najvišjih koncentracij (1000 mg/L) lahko prišlo do možnih interferenc zaradi absorbcije molibdenovih anorganskih spojin v območju 570 nm, torej v območju, kjer absorbira tudi formazan. Absorpcija testiranih spojin MoS_2 in MoO_3 je potencialno vplivala na rezultat MTT testa, in sicer je bila izmerjena absorpcija lahko posledica absorpcije formazana in absorpcije nanospojin, zato je pri interpretaciji rezultatov pomembna previdnost.

S primerjavo citotoksičnih rezultatov za 24, 48 in 72 urne teste smo opazili, da čas izpostavljenosti vzorcem signifikantno vpliva na citotoksičnost spojin TiO_2 PC500 in MoS_2, pri čemer je daljši čas izpostavljenosti (72 ur) izzval višje učinke kot krajši (24 ur). V primeru 24 urne izpostavljenosti MoS_2 spojinam ni bilo opaženih signifikantnih citotoksičnih učinkov, medtem ko so bili ob 48 urni izpostavljenosti signifikantni toksični učinki zabeleženi pri koncentraciji 100 mg/L, ob 72 urni izpostavljenosti pa že pri 10 mg/L. Pri TiO_2 PC500 nanodelcih je 48 urni test pokazal signifikantno toksičnost večih metod priprave dispezij pri koncentraciji 1000 mg/L kot ob 24 urni izpostavitvi pri enaki koncentraciji. 72 urni test pa je signifikantne citotoksične učinke izzval že pri koncentraciji 100 mg/L.

Različne metode priprave disperzije vplivajo na različno stopnjo aglomeracije in reaktivnost, kar igra pomembno vlogo pri toksičnih učinkih in njihovi interpretaciji (Somasundaran in sod., 2010; Magdolenova in sod. 2012; Laban in sod., 2010; Malhi, 2012).

Citotoksičnost v naši raziskavi je odvisna od metode priprave disperzije, pri čemer so disperzijski protokoli z manjšo aglomeracijo (sonifikacija+proteinski stabilizator ali samo sonifikacija) pokazali signifikantno višje citotoksične učinke kot disperzije, ki so vsebovale večje aglomerate (pripravljene z metodo mešanja). Tak zaključek velja za vse testirane nanomateriale.

Ugotavljanje genotoksičnosti

Celice, izpostavljene koncentraciji 1000 mg/L TiO_2 P25 nanodelcev, so pokazale signifikantno aktivacijo DNK popravljalnih mehanizmov (H2A.X fosforilacijo), razen v primeru uporabe optimalne metode priprave disperzije, kjer smo poleg sonifikacije dodali še proteinski stabilizator disperzije (albumin). TiO_2 PC500 nanodelci niso pokazali genotoksičnosti pri analizi deleža pozitivnih jeder H2A.X, vendar pa je bilo opaženo signifikantno povišanje intenzitete H2A.X signala pri disperziji, pripravljeni z mešanjem. Molibdenove spojine niso aktivirale DNK popravljalnega markerja (fosforilirane oblike H2A.X) v našem eksperimentu.

Če primerjamo genotoksične učinke TiO_2 spojin med posameznimi metodami priprave disperzij, lahko zaključimo, da pri optimalnih disperzijah, ki vključujejo dodatek proteinskega stabilizatorja, ni prišlo do genotoksičnih učinkov, kar je v skladu z rezultati objavljenih študij (Toyooka in sod. 2012; Magdolenova in sod., 2012), kjer disperzije z dodanim albuminom, ki ustvari ovoj okoli delcev in tako zmanjša reaktivnost površja le-teh, niso povzročile škodljivih učinkov v različnih genotoksičnih testih.

Rezultati testiranja ekotoksičnosti

TiO_2 nanodelci so pokazali visoko inhibicijo bioluminiscence bakterije *Vibrio fischeri* pri testirani koncentraciji 1000 mg/L, kar nakazuje na visoko ekotoksičnost teh spojin v primeru tako visokih koncentracij (za TiO_2 P25 98.86% za optimalno metodo disperzije, 99.09% za disperzije, pripravljene s sonifikacijo, in 97.85% za disperzije, pripravljene z mešanjem; za PC500 pa 94.94% za optimalno metodo disperzije, 92.67% za disperzije, pripravljene s sonifikacijo, in 84.60% za disperzije, pripravljene z mešanjem). Pri spojini P25 je bilo moč zaznati signifikantne razlike v toksičnosti med pripravo disperzije, ki je vključevala sonifikacijo in tisto, ki je vključevala mešanje. V nasprotju z našimi izsledki pa so pretekle študije večinoma poročale o nizki ekotoksičnosti TiO_2 nanodelcev in majhni inhibiciji bioluminiscence (Pereira in sod., citirano po Lopes in sod., 2012; Lopes in sod., 2012; Heinlaan in sod., 2008; Garcia in sod., 2011; Velzeboer in sod., 2008).

Vzrok za razlike med našimi rezultati in rezultati preteklih študij lahko izhaja iz motnosti vzorcev, ki povzroča interference z merjenjem intenzitete luminiscence. Naši vzorci so bili zelo motni, saj smo za testiranje uporabili visoko koncentracijo 1000 mg/L. Če bi se želeli izogniti interferenci zaradi motnosti, bi bilo potrebno vzorce pred merjenjem centrifugirati ali filtrirati, za kar bi morali uporabiti luminometer, opremljen z razpršilnikom, regulatorjem in mešalnikom (Parvez in sod., 2006). Za bolj podroben uvid v ekotoksičnost teh materialov bi bilo potrebno študije toksičnosti ponoviti z večjim številom ponovitev.

Molibdenove spojine so pokazale določeno stopnjo ekotoksičnosti. V primeru MoO_3 so inhibicije znašale 53.22% za optimalno metodo disperzije, 57.02% za disperzije, pripravljene s sonifikacijo, in 44.72% za disperzije, pripravljene z mešanjem, medtem ko so vrednosti za MoS_2 znašale 89.55% za optimalno metodo disperzije, 81.43% za disperzije, pripravljene s sonifikacijo, in 61.82% za disperzije, pripravljene z metodo mešanja.

Izračun statističnih razlik med posameznimi metodami priprave disperzije je bil nemogoč zaradi premajhnega števila doslednih ponovitev. Za dosego bolj relevantnih rezultatov bi morali testiranje ponoviti.

Prisotnost nanomaterialov v okolju

Proučevanje toksičnosti nanomaterialov je pomemben korak pri določevanju ocene tveganja. Poleg določanja toksičnosti, ocena tveganja zahteva tudi razumevanje mobilnosti, persistence in biološke dostopnosti nanomaterialov v okolju. Analiza porazdelitve velikosti v disperziji je pokazala, da so bili vsi nanomateriali močno aglomerirani. Aglomerati bodo tako manj mobilni in razpršeni in posledično manj toksični (Nowack in Bucheli, 2007).

Testirani nanomateriali so v naši raziskavi pokazali, da imajo toksične učinke na HEK 293 celično linijo in bakterijo *Vibrio fischeri*, vendar le pri uporabi visokih koncentracij, ki smo jih testirali. Naslednji potreben korak pri določitvi ocene dejanskega tveganja je določitev predvidenih koncentracij in izpostavljenost nanomaterialov v realem okolju.

Danes obstaja zelo malo analitskih metod za merjenje nanomaterialov v naravnih sistemih, kar botruje velikemu pomanjkanju podatkov o njihovih koncentracijah v okolju (Nowack in Bucheli, 2007). Podatki o proizvodnji in predvidenih koncentracijah so skopi

in večinoma osnovani na modelih. Z enim takih modelov na primeru Švice je bila izračunana predvidena okoljska koncentracija različnih TiO_2 nanodelcev v okolju, ki je znašala od $1.55*10^{-6}$ µg/L (za realistični scenarij) do 4 mg/L (za scenarij visoke izpostavljenosti) (Müller, 2007). Če primerjamo koncentracije z našimi rezultati, lahko zaključimo, da noben od testiranih TiO_2 nanodelcev ni pokazal toksičnosti pri teh koncentracijah.

Za molibdenove spojine do danes ni dostopnih nobenih podatkov o predvidenih koncentracijah teh spojin v okolju. Naša študija je pokazala, da se citotoksičnost pojavi med koncentracijami 1 in 10 mg/L za MoS_2 in med 10 in 100 mg/L za MoO_3. Na podlagi teh rezultatov predlagamo nadaljnja testiranja v tem koncentracijskem območju.

Poleg že v uvodu omenjene potrebe po standardizaciji protokolov testiranja toksičnosti nanomaterialov je izrednega pomena tudi potreba po definiciji referenčnih materialov in lastnosti nanomaterialov, ki bi morale biti del obvezne karakterizacije pri okoljskih in toksikoloških študijah. Stone in sod. (2010) predlagajo, da bi morali biti protokoli testiranja prilagojeni tistim lastnostim, ki igrajo najpomembnejšo vlogo pri vedenju delcev v okolju in v organizmih.

V naši študiji smo uspeli potrditi odvisnost rezultata testov toksičnosti od metode priprave disperzije, časa izpostavljenosti in primarne velikosti, kar lahko služi kot osnova za prihodnji razvoj standardiziranih protokolov za testiranje toksičnosti nanodelcev.

ZAKLJUČEK

Disperzija nanomaterialov ne vodi v porazdelitev velikosti, kot je primarno deklarirana s strani proizvajalca. Glede na dobljene rezultate sekundarne karakterizacije so bili vsi nanomateriali v naših disperzijah močno aglomerirani. Med tremi disperzijami, ki smo jih pripravili z različnimi metodami, smo opazili signifikantne razlike v porazdelitvi velikosti.

Citotoksičnost nanomaterialov je bila odvisna od vrste nanomateriala, velikosti (relativne površine), kristalinične faze (za TiO_2 vzorce), časa izpostavljenosti in metode priprave disperzije. Čeprav so vsi parametri med seboj povezani, pa je vsak v določenem obsegu vplival na rezultat MTT testa.

Testiranje genotoksičnosti je pokazalo, da so TiO_2 nanodelci pri visokih koncentracijah (>1000 mg/L) pokazali signifikantno aktivacijo DNK popravljalnih mehanizmov. Metoda disperzije, ki je vključevala dodatek albumina kot proteinskega stabilizatorja edina ni inducirala signifikantnih genotoksičnih učinkov med vsemi tremi testiranimi protokoli priprave disperzije. Molibdenove spojine v naši raziskavi niso bile genotoksične, saj niso aktivirale DNK popravljalnega markerja-fosforilirane oblike H2A.X.

Z uporabo testa inhibicije luminiscence za testiranje ekotoksičnosti smo pri testiranju TiO_2 spojin dobili visoke vrednosti inhibicije, pri testiranju molibdenovih spojin pa so bile te vrednosti manjše. Zaradi možnih interferenc motnosti in premalo ponovitev so za potrditev sedanjih rezultatov potrebni še nadaljnji eksperimenti.

Metoda priprave disperzije je v nekaterih primerih vplivala na rezultat toksikoloških študij (manjša aglomeracija-večja citotoksičnost, dodatek albumina-manjša genotoksičnost), pri čemer pa so imeli znaten vpliv na izid študij tudi vrsta

nanomateriala, testirana koncentracija ter vrsta uporabljenega testa. Naše delo demonstrira kompleksnost problema in možne težave pri pridobivanju relevantnih in primerljivih rezultatov, predvsem zaradi skopega znanja o fizikalno kemijskih lastnostih ter vedenju nanomaterialov v okolju in bioloških modelih.

Uvedba standardiziranih metod testiranja toksičnosti in proučevanje obnašanja nanomaterialov v okolju bi morala spadati med prednostna področja nanotehnologije, pri čemer je potrebno poudariti, da trenutni zakoni močno zaostajajo v primerjavi s hitro rastočo proizvodnjo in uporabo, ki ustvarja dobiček brez upoštevanja škodljivosti za zdravje ljudi in posledic v okolju.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

There was a dramatic increase in research, technology, and production of nanomaterials in recent years. Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2007a) defined nanomaterials (NMs) as any form of a material that is composed of discrete functional parts, many of which have one or more dimensions of the order of 100 nm or less.

Due to their small size, NMs exhibit relative surface areas that are greater than the corresponding bulk forms; in addition, the small size often results in higher reactivity and altered surface properties that can be exploited in a variety of applications (Stone et al., 2010). The fraction of the atoms at the surface is increased compared to microparticles or bulk. Besides large surface area, NMs also have a high particle number per unit mass (Buzea et al., 2007).

While the number of NMs types and applications continues to increase, studies to characterize their effects after exposure and to determine their toxicity are few in comparison. The range of approaches and methods used to reach conclusions regarding the effects of manufactured NMs has led to different results. This inconsistency indicates a need for standardized tests in order to get comparable results in screening NMs for potential adverse effects (Oberdörster et al., 2005). There is, therefore, a major need to develop or to revise appropriate protocols to test the *in vitro* toxicity of NMs, as recommended in the Organisation for Economic Co-operation and development (OECD, 2009b) guidelines.

The mechanisms of toxic effects of NMs may be dominated especially by characteristics specifically introduced in order to meet the intended function of the product, possibly including surface reactivity and quantum effects. Therefore, any unpredicted interactions between NMs and biological systems may depend on their unique physical and chemical properties and their multiple functionalities that are largely dependent on the dispersion media and the temporal evolution of NMs properties. These notions have been often highlighted by the SCENIHR (2007a, 2009).

The interpretation of the data in the reviewed literature is thus hampered by various limitations including the differences in the dispersion preparation methods, different toxicity assay types and the use of non-standardized methods with different exposure times, concentrations and cell lines. Additionally there is a lack of information in many studies when it comes to primary and secondary characteristics of NMs as well as dispersion details.

Nanoparticles (NPs) in physiological solutions with salt concentrations and pH values compatible with biological studies form micrometer-sized agglomerates which can exert different biological effects and display different properties compared to well-dispersed NPs.

As the different testing conditions affect NMs properties directly and consequently also the toxicology data, it becomes of utter most importance, to standardize particle dispersion methods and investigate the impact of different dispersion preparation methods on the cytotoxicity, genotoxicity and ecotoxicity of selected NMs.

In this thesis, TiO_2 Degussa P25 and TiO_2 Millennium PC500 NPs were used for the comparison of the results with the current literature. Further analysis explored

biological effects of other nanoproducts as MoS_2 nanotubes and MoO_3 nanowires. Selected NMs were primarily characterized for their physical and chemical characteristics.

Three different dispersion protocols were used when preparing NPs dispersion in order to achieve dispersions with different stability and agglomeration states and to further compare the toxicity of such dispersions. First dispersion protocol included sonication and addition of a protein stabilizer in order to achieve optimal dispersion (Bihari et al., 2008). Second dispersion protocol included only sonication without adding a dispersion stabilizer, while the third dispersion was prepared using only stirring.

Secondary characterization of NMs obtained from different dispersion preparation methods was applied using Dynamic Light Scattering (DLS) analytical techniques in order to determine the size distribution and confirm the differences in agglomeration rate among different dispersion protocols.

The dispersion protocols were tested using current methods of cytotoxicity, genotoxicity and ecotoxicology: Human Embryonic Kidney 293 cells were used to assess if acute and chronic exposure with NMs interferes with basic cell metabolism, growth and cytotoxicity (by MTT colorimetric assay) and activation of the genotoxicity biomarker histone H2A.X phosphorylation. From the ecotoxicological perspective, the same NMs were tested for the inhibition of the luminescence on the testing bacteria *Vibrio fisheri* using set-up LUMIStox procedure. All the protocols were already developed in the laboratory.

Diploma thesis aims

Firstly we set out to study secondary characterization of selected Ti- and Mo-based nanomaterials (TiO_2 P25 and TiO_2 PC500 nanoparticles, MoS_2 nanotubes and MoO_3 nanowires) obtained from three different dispersion preparation methods by using Dynamic Light Scattering technique, to determine their size distribution and confirm the differences among different dispersion protocols. Further on, we aim to determine the cytotoxicity (by using MTT cell viability assay), genotoxicity (by using H2A.X histone phosphorylation assay) and ecotoxicity (by using *Vibrio fischeri* bioluminescence inhibition assay) of tested nanomaterials. Additionally, we will discuss, whether a dispersion state impact different nanomaterials cytotoxicity, genotoxicity and ecotoxicity results by comparing the toxicity assays outcomes. Finally we will try to demonstrate the connection between particle size distributions obtained from secondary characterization and toxicity, taking into account also possible impacts of exposure time, crystalline size and phase (for TiO_2 samples).

2 THEORY

2.1 Nanoparticles and nanotechnology

2.1.1 Definitions

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2007a) has established a framework for relevant definitions concerned with nanoscience, nanotechnologies and the products of nanotechnology.

The main word in the hierarchy of terminology in nanotechnology and nanoscience related to size is 'nanoscale'.

Nanoscale: A feature characterised by dimensions of the order of 100 nm or less.

The following definitions are based on this concept of the nanoscale, and the characteristics required for specific functionality at this scale.

Nanostructure: Any structure that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less.

Nanomaterial (NM): Any form of a material that is composed of discrete functional parts, many of which have one or more dimensions of the order of 100 nm or less.

Engineered nanomaterial (ENM): Any material that is deliberately created such that it is composed of discrete functional parts, either internally or at the surface, many of which will have one or more dimensions of the order of 100 nm or less.

On 18 October 2011 the European Commission adopted the Recommendation on the definition of a NM. According to this Recommendation a "nanomaterial" means:

A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1-100 nm.

In practice, the products of nanotechnology rarely consist of a single entity with one or more dimensions of 100 nm or less, or of large numbers of identical entities with identical sizes. Rather, they consist of very large numbers of similar but non-identical entities, as, for example, in a powder. Rarely will the sample be monodisperse or homogeneous. This is important factor for risk assessment purposes, since a sample has to be carefully and accurately characterised, and described by representative size distributions of its components (SCENIHR, 2007a).

In nanotechnology the term '**nanoparticle**' is used as a collective term for any material consisting of discrete entities with one, two or three dimensions of the order of 100 nm or less. For risk assessment purposes it is preferable to consider a nanoparticle to have a comparable scale in all three dimensions. SCENIHR (2007a) recommended the following terms to be used:

Nanosheet: A discrete entity which has one dimension of the order of 100 nm or less and two long dimensions.

Nanorod: A discrete entity which has two dimensions that are of the order of 100 nm or less, and one long dimension.

Nanoparticle (NP): A discrete entity which has three dimensions of the order of 100 nm or less.

Nanoparticulate matter: A substance comprising of particles, the substantial majority of which have three dimensions of the order of 100 nm or less.

Of all the possible configurations of nanostructured materials, it is the NPs that are by far the most significant as far as human health and the environment are concerned. SCENIHR (2007a) suggested that in order to facilitate risk assessment with nanoparticulate products, the behavior of the NPs themselves within the various compartments of the environment have to be considered, and certain terms are important for this purpose:

Coalescence: The formation of a new homogeneous entity out of two initial ones, e.g. after the collision of two NPs.

Agglomerate: A group of particles held together by weak forces such as van der Waals forces, some electrostatic forces and the surface tensions.

Aggregate: A group of particles held together by strong forces such as those associated with covalent or metallic bonds.

Degradation: A change in the chemical structure, physical properties or appearance of a material.

2.1.2 Nanoparticles properties and classification

According to Buzea et al. (2007), there are two primary factors that cause NMs to behave significantly differently than bulk materials (affecting the chemical reactivity of materials, as well as their mechanical, optical, electric, and magnetic properties). These are:

- Surface effects (causing smooth properties scaling due to the fraction of atoms at the surface). The fraction of the atoms at the surface in NPs is increased compared to microparticles or bulk. Compared to microparticles, NPs have a very large surface area and high particle number per unit mass. As compared by Buzea et al. (2007) the ratio of surface area to volume (or mass) for a particle with a diameter of 60 nm is 1000 times larger than a particle with a diameter of 60 μm. As the material in nanoparticulate form presents a much larger surface area for chemical reactions, reactivity is enhanced roughly 1000-fold.
- Quantum effects (showing discontinuous behavior due to quantum confinement effects in materials with delocalized electrons) describe the physics of electron properties in solids with great reductions in particle size. Quantum effects can begin to dominate the behavior of matter at the nanoscale affecting the optical, electrical and magnetic behavior of materials.

Materials reduced to the nanoscale can suddenly show very different properties compared to what they show on a macroscale. For instance, opaque substances become transparent (copper); inert materials become catalysts (platinum); stable materials turn combustible (aluminum); solids turn into liquids at room temperature (gold); insulators become conductors (silicon) (Pertsov, 2008).

Buzea et al. (2007) classified NPs based on their dimensionality, morphology, composition, uniformity, and agglomeration (Figure 1).

Dimensionality

As shape, or morphology, of NPs plays an important role in their toxicity, it is useful to classify them based on their number of dimensions.

- 1D NMs. Materials with one dimension in the nanometer scale are typically thin films or surface coatings, and include the circuitry of computer chips and the antireflection and hard coatings on eyeglasses.
- 2D NMs. Two-dimensional NMs have two dimensions in the nanometer scale. These include 2D nanostructured films, with nanostructures firmly attached to a substrate, or nanopore filters used for small particle separation and filtration.
- 3D NMs. Materials that are nanoscaled in all three dimensions are considered 3D NMs. These include thin films deposited under conditions that generate atomic-scale porosity, colloids, and free NPs with various morphologies.

Morphology

Morphological characteristics to be taken into account are: flatness, sphericity, and aspect ratio. A general classification exists between high- and low-aspect ratio particles. High aspect ratio NPs include nanotubes and nanowires (in our case MoS_2 and MoO_3), with various shapes, such as helices, zigzags, belts, or perhaps nanowires with diameter that varies with length. Small-aspect ratio (TiO_2 as an example) morphologies include spherical, oval, cubic, prism, helical, or pillar. Collections of many particles exist as powders, suspension, or colloids.

Composition

NPs can be composed of a single constituent material or be a composite of several materials. The NPs found in nature are often agglomerations of materials with various compositions, while pure single-composition materials can be easily synthesized today by a variety of methods.

Nanoparticle uniformity and agglomeration

Based on their chemistry and electro-magnetic properties, NPs can exist as dispersed aerosols, as suspensions/colloids, or in an agglomerate state. For example, magnetic NPs tend to cluster, forming an agglomerate state, unless their surfaces are coated with a non-magnetic material. In an agglomerate state, NPs may behave as larger particles, depending on the size of the agglomerate.

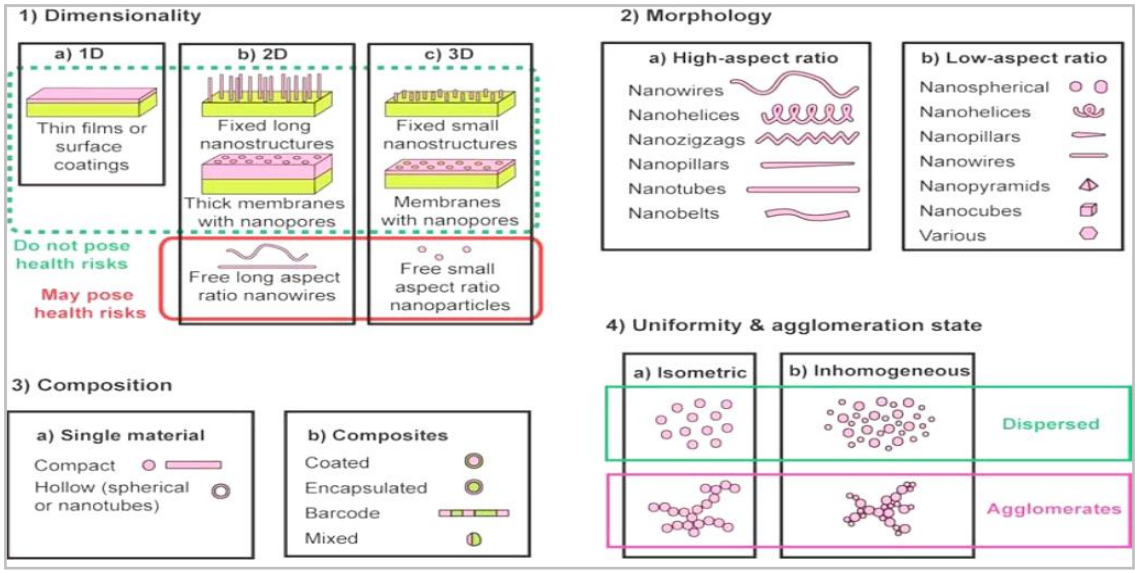

Figure 1: *Classification of nanostructured materials based on nanostructure dimensions, morphology, composition, uniformity and agglomeration state (Buzea et al., 2007: MR27).*

It is evident that NP agglomeration, size and surface reactivity, along with shape and size, must be taken into account when deciding considering health and environmental regulation of new materials (Buzea et al., 2007).

2.1.3 Applications

NMs properties can be exploited in a variety of consumer products such as paints, cosmetics, medicines and food, as well as in applications which directly release NPs into the environment, such as remediation techniques of polluted environments (Aitken et al., 2006).

Nanotechnologies are enabling manipulating matter at the atomic scale and to exploit new properties and functionalities for new applications (SCENIHR, 2007a). According to Buzea et al. (2007), these properties have revealed enormous prospects for progress in both life sciences and information technology.

Examples of nanotechnology applications according to OECD (2009a):

- **Electronics and communications** (data storage media with very high recording densities, new flat-panel plastic display technologies, new materials for semiconductors that increase processing speeds...).
- **Materials and construction** (super-hard and tough drill bits and cutting tools, “smart” magnetic fluids for vacuum seals and lubricants, scratch-proof or non-wettable surfaces, anti-bacterial construction material, self-cleaning and reactive eco-efficient windows…).
- **Machinery and tools** (sensitive sensors to detect incipient failures and actuators to repair problems, chemical-mechanical polishing with NPs, self-assembling of structures from molecules…).
- **Pharmaceuticals and health care** (miniaturized diagnostics that could be implanted for the early diagnosis of illnesses, nanoscale coatings to improve the bioactivity and biocompatibility of implants, ultra-precise drug delivery systems, sensors for labs-on-a-chip, materials for bone and tissue regeneration…).

- **Environment and water** (enhanced membranes for water purification, nanostructured filters for removing pollutants from industrial effluents, improved remediation methods (*e.g.* photo-catalytic techniques)).
- **Energy** (new types of batteries, artificial photosynthesis for clean energy, efficient low-cost photovoltaic solar cells (*e.g.* solar "paint"), safe storage of hydrogen for use as a clean fuel…).

Lewinsky et al. (2010) pointed out, that while the number of NP types and applications continues to increase, studies to characterize their effects after exposure are few in comparison.

2.2 Physical-chemical characterization and analysis

The mechanisms of toxic effects of engineered NPs may be dominated by those characteristics specifically introduced in order to meet the intended function of the product of interest. Therefore, any unpredicted interactions between NPs and biological systems may depend on their unique physical and chemical properties and their multiple functionalities (SCENIHR, 2009).

Properties such as the dynamics of dispersion, the rate of dissolution, the characteristics of NP aggregates, the surface area and the potential to adsorb substances onto NP surfaces are all relevant to the behavior of, and responses to, NPs in biological and ecological systems (SCENIHR, 2007b).

According to Lai (2012), NPs toxicity can be attributed to:
- nonspecific interaction with biological structures due to their physical properties (size and shape), bio-persistence, and
- specific interaction with macromolecules through their surface properties, or alteration of ionic components (release or recruitment of ions) and consequent toxic effects.

Figure 2 shows some physical and chemical factors that can influence biological effects and pathogenesis of NMs (Lai, 2012).

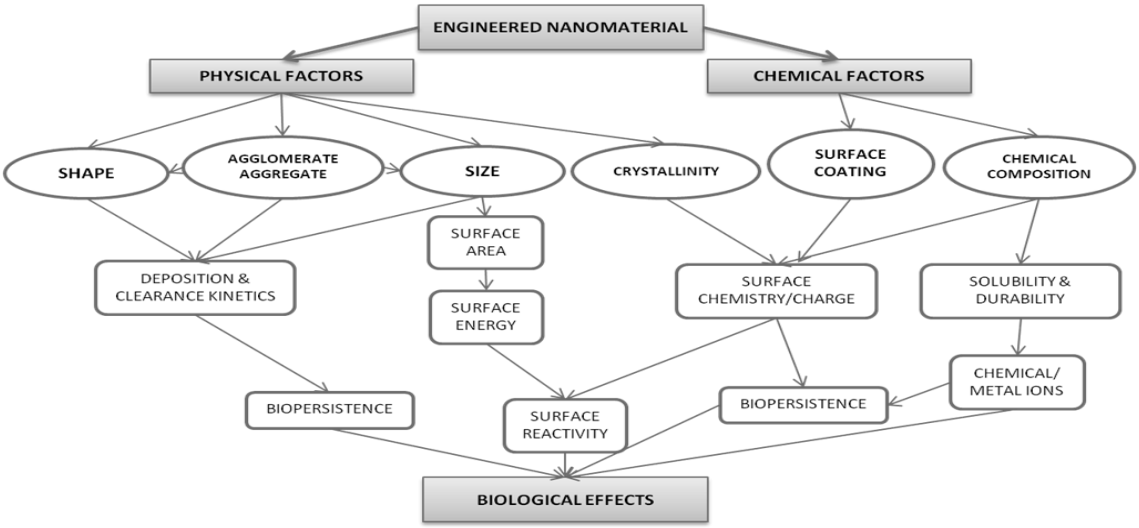

Figure 2: *Some physical and chemical factors that can influence biological effects of NMs (Lai, 2012: 3).*

Due to the size and material specific temporal evolution of some NMs, potentially toxic NMs need to be characterised both 'as manufactured' (primary characterization) and 'as delivered' (secondary characterization) in biological systems (in the various possible forms), or to a human in a specific application, or to a particular ecosystem of concern. Primary characterization provides information for the material safety data sheet of the product itself, while secondary characterization in biological systems is needed as properties of NMs may change considerably, notably the size distribution due to agglomeration/aggregation of the particles (SCENIHR, 2009).

SCENIHR (2007a and 2007b) highlighted that in any study involving NMs, it is important that the sample being characterized is representative of the substance, and that both particle size and shape characteristics should be measured in the most relevant dispersed state.

Discussions are currently ongoing concerning the various characteristics of NPs which need to be measured (Thomas et al., 2006; OECD, 2008; SCENIHR, 2007a; SCENIHR, 2007b; SCENIHR, 2009).

The main parameters of interest with respect to NP safety according to OECD (2008) are:

- **Physical properties:** size, shape, specific surface area, aspect ratio, agglomeration/aggregation state, size distribution, surface morphology/topography, structure including crystallinity and defect structure, solubility.
- **Chemical properties:** surface chemistry (composition, charge, tension, reactive sites, physical structure, photocatalytic properties, zeta potential), composition of NM (including degree of purity, known impurities or additives), phase identity, structural formula/molecular structure, hydrophilicity/lipophilicity.

Many of the instruments used for the characterization of larger particles can be used for NMs, although some specific modifications may also be needed. Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM), equipped with a variety of analysis tools are valuable for the determination of some NPs characteristics, especially morphology and surface chemistry. Normal powder characterization tools like X-ray diffraction (XRD), Inductively Coupled Plasma with Mass Spectrometry (ICP-MS) or Atomic Absorption Spectrometry (AAS) may be used for the determination of elemental composition with NMs, while BET (Brunauer, Emmett, Teller) method can be used for the determination of surface area of the particles (SCENIHR, 2007b).

Among the most prominent physical-chemical properties is size distribution in the suspensions. A number of methods are available to measure size distribution, such as dynamic light scattering (DLS) (Lamberty et al., 2011). This technique is reported by Couteau et al. (2010) to be most commonly used for the size distribution of NPs in the suspensions. In this type of measurement, the calculation of the particle size distribution is based on a kind of light intensity weighing.

2.2.1 Dynamic Light Scattering (DLS)

Dynamic Light Scattering method measures the size of NPs in a dilute liquid medium on the base of the hydrodynamic properties of the particles in a suspension. If the particle size is small enough, the erratic nature of the collisions of the solvent molecules with the particles will result in a random motion of the particles in the

suspending medium (Brownian motion). When shining a laser beam into the suspension, the intensity of the scattered light will fluctuate due to the Brownian motion of the particles. These fluctuations can be analyzed either in the time or the frequency domain, enabling the evaluation of the average diffusion coefficient of the particles (Couteau et al., 2010).

Lately, given the wide range of state-of-the-art NM characterization analytical techniques, multidisciplinary collaborations are strongly recommended (Oberdörster et al., 2005).

2.2.2 Properties of TiO_2 nanoparticles (Degussa P25 and Millennium PC500) used as a reference material

In our experiment we used commercial TiO_2 NPs as a reference material. TiO_2 NPs are the most studied, both for historical reasons (whereby the size dependency of particulate toxicity was first realized for TiO_2) and due to its widespread application within consumer products (such as sunscreens) (Johnston et al., 2009).

Powdered titanium NMs have been widely studied for the environmental and energy applications, including photocatalytic treatment of wastewater, pesticide degradation and water splitting to produce hydrogen (Gupta and Tripathi, 2011).

Many variables can affect photocatalytic properties of these materials in an aqueous suspension such as titanium phase composition, particle (crystallite) size distribution, the aggregation of primary particles giving rise to various pore (void) size distributions, surface area, surface adsorption properties, surface and total TiO_2 content of mixed oxides, etc. (Gun'ko et al., 2009).

Primary properties of TiO_2 NPs used in our study, such as BET surface area, crystalline form and size, aggregate radius, pore size and zeta potential are presented in Table 1.

Table 1: *Characteristics of TiO_2 samples (Gumy et al., 2006: 1378 and Nguyen et al., 2005: 5761).*

Characteristics/TiO_2 sample	**Millennium PC500**	**Degussa P25**		**Characterization methods**
BET Surface area (m^2/g)	335	50		Gas adsorption measurements
Crystalline form	Anatase (>99.5%)	Anatase (80%)	Rutile (20%)	X-ray diffraction measurements (XRD)
Crystalline size (nm)	8±3	24±2	37±3	Transmission electron microscopy
Aggregate radius (nm)	600-700	200-215		Electroacoustic spectroscopy
Pore size (Å)	104	54		Transmission electron microscopy
Isoelectric Point (IEP)-Zeta potential	6.2	7.0		Electroacoustic spectroscopy

Degussa P25 and Millennium PC500 photocatalysts are produced by different routes. P25, which generally contains 99.5% pure TiO_2, is produced in a high-temperature (greater than 1200 °C) process by flame hydrolysis of $TiCl_4$ in the presence of hydrogen

and oxygen. PC500 on the other hand is produced by the sol-gel method (Nguyen et al., 2005).

PC500 contains mainly anatase (>99.5%) while P25 consists of 80% anatase and 20% rutile. PC500 has smaller crystallite size and a surface area almost sixfold that of P25 (Gumy et al., 2006).

For purpose of toxicity testing it is important to determine the particle size in the dispersion (secondary size) using different analytical methods (DLS for example) rather than primary particle size given by the manufacturer.

When comparing the dispersive particle size, both particles were found to be aggregated with PC500 being more compact compared to P25. This was observed by Nguyen et al. (2005) under the TEM as shown in Figure 3.

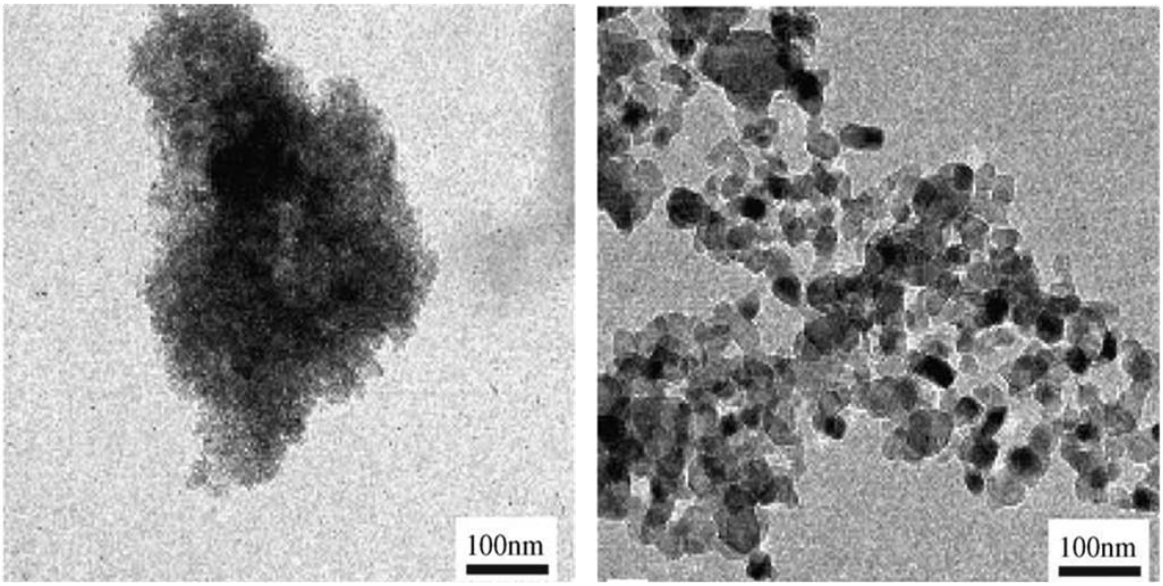

Figure 3: *TEM images of the different TiO_2 powders in suspension PC500 NPs (left) and P25 NPs (right) (Nguyen et al., 2005: 5762).*

P25 and the PC500 had similar zeta potentials (the overall charge that the particle acquires in a particular medium) at pH 3.5 even though they were prepared by different methods (Nguyen et al., 2005).

TiO_2 NPs in the environment

According to Müller's risk assessment of NPs in the environment there were many products found, containing TiO_2 NPs (referring to the situation in Switzerland). Ways of particle release were also considered (see Table 2) (Müller, 2007).

The likelihood of release is mainly determined by the way NPs are incorporated in the material. NPs fixed in material are likely to be released only during manufacture and disposal (Müller, 2007).

Table 2: *TiO_2 products: characterization, concentration and ways of particle release (Müller, 2007: 50-53).*

	Characterized by	Concentration	Example	Ways of particle release
Cosmetics	high usage (daily/weekly), release mainly into wastewater	less than 30'000 ppm; applied on skin at a concentration of 1 mg/cm^2 or less	sunscreens	The main fraction of TiO_2 NPs ends up in the Sewage Treatment Plant (STP) (95%) and a minimal amount (5%) is disposed with the packing to the Waste Incineration Plant (WIP). Cosmetics are washed off directly or stick to clothes from which they are also washed out.
Coating/cleaning agents	high usage (daily/weekly) release mainly into wastewater	20-500 ppm	air sanitizer, self cleaning coating (liquid), cleaner, coating for cars	Most TiO_2 NPs from liquids is released during the application (95%) of which an estimated percentage of 5% of the TiO_2 NPs applied remains airborne while 90% are washed off to the STP. 5% are disposed to the WIP with the packing.
Sporting goods/plastic	low abrasion, release mainly when disposed	/	tennis/squash rackets, golf club, computer mouse, air purifier/filtration	NPs in sporting goods and plastics are well integrated in the material. The abrasion of particles is likely to be very small during use (5%). 95% of the particles are released when the article is disposed.
Energy production/ storage	low to medium usage, release mainly during recycling	ca. 75 grams for a fibril wet solar cell	battery, solar cells	TiO_2 NPs in batteries is indeed free, but protected by a case and thus not released until the disposal of the battery. Particles in solar cells are bound in the material and abrasion is unlikely during use. Articles in this category are recycled to 75% (solar cell 100%, batteries 67%), the rest ends up in the WIP.
Metals	low to medium usage, release mainly during recycling	/	air purifier/conditioner/filter, pans, pots, fishing rod, knifes, hair dryer	The majority of the particles in metals will be recycled with the metal product (90%). Only 5% of the particles are expected to be released during the use through abrasion. The remaining 5% go to the WIP.
Paint	low wastage but relatively high quantities applied, release when disposed	/	paint (bath, kitchen)	Outdoor paint applied on house fronts is mainly released through wash off with the rain (95%) to STP when the house connects to pavement (50%) or directly to the soil for house front towards backyards and greenery (50%). In the case of indoor paint, the abrasion and release during use is negligible. It is assumed that the entire amount is disposed to a landfill at the time of the breakdown of the house.

According to the Müller's model based on exposure modeling, the 35 t (according to realistic scenario) and 400 t (according to high exposure scenario) of TiO_2 NPs used in Switzerland are distributed to the environmental compartments. Predicted environmental concentrations (PEC) were calculated for each of the compartments. The data obtained for realistic and high exposure scenario in this model are presented in Table 3 (Müller, 2007).

Table 3: *Calculation of the predicted environmental concentration (PEC) for the three environmental compartments according to Müller's model (Müller, 2007: 56, 59).*

	Amount of TiO_2 NPs (µg)		PEC (µg/L)	
Scenario	Realistic scenario	High exposure scenario	Realistic scenario	High exposure scenario
Air	$6,2*10^{10}$	$1,7*10^{12}$	$1,50*10^{-6}$	$4,22*10^{-5}$
Water	$2,7*10^{12}$	$5,9*10^{13}$	0,73	15,83
Water Vol. Affected By Wastewater	$2,7*10^{12}$	$5,9*10^{13}$	180	3933,33
Soil	$2,2*10^{12}$	$2,6*10^{13}$	0,61	7,26

2.2.3 Properties of inorganic nanotubes (MoS_2) and nanowires (MoO_3)

Inorganic nanotubes represent a quickly developing field. The important knowledge accumulated in the scientific groups during the research of carbon nanotubes has facilitated the understanding of inorganic nanotubes and enabled fast development in their synthesis and possible applications. Although they share some geometrical similarities with carbon nanotubes, inorganic nanotubes represent a unique system with important features, from the growth mechanisms to the physical and chemical properties (Remškar and Mrzel, 2004).

Six families of inorganic nanotubes have been synthesized up to now. The list, according to Remškar (2004), is as follows:

- Transition metal chalcogenide NTs (**MoS_2**, WS_2...)
- Oxide NTs: transition metal oxides (**MoO_3**, TiO_2, ZnO...)
- Transition metal halide ($NiCl_2$)
- Mixed-phase and metal-doped NTs
- Boron and silicon based NTs (BN, BCN...)
- Metal NTs (Au, Co, Fe, Cu...).

The most important methods for growing inorganic nanotubes can be divided broadly as follows (Remškar, 2004):

- Sulfurization
- Decomposition of precursor crystals
- Template growth
- Precursor-assisted pyrolysis
- Misfit rolling
- Direct synthesis from the vapor phase.

Due to their cylindrical hollow geometry, inorganic nanotubes have a low mass density, a high porosity and an extremely large surface to weight ratio. Their potential applications range from high porous catalytic and ultralight anticorrosive materials, atomic probes and field emitters, to high-temperature strengthening fibres. The helical structure of undoped tubes with semiconductor behavior and their optical activity enable possible applications in non-linear optics and in solar cell technology (Remškar and Mrzel, 2004).

MoS_2 nanotubes with hollow fullerene-like structure

The MoS_2 NMs have shown important applications as solid lubricants, electron devices, catalysts, super shock absorbers, etc. Particularly in the field of tribology, ultra low friction properties have been observed. As lubricants, the weak interatomic interactions (van der Waals forces) between the MoS_2 molecular layers in the form of plate-like crystals allow easy, low-strength shearing in vacuum, but their tribological properties remain poor in the presence of humidity or oxygen, limiting their technological applications (Remškar et al., 2007).

MoS_2 are extremely non-isotropic solids with a layer type structure. The weak interaction holding the layers is predominantly of the van der Waals type. The molecular layer S-Mo-S shows a trigonal symmetry. The transition metal atom is co-ordinated by six sulphur atoms situated at the corners of a trigonal prism (Remškar and Mrzel, 2004).

Compared with traditional 2H-MoS_2, MoS_2 nanotubes with hollow fullerene-like structure eliminate the fringe dangling bonds, which made MoS_2 nanotubes nearly not to be oxidized in humid air and high temperature, their fullerene-like structure nearly not to be destroyed. Therefore they improve the chemical stability and property of friction and wear (Sun and Li, 2010).

The nanotubes serve in two roles: as nanoreactors and afterwards as nanocontainers. Due to very thin walls, which break under short ultra sound agitation, the fullerene like particles can be released in a control way. This special morphology answers also many current questions regarding safe production, storage and transport of NMs (Remškar et al., 2007).

Transmission electron microscopy (Figures 4) demonstrates the general encapsulation of MoS_2 fullerenes inside the MoS_2 nanotubes. The morphology of the nanowires was preserved (Figure 4A), but slightly modulated in diameter. The nanotube walls are relatively thin, in average of 13 nm +/- 5 nm, i.e., from 13 up to 29 molecular layers, and varies slightly from a tube to tube, while some fullerene-like particles exceed several hundred nanometers. As an example, the wall of the nanotube, 12.6 nm in thickness, shown in Figure 4B, is of the constant value along the 1 μm length of the 350 nm-diameter tube, but the size of the encapsulated fullerenes inside the same segment, ranges from 285 nm (a) down to 40 nm (b) (Remškar et al., 2007).

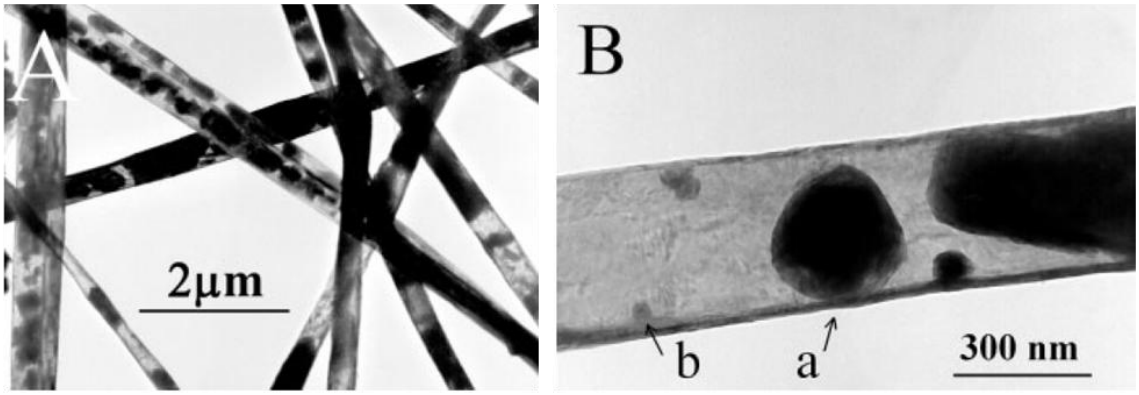

Figure 4: *A) a general view of the MoS_2 nanotubes with encapsulated MoS_2 fullerene-like NPs; B) single MoS_2 fullerenes and their aggregates inside a thin-walled MoS_2 nanotube (Remškar et al., 2007: 4277).*

The spherical MoS_2 NPs grow in a confined geometry of nanotube reactors, which limits the problem of agglomeration of these technologically important materials. Subsequently, the nanotubes serve as nanocontainers and prevent undesired release of NPs into the atmosphere. The unique morphology without a strict analogy in material science deserves a special name, “mama-tubes” as an example (Remškar et al., 2007).

Although the use of nanotubes as nanoreactors or as safe nanocontainers has been predicted, testing has not yet been conducted, giving its low quantity production until now. MoS_2 in applications as lubricants, in catalysis, in polymer composites, in solar energy systems and in other industrial products, gives hope for a wide use of MoS_2 nanopods (Remškar et al., 2007).

MoO_3 nanowires

MoO_3 is a wide-gap semiconductor having a energy difference (gap) between the top of the valence band (the highest range of electron energies in which electrons are normally present at absolute zero temperature) and the bottom of the conduction band (the range of electron energies, higher than that of the valence band, sufficient to free an electron from binding with its individual atom) of about 3 eV and is therefore attracting recent attentions. Molybdenum oxides can be good catalyst and fabrication of their NPs is being intensively investigated seeking for their use as a substitute for rare metals. Molybdenum oxides also serve as a gas-sensing material (Suemitsu and Abe, 2010).

As an example, MoO_3 is used as a catalyst in hydrogen production. The most common cathode material for hydrogen evolution in the electrolysis is platinum due to its high electroactivity. Yet, high price and limited supply of Pt are serious barriers for wider use of water electrolysis. Thus development of economic, but active cathode materials is in order in this field. One-dimensional MoO_3 showed outstanding electroactivity in hydrogen evolution reaction (Phuruangrat et al., 2009).

Nanostructures of Mo oxide, therefore, will definitely enrich the applications of this material through increase of its relative surface area, which may include highly sensitive gas sensors and highly efficient catalysts (Suemitsu and Abe, 2010).

SEM images of the MoO_3 nanowires are shown in Figure 5: The diameter of the nanowires is in the 80-150 nm range, with length in the 5-15 μm range (Rao and Govindaraj, 2001).

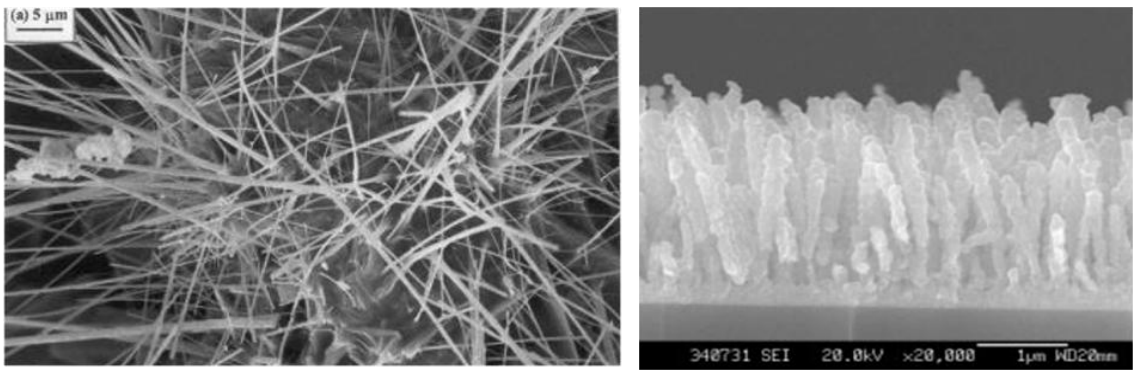

Figure 5: *SEM images of MoO_3 nanowires (Rao and Govindaraj, 2001: 388; Zhou et al., 2003: 1837).*

MoO_3 has several polymorphs, such as the thermodynamically stable, metastable, and hexagonal metastable MoO_3 (Mai et al., 2011).

2.3 Nanomaterials toxicity assay

Due to the rapid expansion of nanotechnology and the increasing range of NMs under production and development, it is essential that the potential impacts on human and environmental health are addressed (Stone et al., 2010).

The range of approaches and methods used to reach conclusions regarding the effects of manufactured NMs and ultrafine particles has led to different results. This inconsistency indicates a need for standardized tests in order to get comparable results in screening NMs for potential adverse effects. As the field of nanotoxicology continues to grow, standard toxicology tests will aid those entering the field and allow for better comparisons and conclusions in determining the toxic effects of NMs (Oberdörster et al., 2005).

Once a NP enters a cell, alteration of cell biology and potential long-term toxicity effects could occur through one or a combination of four possible mechanisms (Puzyn et al., 2011):

- the release of chemical constituents from NMs (ion release);
- the size and shape of the particle, which produces steric hindrances or interferences with the important binding sites of macromolecules;
- the surface properties of the material, such as photochemical and redox properties;
- the capacity of NMs to act as vectors for the transport of other toxic chemicals to sensitive tissues.

Some substances that may be deemed to be of low risk in bulk form may present significant risk when in nanoparticulate form. SCENIHR (2007b) suggested that attention should be paid to factors such as:

- physical parameters such as number concentration and surface area are likely to be more significant than mass concentration in the determination of exposure,
- NPs may agglomerate and disagglomerate in different ionic and oxidative environments, such processes affecting their properties,
- impurities within, and adsorbed species on the surface of NMs may have significant effects on risks,
- biological processes involving NMs, including cellular uptake, translocation, subcellular localization and toxicological mechanisms are still largely unknown and depend on the particle types and surface layer.

The knowledge is still lacking regarding the cellular mechanisms upon cell-NP interaction, namely uptake, biological fate, effects and modes of action of NMs *in vivo*, in species other than rodent and mammalian models used for occupational toxicology, and therefore especially in relation to environmental modes, including exposure routes other than via the air (e.g., waterborne exposure, sediment exposures). Many standardized protocols are available to assess the hazards of substances released into the environment, but while these have been developed for standard chemicals they are not always appropriate for NMs, potentially leading to misleading effects. Modifications

of such protocols are required for NMs, which then brings into question the relevance and reproducibility of the existing protocols (SCENIHR, 2009).

Equally, any potential deleterious effects have to be assessed to understand environmental impacts and potential effects on human health and/through the environment. This requires linking of physical-chemical characteristics of NPs to their behavior in biological environment (Stone et al., 2010). Moreover, to elucidate the modes of toxicity action, NMs complemented with their fate and behavior in the environment, Stone et al. (2010) pointed out, that it is essential to characterize the materials used in the different studies as far as possible and necessary.

2.3.1 Nanomaterials dispersion preparation for biological studies

When testing NMs for biological studies, physical-chemical properties are largely dependent on the surrounding media and the temporal evolution of the NMs (SCENIHR, 2009).

For biological safety evaluation, manufactured NMs need to be dispersed in an appropriate media (with physiological salt concentrations). The interaction between these media and the NMs can have a profound influence on the behavior of the dispersion (SCENIHR, 2009).

Different processes, such as dissolution, agglomeration (SCENIHR, 2009), aggregation, adsorption and surface modification of some NPs can occur in culture medium or biological fluids, affecting their physical-chemical properties, and thus cellular uptake, subcellular localization, and toxic effects (see Figure 6) (Lai, 2012).

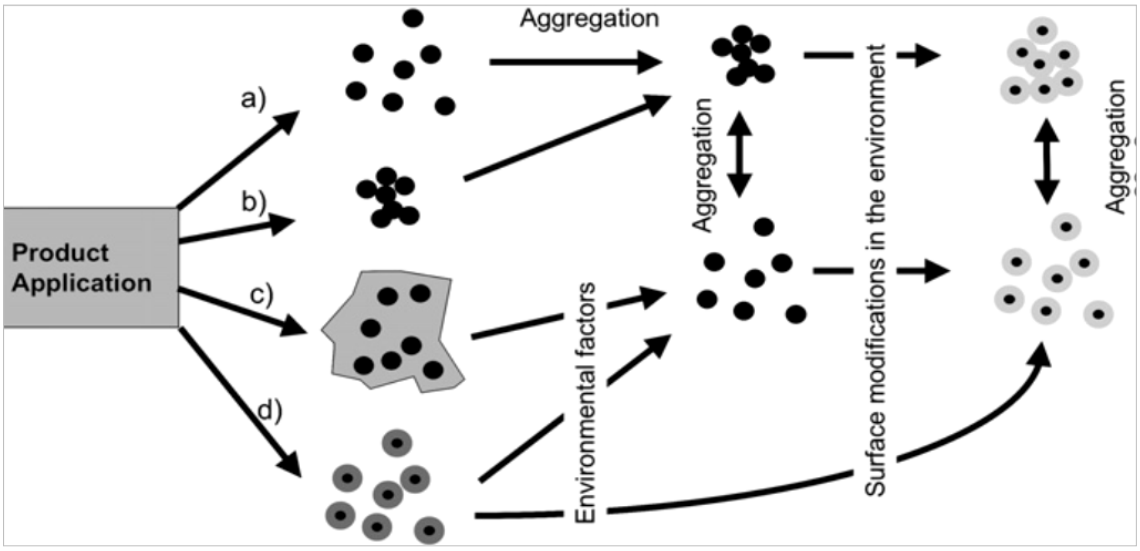

Figure 6: *a) release of free NPs, (b) release of aggregates of NP, (c) release of NPs embedded in a matrix and (d) release of functionalized NPs (Nowack and Bucheli, 2007: 10).*

It is well known from colloid science that NPs can form agglomerates or aggregates, especially when they are kept as powder under dry conditions (SCENIHR, 2009).

Agglomeration/aggregation is particle-size dependent and results in efficient removal of small particles in environmental systems. To quantify the stability of NP in the environment there is a need to predict the stability of their dispersion and their tendency to agglomerate/aggregate or interact with other particles (Nowack and Bucheli, 2007).

In many applications NP are embedded in a matrix and release of NPs will occur through release of matrix-bound NPs. As many NPs are functionalized, release of functionalized NPs is also possible (see Figure 6). In the environment the released NPs are affected by environmental factors such as light, oxidants or microorganisms (Nowack and Bucheli, 2007).

The total surface area (total surface area per unit of mass) is an important parameter for interactions with biological systems (SCENIHR, 2009).

Several studies have made suggestions as to how best disperse the NPs in test media.

Optimized dispersion of NPs for biological studies

Whether NMs should be tested as well-dispersed suspensions is an ongoing debate. An ecological argument could be made that dispersants should not be used at all, but this must be offset by the experimental errors generated when solution handling is poor and the exposure is heterogeneous. The alternative of using sonication to create dispersions may be criticized equally for a lack of environmental realism. Sonication is certainly not a natural phenomenon, and the shear forces at water interfaces can have a profound effect on aggregation rates (Handy et al., 2012).

Some of the practical considerations of dispersion methods for test media are summarized in Figure 7. Although natural dispersants may be regarded as more ecologically relevant than synthetic dispersants, any dispersing agent has the potential to change the dynamics of NM behavior. Furthermore, any surface coating of the NM with the dispersant may alter how the NM interacts with the cell surfaces of the test organism and therefore alter bioavailability (Handy et al., 2012).

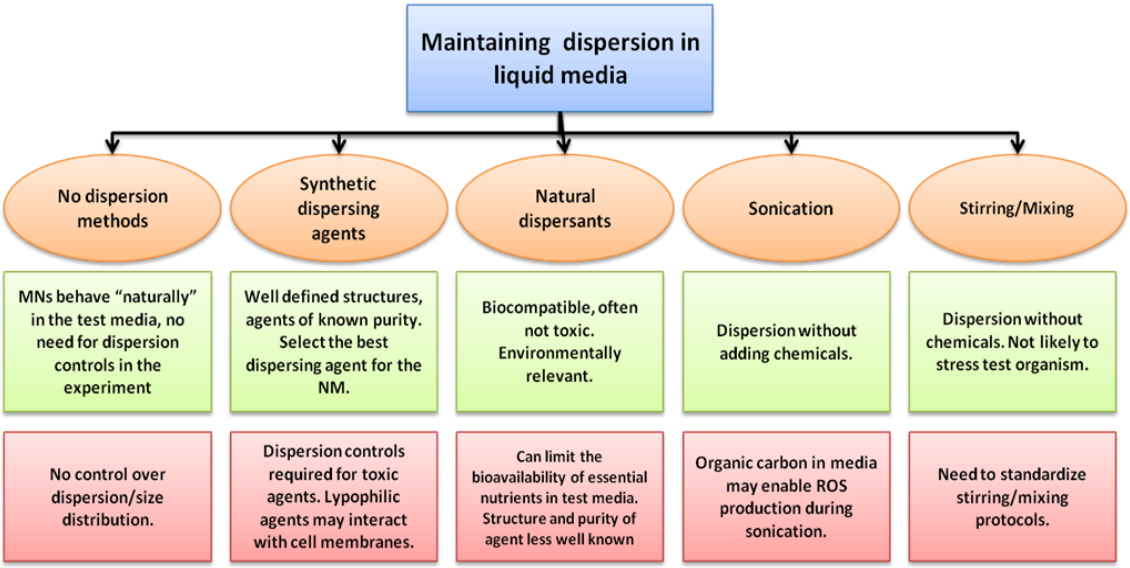

Figure 7: *Some advantages (green) and disadvantages (red) of dispersion methods used in toxicology (ROS-reactive oxygen species) (Handy et al., 2012: 17).*

In order to assess the worst case scenario for NPs, we must observe situations where they exist in a solution with limited agglomerates. To limit agglomeration, the most common methods employed are the use of solvents or surfactants (dispersion stabilizer), sonication, or stirring.

Particles in general and NPs specifically, diffuse, settle, and agglomerate in cell culture media as a function of systemic and particle properties: media density and viscosity and particle size, shape, charge and density, for example. Cellular dose then is also a function of these factors as they determine the rate of transport of NPs to cells in culture (Teeguarden et al., 2007).

Different methods have been published on how to avoid the formation of agglomerates of NPs dispersed in physiological solutions. The importance of the correct ultrasound energy as well as the use of dispersion stabilizers was emphasized for the optimal deagglomeration of NPs (Pohl et al., 2004; Mandzy et al., 2005 and Buford et al., 2007).

Pulmonary surfactants, detergents like Tween, bronchoalveolar lavage fluid, albumin or serum are used as NPs dispersion stabilizers in physiological solutions (Buford et al., 2007).

The stability of particle dispersions depends on the balance between attractive and repulsive forces between the particles. In principle, there are two ways to prepare stable dispersions: electrostatic and steric stabilization. With electrostatic stabilization, the zeta potential of the particles provides the repulsive force. The zeta potential of the particles, however, strongly depends on the pH and the electrolyte concentration of the dispersion. At physiological pH and electrolyte concentration, the zeta potential of the particles is not high enough to stabilize the dispersion, and the NPs form agglomerates (Bihari et al., 2008).

Therefore, steric stabilization is used for NP dispersion stabilization in physiological solutions, where a stabilizer is added to the dispersion adsorbing onto the particle surfaces and preventing them from coming close to one another (Bihari et al., 2008).

Required amount of stabilizer depends on the total surface area of the particles in the dispersion. When NPs get into the circulation they get in contact first with albumin and other serum proteins. Optimized dispersion method uses also albumin or serum, thus NPs dispersed with this method are covered with the same proteins NPs encounter in the circulation (Bihari et al., 2008). However, as a result “NP-protein corona” upon incubation of NP with proteins (Lynch and Dawson, 2008) is formed. In this way, rather than the single NP itself, it is the dynamic corona of associated biomolecules that defines the biological behavior of NP (Lynch and Dawson, 2008).

The corona equilibrates with the surroundings, with high abundance proteins binding initially, but being replaced gradually by lower abundance, higher affinity proteins. This complicates the measurement of such a protein corona. A considerable portion of the true biologically relevant biomolecules (proteins) will be associated with the NPs for a sufficiently long time that they are not affected by the measurement processes -the so-called “hard-corona” (SCENIHR, 2009).

Serum albumin has been shown to induce uptake and anti-inflammatory responses in macrophages, which were not present when the particles were pre-coated with surfactant to prevent albumin binding (SCENIHR, 2009).

Oberdörster et al. (2005) recommended that NMs preparation methods should include the selection of appropriate dispersion media, methods of dispersion in the medium and agglomeration state within the medium. However, specific preparation techniques are not recommended, as these will depend on the material and test protocols being used.

Following aspects are important to consider for the preparation of NP optimal dispersions in physiological solutions (see Figure 8) (Bihari et al., 2008). The optimal sequence is:

- to sonicate the NPs in distilled water (usage of a sonication energy should be high enough for deagglomerating the particles (>4.2 × 10^5 kJ/m^3)),
- to add the stabilizer (addition of high protein content solution, containing albumin or serum as stabilizers, at a concentration that is sufficient to cover NPs (1.5 mg/mL human serum albumin (HSA) for NP dispersions concentration less than 0.2 mg/mL)),
- finally to add buffered salt solution to the dispersion.

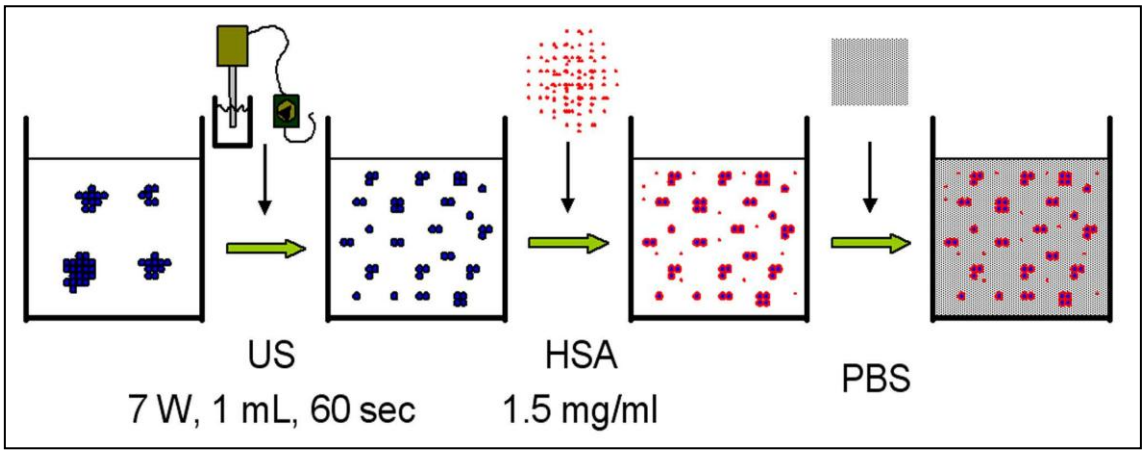

Figure 8: *Preparation steps of NP optimal dispersion (Bihari et al., 2008).*

Optimized dispersion method presented here appears to be effective and practicable for preparing dispersions of NPs in physiological solutions without creating large agglomerates (Bihari et al., 2008).

Protocols may vary between the different NMs. It seems obvious that there should be a best attempt to render the NPs in a size that is relevant to the expected consumer/population exposure (SCENIHR, 2009).

2.3.2 Cytotoxicity, genotoxicity and ecotoxicity investigations on TiO_2 nanoparticles published in scientific literature to-date

Toxicity of TiO_2 NPs is still a matter of debate, as different studies showed ambitious results of toxicity. From the evidences obtained so far, TiO_2 NPs induced toxicity is important since bulk TiO_2 has been considered as a safe material and has been used widely.

The main mechanism of toxicity of NPs is thought to be via oxidative stress that damages lipids, carbohydrates, proteins and DNA. Lipid peroxidation is considered

most dangerous as leading to alterations in cell membrane properties which in turn disrupt vital cellular functions (Heinlaan et al., 2008).

There is some evidence that TiO_2 NPs can enter the body through inhalation, ingestion, dermal penetration and injection. First alarming results of Afaq et al. (1998) showed extensive lung alveolar uptake of TiO_2 NPs and inflammation, fibrosis and pulmonary damage. According to Toyooka et al. (2012) the toxic effects are mainly due to alveolar macrophages and polymorphonuclear leukocytes, which produce excessive amounts of mediators as reactive oxygen species (ROS), proteases, cytokines, etc. and damage surrounding tissue.

Several studies (Chen et al., 2007; Dunford et al., 1997; Gurr at al., 2005; Li et al., 2009; Magdolenova et al., 2011; Nakagawa et al., 1997; Rahman et al., 2002; Saquib et al., 2011; Toyooka et al., 2012 and Wang et al., 2007) have indicated that TiO_2 NPs to be cytotoxic, genotoxic or ecotoxic. This issue has not been proven in other studies (Bhattacharya et al., 2009; Heinlaan et al., 2008; Linnainmaan et al., 1997; Lopes et al., 2012; Rehn et al., 2003; Theogaraj et al., 2007 and Warheit et al. 2007) that found less toxicity for these materials.

The interpretation of the data presented in the literature review (see Table 4: Literature review on different toxicity assays results on TiO_2 NPs published to-date) is hampered by various limitations, including the differences in the preparation methodology used within one assay type, the use of non-standardized methods with different primary cells or cell lines, and by the sometimes the lack of information on possible contaminants.

A major limitation in concluding whether a certain NM is toxic is the scarce description and minimal primary and secondary characterization of the NM samples used in the various studies (SCENIHR, 2009).

When agglomeration of NPs occurs, some reports have suggested that the primary particle size cannot be taken as the practical size for assessing their toxicity (Hsiao and Huang, 2011).

One potential cause of inconsistencies is the difficulty in appropriately delivering the NMs to the testing systems. Most available *in vitro/in vivo* cytotoxicity, genotoxicity and ecotoxicity studies have been performed at high particle concentrations. In *in vivo* situations, this may be associated with marked inflammatory and proliferative responses, and hence may obscure and/or modify cytotoxicity and genotoxicity and even carcinogenicity readouts. In addition, various assays with different primary cells and cell lines were used which did not always show consistent results. Such inconsistencies may depend on physicochemical characteristics of the testing material such as size, shape, aggregation/agglomeration state, surface properties, contaminants present and the cell type used (SCENIHR, 2009).

In conclusion, more work should be done to validate and standardize methods to be further used for screening NMs toxicity.

Table 4: *Literature review on different toxicity assays results on TiO_2 NPs published to-date.*

Ref	Material	Characteristics/preparation	Cell line/ testing organism	Cytotoxicity assay	Results
Bhattacharya et al., 2009	- TiO_2 anatase	- +48.8 mV charge and the average particle hydrodynamic diameter proved to be 91 nm, - spherical in shape	- IMR 90 (human bronchial fibroblasts) and BEAS-2B cells	- comet assay, - Trypan blue assay, - Oxidative DNA damage-adduct formation	- TiO_2 NPs did not induce DNA breakage measured by the Comet-assay in both cell types - TiO_2-NP did not induce cytotoxic effects in BEAS-2B cells up to a tested concentration of 50 $\mu g/cm^2$ whereas significant cytotoxic effects were observed in IMR90-cells - high level of DNA adduct formation was observed in IMR-90 cells exposed to TiO_2 NPs
Chen et al.,2007	- TiO_2 NPs	- TiO_2 NPs were prepared with the final concentration of 0.1 mg mL^{-1}	/	- after irradiation by UV detection of DNA damage by electrochemical method-substrate electrode: DNA and TiO_2 NPs co-modified onto gold electrode, reference electrode: saturated calomel	- DNA damage caused by photovoltaic effect of nano-titanium dioxide (DNA has been oxidatively damaged by the ROS that is produced from the TiO_2 NPs)
Dunford et al., 1997	- TiO_2 (20-50nm in diameter) and ZnO from sunsreens	- TiO_2 samples were extracted from over-the-counter sunscreens by washing with organic solvents (methyl cyanide, acetone, chloroform), - their anatase and rutile contents were determined by X-ray diffraction methods	- human MRC-5 fibroblasts	- Agarose Gel Electrophoresis of supercoiled plasmid - Alkaline Comet Assay in human MRC-5 fibroblasts +/- irradiation from solar simulator	- positive in Agarose Gel Electrophoresis of supercoiled plasmid and in Comet Assay after combined treatment with sunscreen extract + irradiation (sunscreen TiO_2 and ZnO can catalyse oxidative damage to DNA in vitro and in cultured human fibroblasts)
García et al., 2011	- TiO_2 NPs	- concentration (mg/mL): 1.12 - mean size (nm): 7.5 - shape:Shapeless Spherical - Zeta potential (mV): −42.5 - Stabilizer:TMAOH - Stabilizer concentration (mM): 10 - pH (original) : 10	- *Daphnia magna* - *Vibrio fischeri*	- *Daphnia magna* assays - Microtox bioluminescence test	- titanium dioxide NPs were toxic to *D. magna* having values of LC_{50} (0.016 mg/mL) - titanium dioxide NPs showed practically no toxicity in bioluminescence test, and only an inhibition of 21% of the light emitted was detected at the maximum concentration
Gurr at al., 2005	- TiO_2 anatase at 10 (Hombikat) and 20 (Millennium PC500) nm in diameter	- sterilized by heating to 120°C for 2 h, and then suspended in sterilized phosphate-buffered saline to a desired concentration and kept in 4 °C until used	- human bronchial epithelial cells BEAS-2B	- lipid peroxidation (Melanodialdehyde (MDA)), - MTT, - Measurement of nitric oxide and hydrogen peroxide	- in the absence of photoactivation induced lipid peroxidation (results indicated that treatment with 10 µg/mL anatase-sized TiO_2 increased the cellular MDA level), - effect on cell growth (the IC_{50} was estimated to be 6.5 µg/mL), - micronuclei formation and increased hydrogen peroxide and nitric oxide production (10 µg/mL was added to cell cultures)

(to be continued)

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Ref	Material	Characteristics/preparation	Cell line/ testing organism	Cytotoxicity assay	Results
Heinlaan et al., 2008	- TiO_2 NPs sizes of 25-70 nm	- stock suspensions in Milli-Q (40 g L^{-1}) were sonicated for 30 min and stored in the dark at +4 °C	- *Vibrio fischeri* - *D. magna,* *T. platyurus*	- *Vibrio fischeri* bioluminescence assay (Flash Assay) - *Crustacean* assays	- suspensions of nano and bulk TiO_2 were not toxic even at 20 g L^{-1} - The tests performed revealed no toxicity of both tested formulations of TiO_2 for *crustaceans T. platyurus* even at 20 g L^{-1} (2% TiO_2). However, 20 g TiO_2 NPs induced 60% mortality of *D. magna*
Hsiao and Huang, 2011	-Commercial: Ishihara ST-21, Degussa P25 Synthesized: TiO_2-Amorphous	- each powder sample was suspended in dimethyl sulfoxide at a concentration of 20 mg mL^{-1} - all stock solutions were dispersed using a 5 W probe sonicator for 90s in an ice bath - Ishihara ST-21(size distribution: 22-38nm) - Degussa P25(size distribution: 18-53nm - TiO_2-Amorphous (size distribution:9-160nm)	- human lung carcinoma epithelial cell line (A549)	- MTT, - sandwich ELISA protocol for the human IL-8 assay with exposure times of 12, 24, and 72 h	- cytotoxicity of TiO_2 samples decreased in the order TiO_2-Amorphous>ST-21>P25 after exposure for both 12 and 72 h - amorphous TiO_2 and ST-21 NPs exhibited greater potential to induce more pro-inflammatory factor in human cells than did the P25 NPs - although the ST-21 and P25 NPs had a similar surface area (ca. 68 m^2 g^{-1}), size (ca. 20 nm), and shape (spherical), they induced different toxicity responses, confirming that the phase of a TiO_2 NP affects its cytotoxicity - results revealed that only amorphous-phase TiO_2 NPs induced 50% cell death after exposure for up to 72 h at concentrations of up to 25 µg/mL
Linnainmaa et al., 1997	- TiO_2 P25 and UV-TITAN M160	- TiO_2 P25: Average crystal size 20 nm, uncoated anatase - UV-TITAN M160: average crystal size 20 nm, rutile coated by aluminium hydroxide and stearic acid, - before exposure washed with ethanol to remove the stearic acid to make the particles hydrophobic and suspensable - pigmentary TiO_2 (170 nm, uncoated anatase) - all suspensions ultrasonicated	- rat liver epithelial cells (RLE cells)	- Cytokinesis Block Micronucleus Assay - after 1 h of incubation, half of the slides were irradiated with an UV lamp	- ultrafine TiO_2 samples were non-toxic to RLE cells in 'relevant' treatment concentrations, and none of the dusts induced chromosomal damage measured as the induction of micronuclei in the cell cultures *in vitro* - these results support the evidence, according to which the possible carcinogenic potential of TiO_2 (pigmentary or ultrafine) is not due to direct chromosome-damaging effects of the dusts
Li et al., 2009	- TiO_2 NPs	- suspension was prepared using the culture media and dispersed for 20 min by using a sonicator	- Microglial cell line N9	- MTT, - Hoechst 33258 staining, - Flow cytometric analysis, - FDA and PI double staining were used to observe the viability of cells	- cell density decreased with the increasing of NPs concentration, MTT analysis showed the same result - evidence of apoptosis induced by nano-materials based on morphologic changes in cellular nuclei, membrane impermeability to PI and flow cytometric analysis was presented - after Hoechst 33258 staining, cells were scored as apoptotic - the results of flow cytometry were in accordance with that of the MTT

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Ref	Material	Characteristics/preparation	Cell line/ testing organism	Cytotoxicity assay	Results
Lopes et al., 2012	- TiO_2 NPs	- TiO_2 (particle size\100 nm, 99.9% metal basis) - samples were continuously stirred for at least 24 h in a stirring platform at room temperature	- *Vibrio fischeri* - *Salmonella typhimurium*	- Microtox test - Ames test with strains TA98 and TA100	- no inhibition in bioluminescence of *V. fischeri* was observed at any tested concentration - no mutagenic effects were detected
Magdolenova et al., 2011	- TiO_2 NPs, an anatase/rutile powder of 21 nm (nominal size)	- Dispersion protocol 1: fetal serum in stock solution and sonication 15 min (with agglomerates less than 200 nm) - Dispersion protocol 2: 3 min sonication and no serum in stock solution (large agglomerates) - Shape of particles: Irregular/ellipsoidal - Particle size: 15-60 nm - Crystal structure: Rutile/anatase - Surface area(m^2 g^{-1}): 61	- TK6 human lymphoblast cells, - EUE human embryonic epithelial cells - Cos-1 monkey kidney fibroblasts	- cytotoxicity (by trypan blue exclusion, proliferation activity and plating efficiency assays), - genotoxicity (by the comet assay).	- the TiO_2 NPs dispersion with large agglomerates (protocol 2) induced DNA damage in all three cell lines, while the TiO_2 NPs dispersed with agglomerates less than 200 nm (protocol 1) had no effect on genotoxicity - an increased level of DNA oxidation lesions detected in Cos-1 and TK6 cells indicates that the leading mechanism by which TiO_2 NPs trigger genotoxicity is most likely oxidative stress - results show that the dispersion method used can influence the results of toxicity studies
Nakagawa et al., 1997	- TiO_2: P25 anatase, WA anatase, WR rutile, TP-3 rutile	- P25 anatase-average size 0.021 mm,WA anatase-average size 0.255 mm, WR rutile-average size 0.255 mm, TP-3 rutile-average size 0.42 mm	- mouse lymphoma L5178Y cells, - *Salmonella typhimurim,* - Chinese hamster CHL/IU cells	- Alkaline Comet Assay - Chromosomal aberration assay - Salmonella/Micosome Assay - Mammalian cell mutation assay All tests: After 1 h incubation in the dark, the cells were exposed to UV-vis light for 50 min	- Comet positive after irradiation - Comet negative without irradiation - negative in the Ames Salmonella/Micosome assay with and without irradiation - negative in the mammalian cell mutation assay with and without irradiation - chromosomal aberrations increased after irradiation
Rahman et al., 2002	- Ultra fine (UF) TiO_2 and TiO_2	- the particle size was 20 nm for UF-TiO_2 and > 200 nm for TiO_2 - particles were sterilized by heating to 120°C for 2 h and suspended in phosphate-buffered saline (PBS; 1 µg/µL).	- Syrian hamster embryo (SHE) cells	- Micronucleus assay in Syrian Hamster Embryo Fibroblasts - Agarose gel electrophoresis	- increase of micronuclei in reticulocytes - increase in micronuclei, no significant increase in kinetochorepositive micronuclei - the agarose gel electrophoresis revealed typical apoptotic structures
Rehn et al., 2003	- TiO_2 P25, TiO_2 T805	- TiO_2 P25 (surface hydrophilic) and TiO_2 T805 (surface made hydrophobic) were suspended in physiological saline supplemented with 0.25% lecithin -primary particle diameter 20 nm, but particles were highly aggregated, sonication not leading to primary particles	/	- intratracheal instillation in rats followed by bronchoalveolar lavage for immunological determination of 8-oxoguanine	- no increase of 8-oxoguanine

(to be continued)

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Ref	Material	Characteristics/preparation	Cell line/ testing organism	Cytotoxicity assay	Results
Saquib et al., 2011	-crystalline, polyhedral rutile TiO_2	- size estimated with X-ray diffraction=30.6nm - hydrodynamic size estimated with DLS=13nm particle size and larger aggregates of 152 nm - suspension of 2 mg/mL was prepared in deionized Milli-Q water and sonicated for 15 min at 40W afterwards diluted in the Milli-Q water and cell medium	- human amnion epithelial cell line established as WISH cells	- MTT - NRU(neutral red uptake), - catalase activity, - glutathione (GSH), - ROS generation using fluorescent probe DCFH-DA	- concentration dependent cytotoxic effects at concentration range of 0.625-10 µg/mL. - cells exposed to 10 µg/mL exhibited significant reduction (46.3% and 34.6%) in catalase activity and glutathione (GSH) level, respectively. - treated cells showed 1.87-fold increase in intracellular ROS generation, as compared to the untreated control
Theogaraj et al., 2007	- 8 different classes of ultrafine TiO_2 NPs	- tested materials: 1. crystal anatase 80%, rutile 20%, primary particle size 21 nm; 2. 100% rutile, primary particle size 14 nm; 3. 100% anatase, aggregate size 60 nm; 4. 100% rutile, primary particle size 20 nm, 15 nm, 20–22 nm - 30–150 nm aggregates are expected in the samples tested.	- Chinese hamster ovary (CHO) cells	- Chromosome aberration test in CHO-WBL cells +/- UV irradiation	- none of the titanium dioxide particles tested induced any increase in chromosomal aberration frequencies either in the absence or presence of UV
Toyooka et al., 2012	- 2 kinds of TiO_2 NPs (anatase), whose primary sizes were 5 nm and <5000 nm,	- TiO_2 particles in 1.5 mL microtubes were suspended in DMEM at a final concentration of 20 mg/mL. The tubes were sonicated for 1 min in a bath-type sonicator - BSA-coated TiO_2 particles - secondary size distribution: 250-650 nm and 600-1050 nm	- lung adenocarcinoma epithelial cell line A549	- histone H2A.X phosphorylation test (flowcytometric analysis)	- TiO_2 particles have the ability to phosphorylate histone H2AX, which was more remarkable in smaller particles - flow cytometric analysis showed that the generation was independent of cell cycle phases and cells which incorporated larger amounts of TiO_2 particles had more significant γH2AX - generation of γH2AX was attenuated by coating the surface of TiO_2 particles with bovine serum albumin - generation of γH2AX was independent of ROS
Vevers and Jha, 2008	- TiO_2 75% rutile and 25% anatase	- suspended in PBS or H_2O, sonicated for 12 h in a water bath sonicator following a more powerful upright sonicator for 5 min, - samples were centrifuged at 2,500 rpm - using TEM-mean size = 24.4 +/- 0.5 nm, minimum = 11.8 nm, maximum = 38.5 nm	Rainbow trout gonad (RTG-2) cells	- comet assay, - cytokinesis-blocked micronucleus (MN) assay, - neutral red retention (NRR) assay	- the highest concentration (i.e. 50 µg mL^{-1}) did not produce elevations in DNA damage over 4 h (comet assay), 24 h (modified comet assay) or 48 h (MN assay) exposures in the absence of UVA irradiation, there was a significant reduction in lysosomal integrity over 24 h exposure (NRR assay) - significantly increased level of strand breaks was observed in combination with UVA . NRR assay suggested elevated levels of cytotoxicity when the UVA exposure was carried out

(to be continued)

(continuing from the previous page)

Ref	Material	Characteristics/preparation	Cell line/ testing organism	Cytotoxicity assay	Results
Wang et al., 2007	- Ultra fine (UF) TiO_2 NPs	- particles (99% pure, size by volume 6.57 nm: 100%, by intensity 8.2 nm: 80.4% and 196.5 nm: 19.4%) were suspended in culture medium, and sonicated to ensure a uniform suspension	- WIL2-NS a human B-cell lymphoblastoid cell line	- MTT assay, - the population growth assay, - the apoptosis assay by flow cytometry, - the cytokinesis block micronucleus (CBMN) assay, - the comet assay, -hypoxanthineguanine phosphoribosyltransferase (*HPRT*) gene mutation assay	- significant decreases in viability were seen in the MTT assay at higher doses - treatment with 130 µg/mL UF-TiO_2 induced approximately 2.5-fold increases in the frequency of micronucleated binucleated cells - reduction in the cytokinesis block proliferation index was observed by the CBMN assay - positive comet assay - positive HPRT mutation assay
Warheit et al. 2007	Ultra fine (UF) TiO_2 NPs	Particles (79% rutile; 21% anatase, median size 140 nm, surface 38.5 m^2/g; 90% TiO_2, 7% alumina, 1% amorphous silica) were suspended in water. Particle size distribution results for the ultrafine TiO_2 particle-types were highly agglomerated following dispersion in the phosphate-buffered saline solution	- *Salmonella t*., strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2*uvr*A - Chinese hamster ovary (CHO) cells	- Bacterial Reverse Mutation (Ames) Test using the plate incorporation method. *Salmonella t.* strains and *E. coli* strain were tested in the absence and presence of an exogenous metabolic activation system - chromosomal aberration (CHO) +/- exogenous metabolic activation system	- TiO_2 particles showed no evidence of mutagenicity in this study (Ames negative) - TiO_2 particles did not induce structural or numerical chromosome aberrations in this study

2.3.3 Toxicity investigations on MoO_3 nanowires and MoS_2 nanotubes published in scientific literature to-date

There were very few studies assessing the toxicity of molybdenum inorganic NMs such as MoO_3 nanowires and MoS_2 nanotubes up to-date.

Braydich-Stolle et al. (2005) reported that molybdenum NMs (MoO_3 30 nm) did not affect metabolic activity at exposure for 48h of the C18-4 cells, at least up to a concentration of 40 µg/mL. At higher concentrations (over 50 µg/mL), the molybdenum NPs become significantly toxic. No data about dispersion preparation method were reported.

Hussain at al. (2005) evaluated toxicity of molybdenum compounds (MoO_3 30, 150 nm). Dispersions were prepared using physical mixing and sonication. MTT assay, LDH (lactate dehydrogenase) assay, reduced glutathione (GSH) levels, reactive oxygen species (ROS), and mitochondrial membrane potential (MMP) were assessed under exposed conditions (24 h of exposure). Results showed that MoO_3 had no measurable effect on mitochondrial function at lower doses (10-50 µg/mL), while there was a significant effect at higher levels (100-250 µg/mL). LDH leakage was displayed only at higher doses (100-250 µg/mL). In summary MoO_3 was moderately toxic.

The cytotoxic behavior of MoS_2 NPs on cells was examined by Wu et al. (2011) using the MTT assay. CCC-ESF-1 cell (human embryonic epidermal fibroblast cells) viability was assessed 48 h after exposure to different concentrations of MoS_2 NPs. Cell viability data indicated that the MoS_2 NPs did not significantly affect CCC-ESF-1 cell proliferation up to 3.52 mg/L particle concentration. Two other different human cells were tested for toxicity: A549 cells (lung adenocarcinoma cells) and K562 cells (leukemic cells). The results showed that the MoS_2 NPs were reasonably nontoxic and biocompatible up to the given concentrations.

2.3.4 Cytotoxicity assay using MTT cell viability test

Determining the toxicity of NPs can often involve *in vitro* cell-culture based studies. Compared to animal studies, cellular testing is less ethically problematic, is easier to control and reproduce, and is less expensive.

In the case of cytotoxicity, it is important to recognize that cell cultures are very sensitive to changes in their environment per se, such as fluctuations in temperature, pH, and nutrient and metabolites concentrations. Therefore, controlling the experimental conditions is crucial to ensure that the cell biology measurement corresponds to the effect of the added NPs only, with no effect of the unstable culturing conditions. In addition, because NPs can chemically react with reference dyes and be redox active, it is therefore important that cytotoxicity assay is appropriately controlled (Lewinsky et al., 2008).

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method is one of the most widely used methods to analyze cell proliferation and viability in cytotoxicity assay.

The assay relies on the ability of absorption and metabolism of MTT salts of the examined cells. The principle of the assay, as demonstrated in previous studies, is in

the solute of this salt uptaken into the cells and reduced primarily in the mitochondria to water-insoluble purple formazan (see Figure 9) by NADH reductase and flavin oxidases (Lü et al., 2012).

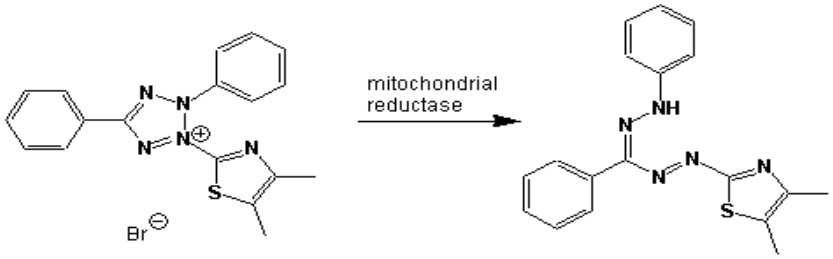

Figure 9: *MTT reduction in live cells by mitochondrial reductase results in the formation of insoluble formazan, characterized by absorbance at 570 nm (Brescia and Banks, 2009).*

MTT formazan crystals are insoluble in water, can be dissolved in dimethyl sulfoxide (DMSO) and are spectrophotometrically measured at wavelength 570 nm. The value of the optic density serves as an estimation of the number of active mitochondria and hence the number of living cells in the samples. An increase or decrease in cell number results in a concomitant change in the amount of formazan, indicating the degree of cytotoxicity of the test material (Lü et al., 2012).

Since *in vitro* cell lines in culture are proliferating due to their cancer origin, they are metabolically more active than non-proliferating (resting) cells. The MTT assay is therefore suitable not only for the determination of cell viability and factor inducing cytotoxicity, but also for the determination of cell activation and proliferation (Berridge et al., 1996).

Although MTT is widely used, it is not always specifically correlated with the actual cell growth and viability, but can display experimental artifacts. Studies investigating the cytological effects of TiO_2 NPs may encounter misleading results when using MTT/XTT (Tetrazolium-Carboxanilide) to measure viability or proliferation (Wang et al., 2011) therefore also positive results should not be overlooked as reported in several studies.

In the research of Lü et al. (2012), it was shown, that MTT itself could induce SH-SY5Y cell death. Secondly, the severity of cell death induced by MTT reduction is closely correlated with the incubation time. Especially at 2 h point, more than 70% of the total cells exhibit apoptosis or cell death. Finally, the main possible mechanism of MTT inducing cell death is the increase of plasma permeability following the exocytosis of intracellular granules and the formation of needle-like crystals.

In addition, superoxide ions can also reduce tetrazolium salts to produce the absorbant formazan end products (Wang et al., 2011).

Moreover, compared with other tests such as LDH (lactate dehydrogenase), MTT cytotoxicity results should be interpreted with caution, because it has also been found that MTT assay can interfere with mesoporous silica NPs in the cells (Fisichella et al., 2009) .

Finally, although there are many controversies about MTT assay, it is still considered the most simple and accurate preliminary *in vitro* method for investigation of effects of various substances (Lü et al., 2012).

2.3.5 Genotoxicity assay using H2A.X histone phosphorylation test

The genotoxic effects of the conventional particles are driven by two mechanisms: direct genotoxicity and indirect (inflammatory processes-mediated) genotoxicity. It is belived that NPs may act via either of these pathways since they may cause *in vivo* inflammation that causes secondary oxidative stress and indirect genotoxicity (Oberdörster et al., 2005).

There is some evidence, that the small size may allow NPs physical injury and to penetrate into sub-cellular, like the mitochondria, and nucleus. The presence of NMs in both compartments opens the possibility for direct DNA damage, interaction with DNA-binding proteins (histones, regulatory proteins etc), and possible oxidative reactions. Besides oxidative stress, additional mechanisms of genotoxicity which may be specific for NMs also need to be considered, such as possible mechanical intercalation and reaction with DNA elements (that may be mutagenic, affecting cell division, and potentially inducing transformation) and release of toxic metal ions (SCENIHR, 2009).

The role of chromatin in the response to genotoxins

Eukaryotic cells store their genetic information in a highly organized nucleoprotein complex termed chromatin. The high degree of compaction of DNA within chromatin places severe constraints on proteins that require access to the DNA template to facilitate gene transcription, DNA replication and DNA repair. As a consequence, eukaryotic cells have developed sophisticated mechanisms to allow chromatin to be rapidly decompacted locally for access by DNA-transcription factors (Moggs and Orphanides, 2004).

One of the principle in which a eukaryotic cell responds to changes in its environment is by altering gene expression to change the complement of expressed proteins and, thereby, respond to stress and adapt to environmental changes. Consequently, it is highly possible that the majority of toxic stressful events results in changes in gene expression (Moggs and Orphanides, 2004).

In response to DNA damage, cells activate DNA damage repair machinery to arrest cell cycle progression and repair the damaged DNA (Kim et al., 2011).

When the DNA damage is too severe to fix or cells cannot properly repair the damaged DNA, the cells undergo apoptotic cell death. The cellular responses-cell cycle checkpoint activation, DNA damage repair, and apoptosis-that occur upon DNA damage are collectively called the DNA damage response. DNA damage response can be considered a cellular protection mechanism that secures the genomic integrity of normal cells (Kim et al., 2011).

Histone modifications serve as markers of toxicity

Genotoxic agents cause rapid activation of protein kinase signaling cascades. The resulting rapid, defensive alterations in control systems and in gene activity require the transmission of a signal directly to the repair mechanisms (Moggs and Orphanides, 2004). **Histone phosphorylation** is one of the most upstream signals in these series of events and is induced by Histone PI3K-like kinases (ATM, ATR, and DNA-PK) (see Figure 10).

The sensing of double-strand DNA is accompanied by the activation of protein kinases that rapidly phosphorylate the specialized histone variant H2A.X and also transduce signals to additional signaling and DNA repair proteins (Moggs and Orphanides, 2004).

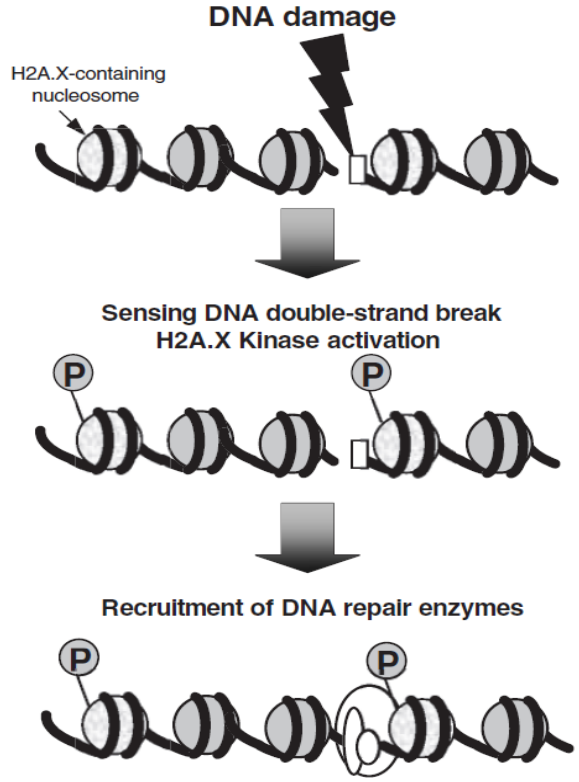

Figure 10: *Histone phosphorylation as early marker of DNA damage (Moggs and Orphanides, 2004: 221).*

H2A.X is a specific subtype of histone H2A, its levels vary from 2-25% of the mammalian histone H2A pool depending on the cell line or tissue examined (Rogakou et al., 1998).

Phosphorylated H2A.X is thought to function by recruiting additional proteins (kinases) that may induce signals to alert DNA repair systems (Moggs and Orphanides, 2004).

The observation that rapid and specific post-translational histone modifications accompany defined cellular responses to certain toxicants suggests that they may serve as useful markers of toxicity (Moggs and Orphanides, 2004).

Phosphorylation of histone H2A.X occurs very rapidly upon the formation of double strand breaks and may therefore act as a sensitive and specific marker for this type of cellular attack. Antibodies that recognize specifically histone H2A.X phosphorylated at position 139 are available commercially and have been used to detect double strand breaks resulting from DNA damage. Persistent DNA damage, correspond to a long staying H2A.X phosphorylation and nuclear morphological changes such as internucleosomal fragmentation or phosphotidylserine externalization, representing hallmarks of apoptosis. Therefore, immunostaining for H2A.X phosphorylation is a sensitive marker for very initial stages of genotoxic stress as well as chronic persistence of apoptosis (Moggs and Orphanides, 2004).

γH2A.X, the phosphorylated form of H2A.X, can be easily visualized as discrete nuclear foci after immunofluorescence staining. Because γH2A.X is de-phosphorylated on completion of DNA double strand brake repair, γH2A.X foci can be used as markers for DNA double strand breaks and DNA damage response. Although it is possible to count γH2A.X foci under a fluorescence microscope, this manual quantification method is difficult to apply to large-scale screening. Despite some intrinsic limitations, flow cytometric analysis has been considered an alternative method for quantifying γH2A.X (Kim et al., 2011). Nevertheless several reports show that the level of γH2A.X as detected by flow cytometry correlates well with the number of DNA strand breaks, the

level of cell death and radiosensitivity (Ismail et al., 2007). Thus detection of γH2A.X appears to be more sensitive marker of DNA damage than cell survival and the detection of double strand breaks by gel-electrophoresis (Toyooka et al., 2012).

The pattern of γH2A.X foci has been studied in cell nuclei after radiation exposure. The results indicate that γH2A.X signal is not uniform:

- first, it is dependent on the type/dose of the stimulus;
- second, γH2A.X focal formation in nuclei is dependent on the intrinsic level of chromatin compaction, regardless of the stimulus type;
- third, not all cell types respond similarly with γH2A.X focal formation.

Comparison of irradiation-induced H2A.X foci in different animal tissues, cell cultures, and primary cells from different individuals indicate however, a high degree of the assay reproducibility and its main dependence on the dose rather than cell type (Redon et al., 2011).

Quantification of γH2A.X refers not only to overall signal intensity at nuclear level, but may also account for difference in foci number, a fact that correlates with the extent of the DNA damage.

2.3.6 Ecotoxicity assay using *Vibrio fischeri* bioluminescence inhibition test

In order to reduce animal testing to a minimum, new testing strategies (involving toxicity assays performed, e.g. on bacteria and non-vertebrate animals) are needed (Mortimer et al., 2008). Furthermore, the ecotoxicology encompasses the test on different biological levels producing a broad impact assessment important to identify global ecological responses.

The main disadvantages associated with animal and plant bioassays are: problems with standardization of the organisms, requirements for special equipment and skilled operators, long duration of the assay and lack of reproducibility (Parvez et al., 2006).

Bacterial-based bioassays in toxicology can quantify: bacterial population growth, substrate consumption, respiration, ATP luminescence and bioluminescence inhibition. The test species used for bioluminescence inhibition assay includes *Vibrio fischeri/ Photobacterium phosphoreum, Vibrio harveyi* and *Pseudomonas fluorescens*; while those used for metabolic inhibition includes *Escherichia coli* and *Pseudomonas putida*. Mixed culture from activated sludge is often used for growth inhibition studies. The popularity of bacterial assays is based on the fact that bacteria are an integral part of the ecosystem and the bacterial assays are relatively quick and simple (Parvez et al., 2006). Finally, they give an instant (acute) response for the ecotoxic evaluation and predicting the toxicity of chemicals in other, higher-up *in vitro* systems.

One of the widely used tests in ecotoxicology is the *Vibrio fischeri* bioluminescence inhibition assay (Mortimer et al., 2008). *Vibrio fischeri* is a marine Gram negative bacterium which has bioluminescent properties. Light production is directly proportional to the metabolic activity of the bacterial population and any inhibition of enzymatic activity causes a corresponding decrease in bioluminescence. The assay provides a measure of sub-lethal response (Parvez et al., 2006).

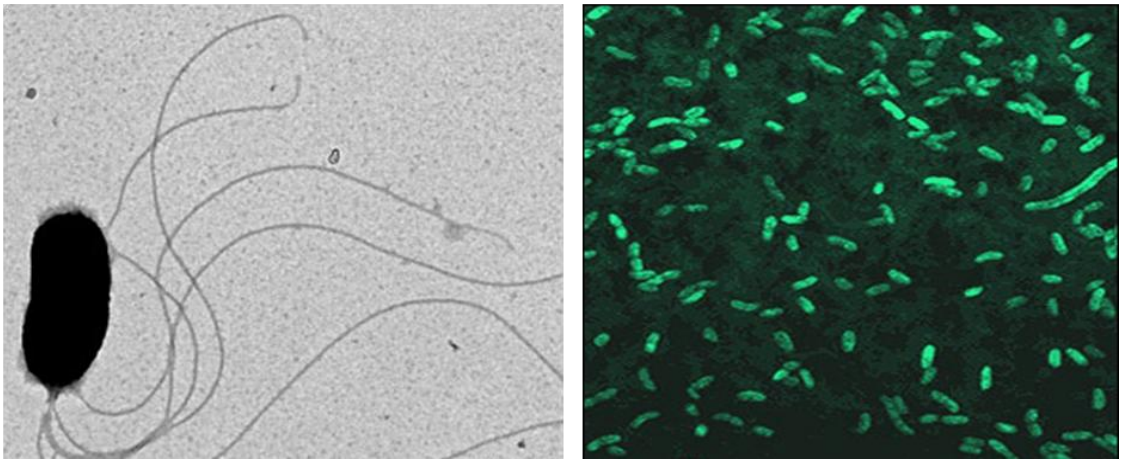

Figure 11: *Marine bacterium Vibrio fischeri and its intrinsic ability of luminescence (right) (Laboratory of Ecotoxicology and LCA).*

The bioluminescence of the *V. fischeri* is a result of a complex chain of biochemical reactions, where reduced flavin mononucleotide ($FMNH_2$-a long-chain fatty acid aldehyde) and luciferase are the key players. This special pathway uses NADH as a cofactor and is intrinsically linked to the central metabolism of the luminescence in certain microorganism (Mortimer et al., 2008). FMN reduces to $FMNH_2$ upon reaction with the reduced form of nicotinamide adenine dinucleotide phosphate (NAD(P)H) in presence of flavin reductase enzyme. Reduced $FMNH_2$ gets oxidized into FMN and H_2O upon reaction with molecular oxygen in the presence of aldehyde and luciferase enzyme. In this reaction blue-green light of wavelength 490 nm is emitted (Parvez et al., 2006).

This bacterial based toxicity test is very rapid and gives promising results comparable to other standard methods. Its benefits of technical simplicity, rapidity and sensitivity showed a high applicability in testing a wide variety of NMs (Zheng et al., 2010).

The assay is often chosen as the first test in a test battery based on speed and cost consideration. The test protocol is simple and was originally applied for aqueous phase samples or extracts (Parvez et al., 2006).

2.3.7 The biological mechanisms driving nanomaterials toxicity

NPs uptake into the cells

The internalization of particles by cells is important because particle uptake may enhance their toxicity due to their interference with normal cellular physiology and function. Particles can be also internalized by cells and not have an impact on cell function, which is likely to be driven by the intracellular location in which they accumulate or the extent of uptake. However, it is also possible that particles act from outside the cell to elicit toxicity that is mediated by interactions of particles with the cell surface and inducing mechanical damage (Johnston et al., 2009).

As reported by Busch et al. (2010), particle incorporation occurs more likely in phagocytic cells, such as monocytes and macrophages; yet for NPs, uptake by non-phagocytic cells has been reported as well recently. While large particles are thought to be taken up by phagocytosis, an actin filament-dependent process, smaller particles

seem to be internalized by other endocytic pathways with as of yet no apparent strict size threshold.

There are two factors that need to be considered for non-phagocytic particle uptake. First, in the *in vitro* experiments, particles are added to the media on top of the cells and therefore gravitational forces may also play a role in particle uptake. To avoid this aspect, many researchers do use compartmentalized tissue culture chambers. Second, proteins and lipids bound to the particle surfaces may impact on the route of particle uptake. However, whether a protein-particle complex can be internalized the same way as proteins is still not clear; certainly the NP-protein complex is much larger than a single protein (Busch et al., 2010).

Uptake of NPs by cells is also influenced by their cell cycle phase (Kim et al., 2012). Although cells in different phases of the cell cycle were found to internalize NPs at similar rates, after 24 h the concentration of NPs in the cells could be ranked according to the different phases: G2/M > S > G0/G1. NPs that are internalized by cells are not expelled from cells but are split between daughter cells when the parent cell divides.

TiO_2 mediated oxidative responses

Oxidative responses exhibited by TiO_2 particles have been a focus of a number of studies, which suggested that oxidative stress drives the inflammation and cytotoxicity (see Table 4: Literature review on different toxicity assays results on TiO_2 NPs published to-date).

Afaq et al. (1998) observed decrease in cell viability of TiO_2 (<30 nm) and concluded that an oxidant driven inflammatory, and cytotoxic response was observed within macrophages on exposure when using 2 mg of TiO_2 per rat. Also Dunford et al. (1997) confirmed oxidative damage to DNA by TiO_2 NPs extracted from sunscreens. Gurr et al. (2005) suggested that TiO_2 at concentration 10 mg/L induced oxidative stress which has cytotoxic consequences. Another study by Wang et al. (2007) concluded that a TiO_2 concentration of 65 mg/L distributes within the brain and elicits oxidative damage.

On the contrary, Toyooka et al. (2012) reported that TiO_2 NPs have the ability to phosphorylate histone H2A.X, but this genotoxicity was independent of ROS production.

It is relevant that the level of oxidative stress, which is related to the duration or concentration of particles administered, drives the nature of the response after exposure to NPs. Specifically, at moderate levels of oxidative stress, inflammatory responses may be stimulated due to the activation of ROS sensitive signaling pathways. At higher levels of oxidative stress, cytotoxicity is evident, as cells are damaged by increased concentration of ROS (Nel et al., 2006).

3 PRACTICAL WORK

3.1 Selected nanomaterials tested in this work

3.1.1 TiO_2 nanoparticles

As reference NPs, two different commercial TiO_2 materials were tested: Millennium PC500 (Crystal company, product code: 12174, Lot number: 6293000234) and Degussa P25 (Degussa AG, CAS reg. number: 13463-67-7, control number: P1S12B3).

3.1.2 Molybdenum inorganic nanotubes and nanowires

As testing materials MoS_2 nanotubes and MoO_3 nanowires were used. Molybdenum inorganic compounds were kindly produced by Dr. Maja Remškar from Jožef Stefan Institute, Ljubljana.

3.2 Dispersion preparation methods

Test dispersions of the TiO_2, MoS_2 and MoO_3 materials were prepared in three different ways. The stock solution was prepared in double deionized water at the concentration of 1000 mg/L, for all 4 materials. Material weighing was performed on an electronic balance (KERN&Sohn GmbH, type: ABJ 120-4M, No.:WB0540125, range: 0.01-120 g, readability: 0.1 mg).

Dispersion protocol 1 (DP1): is the **optimal dispersion preparation** and consisted in NP sonication with the addition of a protein stabilizer (Bihari et al. 2008). Stabilizer is added to the dispersion where it adsorbs onto the particle surfaces and prevents them from coming close to one another (Bihari et al. 2008).

Stock solution (50 mL of dispersion, 1000 mg/L NP) was sonicated (for 15 min) using ultrasonic bath (230V, 50 Hz, type: SONIS 4GT, identification number: 63100013). When powder was dissolved, stock solution (1000 mg/L) was used to prepare serial dilutions at 1, 10 and 100 mg/L final concentrations. 30 µL volume of 1.5 mg/mL albumin stabilizer (Albumin fraction V from bovine serum, Merck KGaA, K21415218 833) was added to 870 µL of each sample. Finally, 1/10 volume of PBS (Phosphate Buffered Saline, pH 7.4, 10x concentrated) was added to each sample, in order to obtain a physiologic solution compatible with cell biology.

PBS solution was prepared by dissolving 80 g of NaCl, 2.0 g of KCl, 14.4 g of Na_2HPO_4 and 2.4 g of KH_2PO_4 in 800 mL distilled H_2O. pH was adjusted to 7.4 and distilled water was added to reach the volume of 1 L. At the end PBS 10x solution was sterilized by autoclaving.

Dispersion protocol 2 (DP2) consisted in NPs sonication: The same as DP1 but without the addition of albumin stabilizer.

Dispersion protocol 3 (DP3) consisted in NP stirring: Stock solution (1000 mg/L NP concentration) was left on magnetic stirrer (IKA basic, 220-240V, 50/60 Hz, 416 W) for 5 h at room temperature. After this time, other concentrations (1, 10 and 100 mg/L) were prepared with the dilution of the highest concentration. On the end 1/10 volume of PBS (100 µL to 870 µL of sample) was added.

Table 5: *Summary of dispersion protocols used in the experiment.*

DP1	DP2	DP3
1. 15 min sonication **2.** albumin stabilizer (albumin/NPs concentration ratio=0.75:1) **3.** 1/10 of PBS 10x	**1.** 15 min sonication **2.** 1/10 of PBS 10x	**1.** 5 h stirring **2.** 1/10 of PBS 10x

3.3 Particle size distribution analysis (Dynamic Light Scattering)

DLS was performed under the supervision of Prof. Dr. Nataša Novak Tušar at the National Institute of Chemistry, Ljubljana.

The principle of DLS is based on the study of the intensity fluctuations of the light diffused by NPs suspended in a medium, due to Brownian motion. These fluctuations are random and related to the translational diffusion coefficient and so to the diameter of the particles.

DLS apparatus consists of a source of radiation, a scatterer and a detector. The laser is a water-cooled spectra-physics argon laser of 488 nm. Target is a solution of NPs in a 1 cm path-length quartz cell. Light is scattered from the region of the sample illuminated by the laser into all angles. In order to obtain a measurable signal it is necessary to collect the light scattered into a certain solid angle with a lens and focus it in the detector. The detection system for the scattered light consists of a polarizer and a gradient index lens optical fiber. The signal is sent to the detector which uses two photomultipliers and a beam splitter cube for setting a correlation detection which is necessary in order to increase the accuracy in the detection of very fast processes. The signal is then analyzed with the acquisition program (Cecere et al., 2003).

3.4 Cell culture methods

3.4.1 Cell culture

Human Embryonic Kidney 293 cells (HEK 293), originally derived from human embryonic kidney cells (ATCC) grow in tissue culture. Cell culture work is performed under a laminar flow hood to maintain sterility.

For experiment, HEK 293 cells were plated at equal density ($2x10^5$ cell/mL; 100 µL volume) in 96-well cell culture plate (flat bottom) and grown in Dulbecco's Modified Eagle's Medium-DMEM medium containing GlutaMAX(TM). HEK 293 cells were maintained in 5 mL of DMEM, catalog no. 10566) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, catalog no. F2442), 200 µL/mL of penicillin (Sigma) and 200 mg/mL streptomycin (Sigma). Cells were incubated at 37°C in an atmosphere of 5% CO_2 for 24 h to allow cells adhesion to the wells surface. For the genotoxicity assay

HEK 293 cells were plated on poly-lysine glass surfaces for immunofluorescence studies, or on black 96-well imaging plates with clear bottoms for high throughput studies (Optical Bottom Plates Nunc).

3.4.2 Exposure with nanoparticles

24 h after plating, the cells were taken out and put in the laminar flow hood in order to maintain sterilized conditions to avoid microbiological contamination. The cells in 90 µL cell culture medium were exposed to 10 µL of each NP sample (1/10 volume) for 24, 48 or 72 h at 37°C in an atmosphere of 5% CO_2. As control, cells were exposed to vehicle only (solutions without NPs).

3.5 Cytotoxicity assay (MTT cell viability test)

After 24, 48 or 72 h of NP exposure, supernatant medium was carefully removed from each well with a multichannel pipette and replaced with 100 µL of MTT-Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich catalog no. M2128, 0.025 mg/mL in cell medium). For this assay, cell medium without phenol red (catalog no. 21063-DMEM, high glucose, HEPES) was used to avoid absorbance measurement interference. Cells were incubated at 37°C for further 1.5 h to allow MTT metabolism. MTT solution was thus discarded and reaction was blocked adding 100 µL of Dimethyl sulfoxide (DMSO, Sigma-Aldrich, catalogue number: D2650) to each well to dissolve the insoluble formazan product, and transform it into a coloured violet solution. Finally, absorbance (optical density) of the solution was measured at 570 nm using Tecan Infinite 200 Automated microplate reader specrophotometer.

3.5.1 Evaluation of the test results

The relative cell viability related to control wells containing culture medium without NPs was calculated by:

$$Cell\ viability = A_{test}/A_{control} * 100 \quad (1)$$

Where:

A_{test} is the absorbance of the test sample

$A_{control}$ is the absorbance of control sample

Absorbance values measured at 570 nm that are lower than the control cells indicated a reduction in the cell metabolism, mitochondria activity and/or in the rate of cell proliferation. Conversely a higher absorbance indicated an increase in the cell proliferation.

3.6 Genotoxicity assay (H2A.X histone phosphorylation test)

3.6.1 Protocol for H2A.X immunofluorescence

After NP exposure the cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, and then washed using PBS for 5 min. Cell membranes were thus permeabilized with a detergent (0.2% Triton X-100 in PBS) and unspecific potentially immunoreactive sites were blocked with a high content protein solution (BSA 5%, 10% serum, 0.2% Triton X-100; blocking buffer) for 30 min at the room temperature. Cells were incubated for 2 h with mouse monoclonal anti-phosphorylated H2A.X (1:50 dilution, Millipore) in blocking buffer. After three times washes in PST, each of 5 min duration, secondary detection was carried out using an Alexa 488-conjugated antibody directed against mouse antibody (dilution 1:500, Invitrogen) in blocking buffer.

The phosphospecific antibody used to detect γH2A.X, does not bind to non-phosphorylated H2A.X. This antibody is detected by addition of a secondary, fluorophore-labeled antibody. The stained γH2A.X can then be analyzed by manual or automated scoring by fluorescence microscopy (Garcia-Canton et al., 2012).

For DNA labeling, cells were stained with DAPI (Sigma) for 5 min. Samples, mounted on a glass coverslip, were thus analyzed with automated Zeiss fluorescence microscope equipped with appropriate filters (“green” Filter: EX BP 470/40, BS FT 495, EM BP 525/50; “Dapi” Filter: set EX G 365, BS FT 395, EM BP 445/50).

3.6.2 High content analysis of immunofluorescence signal

Both nuclear and γH2A.X immunofluorescence signals were analysed using High content analysis (HCA, Molecular Devices) and dedicated software (Metamorph). For nuclear labeling, DNA was stained with Hoechst 33342 for 5 min (Invitrogen). Images of stained cells were acquired from the automated fluorescence microscope of the HCA platform using a 20x objective lens. Images from 9 fields per well were collected. With this method we obtained information about cell number, proliferation and survival, and extent of activation of DNA repair systems.

3.7 Ecotoxicity assay (*Vibrio fischeri* bioluminescence inhibition test)

This method was used to determine acute toxicity on luminescent bacteria in accordance with DIN EN ISO 11348-3. The measuring unit is the natural luminescence of the used microorganism *Vibrio fischeri* NRRL B-11177. The test system measured the light output of the luminescent bacteria after being exposed to the testing sample and compared it to the light output of a control (reagent blank) with no exposure to the testing sample. A difference in light output between the sample and the control is attributed to the effect of the sample on the organisms.

3.7.1 Materials

- Glass cuvettes 50x12 mm (REF 916 912),
- freeze-dried luminescent bacteria in accordance with DIN EN ISO 11348-3,

- Biofix *Lumi* reconstitution solution,
- Biofix *Lumi Medium* for freeze-dried luminescent bacteria (REF 945 608),
- LANGE LUMIStherm+LANGE LUMIStox 300 instrument.

3.7.2 Reactivation of bacteria

Deep frozen vial with Biofix *Lumi* luminescent bacteria was removed from the deep-freeze compartment upon which 1 mL of precooled (+2°C to +8°C) Biofix *Lumi* reconstitution solution was instantly poured to the Biofix *Lumi* luminescent bacteria ("shock thawing"). Luminescent bacteria were dissolved by rigorous shaking of the vial. Reactivated luminescent bacteria were stored for 5 min at a temperature +2°C to +8°C to stabilize.

3.7.3 Dosage

0.5 mL of precooled Biofix *Lumi Medium* for freeze-dried luminescent bacteria (REF 945 608) was transferred to each provided cuvette, followed by an addition of 10 µL reactivated undiluted luminescent bacteria suspension which were incubated for 15 min at temperature + 15°C.

3.7.4 Measuring the luminescence using LUMIStox instrument

Initial luminescence (I_0) was measured after sample solutions were added to the cuvettes in all cases, except for the blanks. Incubations lasted for 30 min at + 15°C, following by the inhibition luminescence (I) measurements. During the measurements, note has to be taken for the stable temperature and accurate time intervals.

3.7.5 Evaluation of test results

The decrease in bacterial luminescence (H (%)) due to addition of samples containing NPs can be determined as follows (DIN EN ISO 11348-3).

Using equation (2), intensity correction factor, which adjusts reference values affected by water dilution, was calculated:

$$F=I_C/I_0 \quad (2)$$

Where:

F is correction factor for exposition of 30 min

I_C is luminescence intensity of control sample after exposition of 30 min, expressed in relative units of luminescence

I_0 is luminescence intensity of control testing suspension measured just before addition of dilution water, expressed in relative units of luminescence

Average value of F of control samples is calculated afterwards. According equation (3) I_C is calculated:

$$I_C = I_0 * F \text{ (average)} \quad (3)$$

Where:

F (average) is average value of F

I_C is corrected I_0 value for tubes with examined sample just before addition of tested sample

Inhibition effect of tested sample is counted by equation (4):

$$H (\%) = (I_C - I / I_C) * 100 \quad (4)$$

Where:

H (%) is inhibition effect of tested sample after exposition of 30 min, in percent

I is luminiscence intensity of tested sample after exposition, in relative units of luminiscence

Average inhibition effect H (average) in percent is calculated for every dilution.

3.8 Statistical data analysis

The results were statistically analyzed using MS Excel software. Firstly F-test two-sample for variances was performed to make a statistical comparison between the variances of two data sets. Null hypothesis and the alternative hypothesis were designed as following:

H_0: there is no significant difference between the two variances.
H_1: larger variance is significantly different than the smaller variance.

Variances were not equal if $p<0.05$. Based on the F-test outcome, type of Student t-test was chosen: type 2 if $p>0.05$ (for two-samples assuming equal variances) or type 3 if $p<0.05$ (for two-samples assuming unequal variances). Student t-test was performed, after they passed normality test ($p<0.05$). Null hypothesis and the alternative hypothesis for Student t-test were designed based on the research question as following:

H_0: there is no significant difference among different NMs/dispersion preparation protocols/concentrations.
H_1: there is significant difference among different NMs/dispersion preparation protocols/concentrations.

The response was considered statistically different at a significance level of $p<0.05$. t-values (or p values) for the t-test were reported throughout. The statistical data obtained were presented on histograms, whereby * indicated $p<0.05$ and ** indicated $p<0.01$.

Standard deviations were calculated for each value and were illustrated on the box plot histograms. All experiments were done using three samples for each concentration and dispersion method testing (cytotoxicity, ecotoxicity) or two samples (genotoxicity) in each treatment, and the results were presented as mean value ± standard deviation.

4 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Particle size distribution analysis (Dynamic Light Scattering)

Particle size distribution analysis results of means of volume, number and area distribution for different NMs and three dispersion preparation methods are presented in Table 7. For the graphical size distribution analysis see ANNEX A (Dynamic light scattering particle size distribution analysis for selected NMs using three different dispersion protocols). In our interest of this data is especially the number distribution where volume distribution data are weighted to the smaller particles in the distribution.

Table 6: *DLS particle size distribution analysis results of means of volume, number and area distribution.*

NPs	P25			PC500			MoO_3			MoS_2		
DP / SIZE	DP1	DP2	DP3	DP1	DP2	DP3	DP1	DP2	DP3	DP1	DP2	DP3
MV(µm)	6,19	3,91	5,87	3,81	2,676	7,22	9,70	6,80	12,59	14,85	14,11	23,09
MN(µm)	0,573	1,060	1,381	0,977	1,000	0,984	0,2400	0,644	1,026	0,2230	2,179	4,39
MA(µm)	1,723	2,162	2,923	2,257	1,983	2,374	1,357	2,078	3,09	1,650	6,64	13,02
SD(µm)	4,59	2,312	3,68	1,794	1,065	3,77	11,47	5,62	13,49	11,29	9,63	15,54

Where:

MV is a mean diameter (in µm) of the "volume distribution" represents the center of gravity of the distribution. MV is weighted (strongly influenced) by a change in the volume amount of large particles in the distribution. It is one type of average particle size or central tendency.
MN describes a mean diameter (µm) of the "number distribution" which is calculated using the volume distribution data and is weighted to the smaller particles in the distribution. This type of average is related to population or counting of particles.
MA presents an average diameter (µm) of the "area distribution" that is calculated from the volume distribution. This area mean is a type average that is less weighted (also less sensitive) than the MV to changes in the amount of coarse particles in the distribution. It represents information on the distribution of surface area of the particles of the distribution (Plantz, 2008).

DLS number distribution data indicated that, for all the tested NMs, optimized dispersion protocol (DP1) that includes sonication and use of highly protein solution as dispersion stabilizer, is the most efficient protocol to maintain the particles dispersion at the minimum aggregation state (see Table 7). Dispersion protocol that produced the largest agglomerates by number distribution was, as expected, obtained by using stirring (DP3) as dispersion preparation method.

Considering the results of volume distribution data, appears however that the smallest agglomerates were obtained with protocol DP2 and not with the optimized protocol DP1. Volume distribution data show the average particle size which is strongly influenced by a change in the volume amount of large particles in the distribution. Although DP1 produced the smallest particles regarding smaller particle distribution (number distribution data), DP2 contained the smallest particles (aggregates) among larger particle distribution (volume distribution data).

Finally, while number distribution was the smallest for the dispersion protocol DP1 (sonication and use of protein stabilizer), dispersion protocol DP2 (only sonication), generated smaller particles per volume distribution. DP3 (only stirring) obtained the largest particles by number distribution as well as by volume distribution when comparing to DP1 and DP2.

When focusing on the smallest particle sizes obtained in our analysis (Table 7), we can conclude, that there was no nano fraction present in our dispersions for all four tested NMs. Sizes of the smallest particles in the distribution are in range of 122-2312 nm, depending on the dispersion method and nanomaterial being used.

Table 7: *The smallest particle sizes obtained with particle size distribution analysis.*

NPs	P25			PC500			MoO_3			MoS_2		
DP / SIZE	DP1	DP2	DP3	DP1	DP2	DP3	DP1	DP2	DP3	DP1	DP2	DP3
MIN (µm)	0.344	0.486	0.688	0.486	0.486	0.486	0.122	0.289	0.409	0.145	0.972	2.312

4.1.2 Cytotoxicity assay (MTT cell viability test)

Selected NPs (TiO_2 P25, TiO_2 PC500, MoS_2 nanotubes and MoO_3 nanowires) dispersed according to protocols DP1, DP2 and DP3 (see Table 6: Summary of dispersion protocols being used in the experiment) were tested for their metabolic and cytotoxic effects on Human Embryonic Kidney (HEK) 293 cells using MTT cell viability test, essentially pointing towards the changes in cell metabolism. The cells were exposed to selected NPs at 1, 10, 100 and 1000 mg/L concentrations for different exposure times (24, 48 and 72 h). Control cells were treated with vehicle only without NPs and were considered to have 100 % activity. All the samples were done in three replicates for each treatment.

The values with a lower absorbance ratio ($A_{test\ average}/A_{control\ average}$) respect to the control indicated reduction in the number of active mitochondria (a fact that correlates with cell death or decrease in metabolic activity) while the higher ratio values pointed towards increase in cell metabolism (including proliferation).

24 h exposure test

Results of MTT cell viability test with the exposure time of 24 h with three different dispersion protocols are presented on Figure 12. There were three samples (triplicates) of the same kind (n=3) in one treatment.

For TiO_2 P25 no significant MTT difference ($p>0.05$) between the tested dispersion protocols compared to the control was observed for the concentrations 1, 10 and 100 mg/L and for all three dispersion protocols. At the concentration 1000 mg/L in case of DP2 significantly (t-test, type 2, $p=0.009$) larger metabolic activity was found which indicates cell proliferation, while the other two dispersion protocols (DP1 and DP3) showed no significant difference ($p>0.05$) at the same concentration when comparing to the control. Statistical analysis showed that there is no significant difference ($p>0.05$) among different dispersion preparation protocols.

TiO_2 PC500 showed no significant difference ($p>0.05$) in comparison to the control at the concentrations 1, 10 and 100 mg/L for all three dispersion protocols. However, at a concentration 1000 mg/L there was a significant (t-test, type 3, $p=9.39*10^{-5}$) decrease in cell viability (decrease in metabolic activity) when using dispersion protocol 2 (DP2), while the other two dispersion protocols (DP1 and DP3) showed no significant difference ($p>0.05$) in respect to the control. Statistical analysis confirmed that at a concentration 1000 mg/L there was a significant difference (t-test, type 3, $p=0.045$) among dispersion protocols when comparing DP2 to DP1 and DP3.

At concentration levels of 1, 10 and 100 mg/L for MoO_3 using three different dispersion protocols, no significant ($p>0.05$) changes in proliferation activity was recorded. At a concentration 1000 mg/L there was a significant decrease (t-test, type 2, $p=0.035$) in metabolic activity for DP1 and significant increase of metabolic activity (proliferation) for DP3 (t-test, type 2, $p=0.025$), while DP2 showed no significant difference ($p>0.05$) when comparing to the control. Statistical analysis confirmed that at the concentration 1000 mg/L there was a significant difference among all three dispersion protocols (DP1 compared to DP2 (t-test, type 2, $p=0.037$), DP2 compared to DP3 (t-test, type 2, $p=0.014$)).

For MoS_2 nanotubes significantly higher metabolic activity (cell proliferation) in comparison to control was observed at the concentration 1 mg/L for DP2 (t-test, type 2, $p=0.025$) and at the concentration 10 mg/L for DP3 (t-test, type 2, $p=0.001$). No significant decrease ($p>0.05$) in metabolic activity was observed in any of the concentrations or dispersion protocols tested.

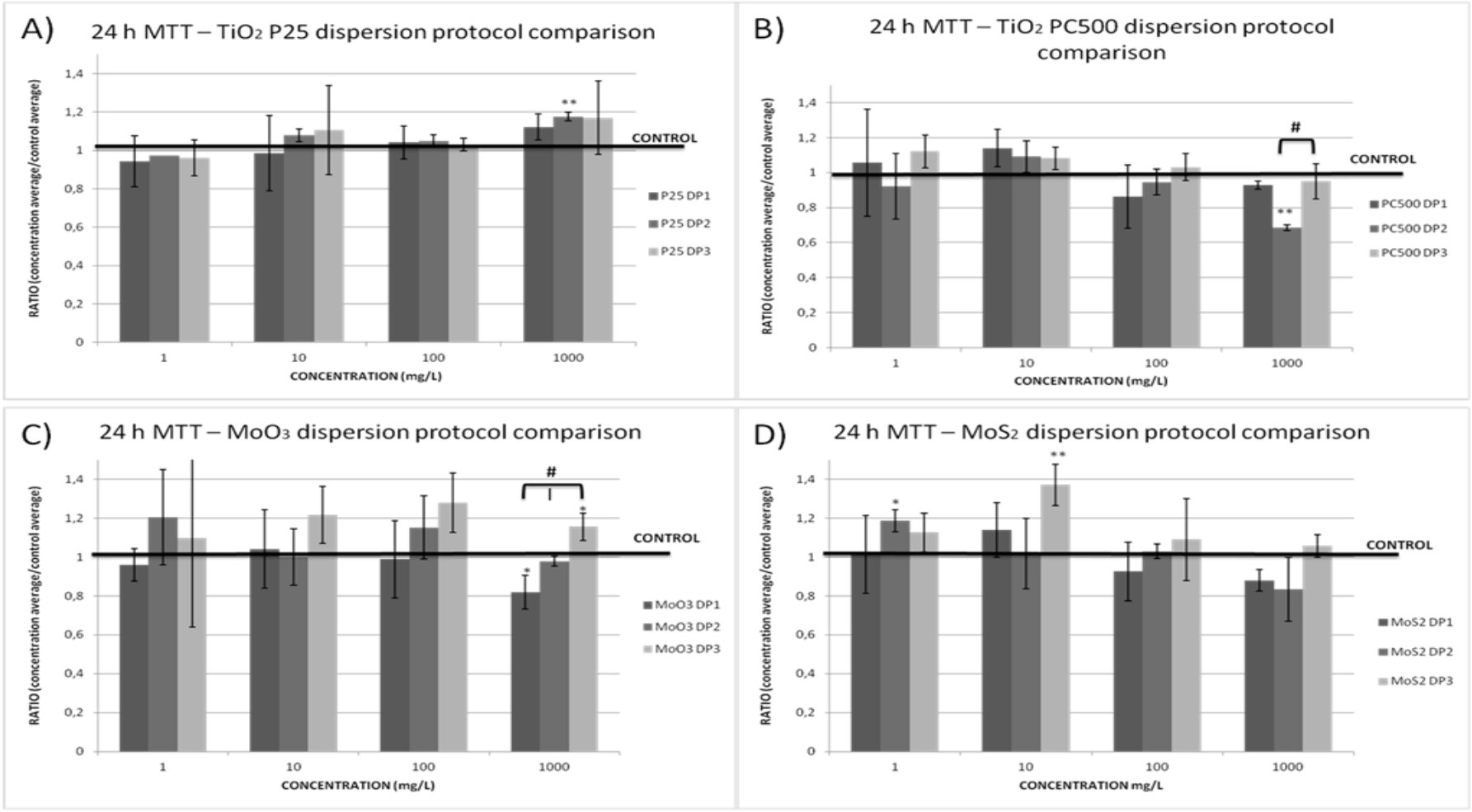

Figure 12: *MTT test showing metabolic activity of HEK 293 cells exposed to selected NPs for 24 h prepared with different dispersion protocols (DP1, DP2 and DP3): A) TiO_2 P25. B) TiO_2 PC500. C) MoO_3 nanowires. D) MoS_2 nanotubes. *p<0.05, **p<0.01 vs. control cells, (n=3)*

48 h exposure test results

Results of MTT cell viability test with the exposure time of 48 h with three different dispersion protocols are presented on Figure 13. For each treatment, there were three samples (n=3) of the same kind tested each time in order to perform statistical analysis.

For the TiO_2 P25 NPs significantly enhanced proliferation activity was observed for the DP3 at the concentration 1mg/L (t-test, type 2, p=0.008). For all the other dispersion protocols and concentrations, no significant difference (p>0.05) in comparison to the control was recorded.

In case of TiO_2 PC500 smaller concentration caused the MTT activity to increase, while higher concentrations had a negative effect of the MTT metabolism. At the concentration 10 mg/L there was a significant increase in proliferation activity of DP3 (t-test, type 2, $p=1*10^{-4}$). Additionally at this concentration also significant difference among dispersion protocols (DP1 and DP2 are statistically significantly different than DP3) was statistically confirmed (DP1 compared to DP3 (t-test, type 2, p=0.001), DP2 compared to DP3 (t-test, type 2, $p=3*10^{-4}$)). Significant decrease in metabolic activity was found at the concentration 1000 mg/L for DP1 (t-test, type 2, p=0.04) and DP2 (t-test, type 2, $p=8.66*10^{-6}$). When comparing MTT activity of different dispersion protocols at the concentration 1000 mg/L a significant difference was found between the protocols DP1 and DP2 in comparison to protocol DP3 (DP1 compared to DP3 (t-test, type 2, p=0.009), DP2 compared to DP3 (t-test, type 2, p=0.003)).

In case of MoO_3 nanowires increased cell metabolic activity was detected for MoO_3 nanowires samples at the lower and a decrease at the higher concentrations. Significantly increased cell proliferation (t-test, type 2, p=0.028) was detected at the concentration 1 mg/L, when using dispersion protocol 2 (DP2). At this concentration no significant difference (p>0.05) was observed between different dispersion protocols. Significant decrease in metabolic activity was observed for DP1 at the concentration 10 mg/L (t-test, type 2, p=0.040), while the other two dispersion protocols showed no significant difference (p>0.05) in cell viability at this concentration compared to the control. The concentration 100 mg/L also showed no significant (p>0.05) cytotoxic or proliferating effects with any of the three dispersion protocols tested. At the concentration 1000 mg/L, DP1 and DP2 showed significant decrease in metabolic activity (cell viability) where DP1 (t-test, type 2, p=0.002) showed higher decrease in cell viability than DP2 (t-test, type 2, p=0.004). Comparing MTT activity of DP1, DP2 and DP3 at this concentration, statistically significant difference between protocols was confirmed (DP1 compared to DP2 (t-test, type 2, p=0.009), DP1 compared to DP3 (t-test, type 2, $p=4*10^{-4}$), DP2 compared to DP3 (t-test, type 2, p=0.008)).

For MoS_2 nanotubes there was no observed difference (p>0.05) in metabolic activity compared to control at the concentrations of 1 and 10 mg/L. However at the concentration 100 mg/L there was a significant decrease in metabolic activity for all three different dispersions protocols (t-test, type 2, p=0.001) for DP1, (t-test, type 2, $p=6.52*10^{-5}$) for DP2, (t-test, type 2, p=0.003) for DP3. DP1 showed the highest decrease in cell viability, followed by DP2 and DP3. When comparing different dispersion protocols, statistically significant difference was found between DP1 and DP3 (t-test, type 2, p=0.030) at concentration 100 mg/L. The same scenario was observed at the concentration 1000 mg/L where a significant decrease in metabolic activity for all three different dispersions protocols was demonstrated (t-test, type 2, $p=5*10^{-4}$) for DP1, (t-test, type 2, $p=4.96*10^{-5}$) for DP2, (t-test, type 2, $p=7*10^{-4}$) for

DP3. DP1 showed the highest decrease in cell viability, followed by DP2 and DP3. When comparing different dispersion protocols, statistically significant difference was found between DP1 and DP3 (t-test, type 2, p=0.013) at concentration 1000 mg/L. Based on the results the threshold value was set between 10 and 100 mg/L.

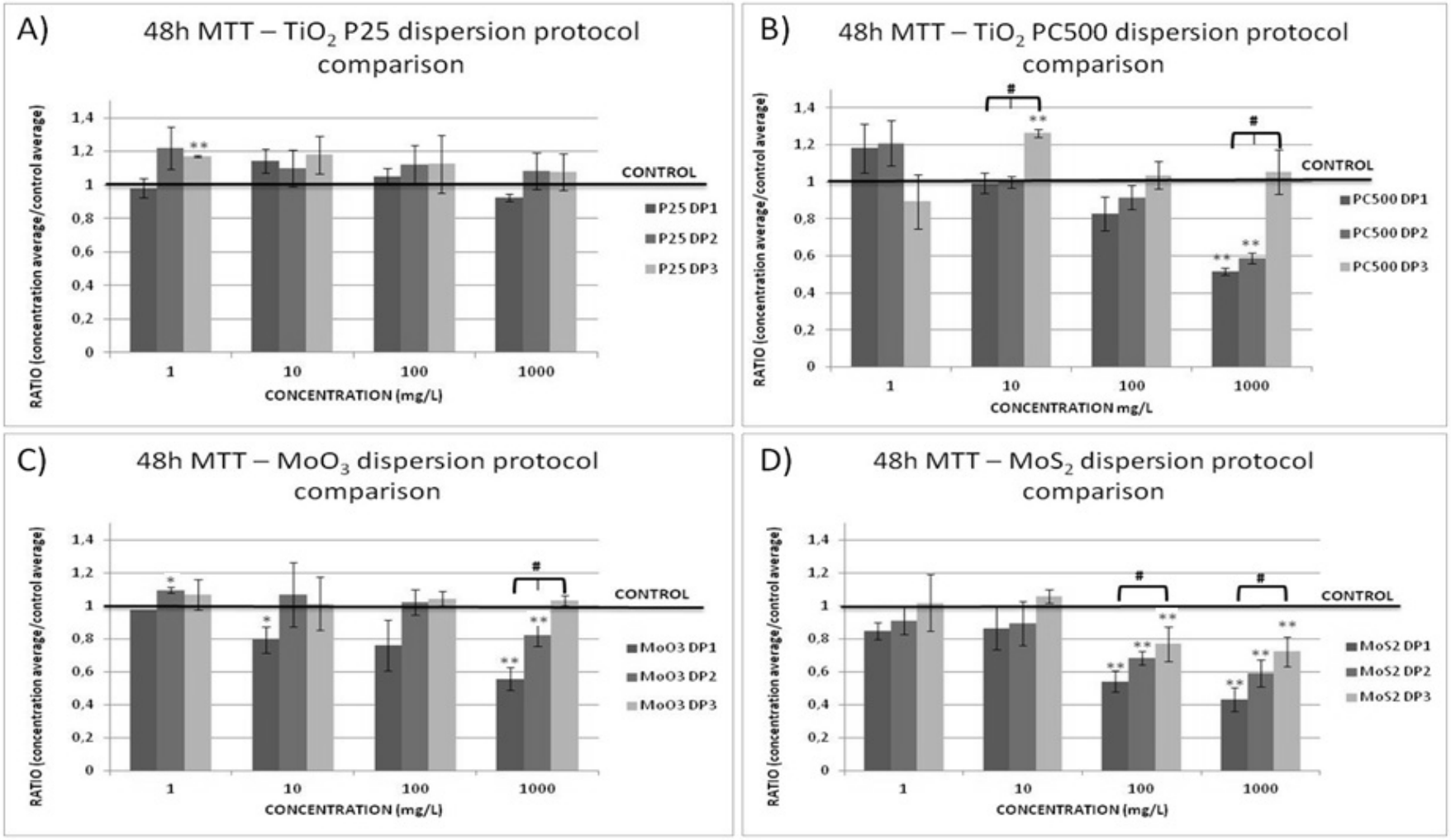

Figure 13: *MTT test showing metabolic activity of HEK 293 cells exposed to selected NPs for 48 h prepared with different dispersion protocols (DP1, DP2 and DP3): A) TiO_2 P25. B) TiO_2 PC500. C) MoO_3 nanowires. D) MoS_2 nanotubes. *p<0.05, **p<0.01 vs. control cells, (n=3)*

72 h exposure test results

Results of MTT cell viability test with the exposure time of 72 h with three different dispersion protocols are presented on Figure 14. Within one treatment, there were three same samples (n=3) analyzed, granting the statistical analysis for the evaluation of the results.

For TiO_2 P25 NPs there was a significant enhancement of metabolic activity in a case of DP1 at the concentration of 1000 mg/L (t-test, type 2, p=0.017). All the other dispersion protocols did not induce any significant (p>0.05) effect on metabolic activity at all concentrations tested. No significant statistical difference (p>0.05) was observed among different dispersion protocols.

TiO_2 PC500 NPs, dispersed according to DP2 showed significant decrease in proliferation activity at the concentration of 100 mg/L (t-test, type 2, p=0.049) as well as at the concentration of 1000 mg/L (t-test, type 2, p=0.003). At the concentration of 1000 mg/L also PC500 NPs, dispersed according to DP1 showed a significant decrease in cell viability (t-test, type 3, $p=2*10^{-4}$). When comparing effects of DP1 and DP2 to effects of DP3 at the concentration level of 1000 mg/L, a statistically significant difference among dispersion protocols was demonstrated (DP1 compared to DP2 (t-test, type 2, $p=3*10^{-4}$), DP2 compared to DP3 (t-test, type 2, p=0.002)), where significant decrease in metabolic activity for DP1 and DP2 was observed, while DP3 showed no significant (p>0.05) effect on metabolic activity.

At the concentration levels of 1, 10 and 100 mg/L, no significant changes (p>0.05) in metabolic activity were found for MoO_3 dispersed with three different protocols. At a concentration of 1000 mg/L, there was a significant decrease in metabolic activity for DP1 (t-test, type 2, p=0.007) and DP2 (t-test, type 2, p=0.002) while DP3 induced no significant decrease (p>0.05) in metabolic activity. In addition, we confirmed significant difference among DP2 and DP3 dispersion protocols (t-test, type 2, p=0.020), while DP1 was not significantly different (p>0.05) to the other two dispersion protocols. Threshold value for DP1 and DP2 dispersion protocols was fitted between the concentration of 100 and 1000 mg/L.

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The results for MoS_2 nanotubes showed a significant decrease in metabolic activity at the concentration level 10 mg/L for DP1 (t-test, type 3, p=0.033) and DP2 (t-test, type 2, p=0.021), at 100mg/L for all three dispersion protocols DP1 (t-test, type 2, $p=5*10^{-4}$), DP2 (t-test, type 3, $p=8*10^{-4}$) and DP3 (t-test, type 2, p=0.013) and at 1000 mg/L for all three dispersion protocols DP1 (t-test, type 2, p=0.003), DP2 (t-test, type 3, $p=6*10^{-4}$) and DP3 (t-test, type 3, p=0.001). At the concentration 10 mg/L and 1000 mg/L statistically significant difference between DP2 and DP3 was observed ((t-test, type 2, p=0.036) at 10 mg/L, (t-test, type 2, p=0.005) at 1000 mg/L), while there was no significant difference (p>0.05) between dispersion protocols at the concentration 100 mg/L. Threshold value for DP1 and DP2 was determined between concentration 1 and 10 mg/L, and between 10 and 100 mg/L for DP3, respectively.

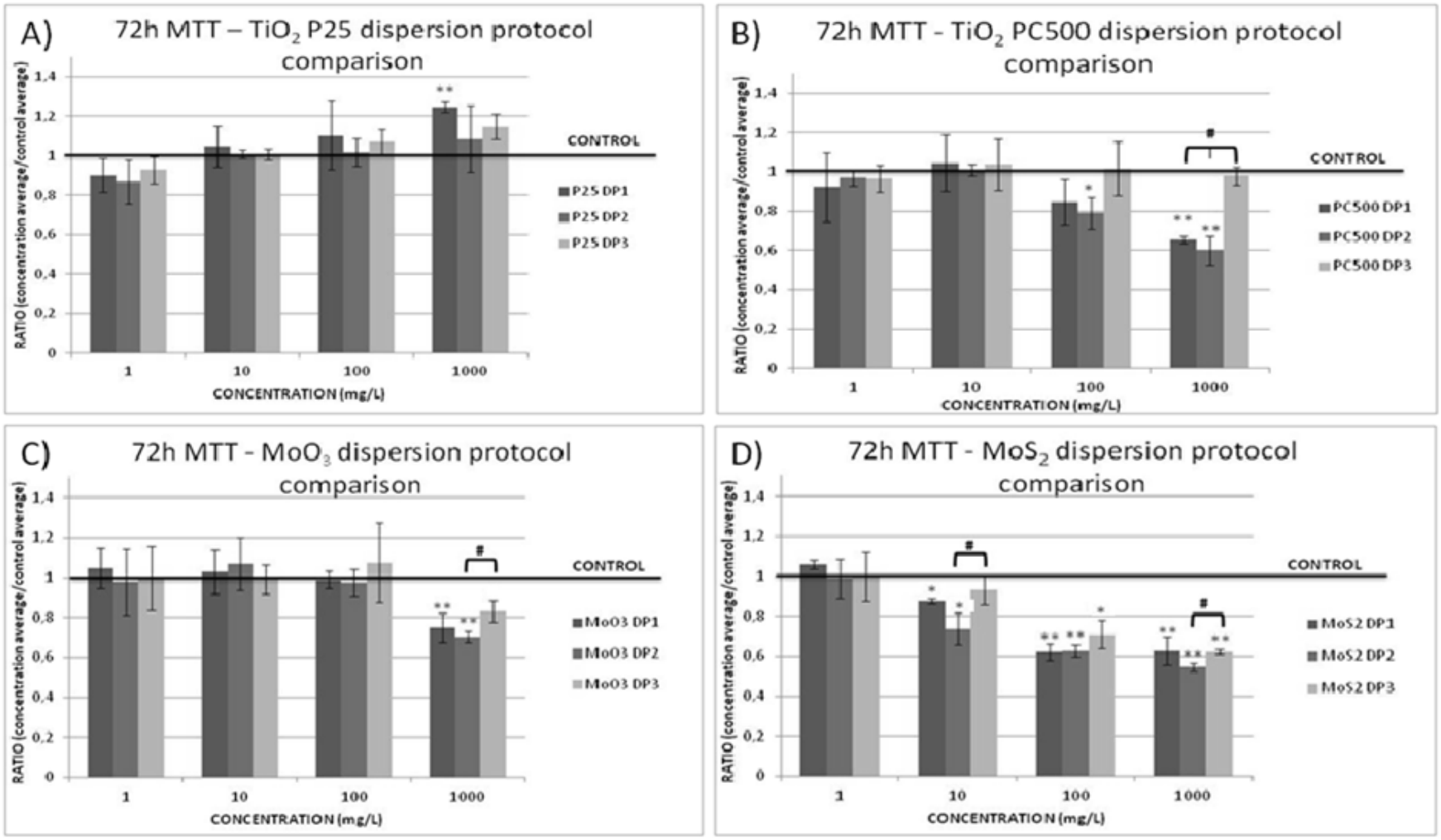

Figure 14: *MTT test showing metabolic activity of HEK 293 cells exposed to selected NPs for 72 h prepared with different dispersion protocols (DP1, DP2 and DP3): A) TiO_2 P25. B) TiO_2 PC500. C) MoO_3 nanowires. D) MoS_2 nanotubes. *p<0.05, **p<0.01 vs. control cells, (n=3)*

Summary of the MTT test results

Table 8: *Summary of the MTT test results for TiO_2 P25 NPs.*

TiO_2 P25 NPs												
Concentration (mg/L)	**1**			**10**			**100**			**1000**		
Dispersion protocol	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**
24 h exposure	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↑	↔
48 h exposure	↔	↔	↑	↔	↔	↔	↔	↔	↔	↔	↔	↔
72 h exposure	↔	↔	↔	↔	↔	↔	↔	↔	↔	↑	↔	↔

Table 9: *Summary of the MTT test results for TiO_2 PC500 NPs.*

TiO_2 PC500 NPs												
Concentration (mg/L)	**1**			**10**			**100**			**1000**		
Dispersion protocol	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**
24 h exposure	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓	↔
48 h exposure	↔	↔	↔	↔	↔	↑	↔	↔	↔	↓	↓	↔
72 h exposure	↔	↔	↔	↔	↔	↔	↔	↓	↔	↓	↓	↔

Table 10: *Summary of the MTT test results for MoO_3 nanowires.*

MoO_3 nanowires												
Concentration (mg/L)	**1**			**10**			**100**			**1000**		
Dispersion protocol	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**
24 h exposure	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓	↔	↑
48 h exposure	↔	↑	↔	↓	↔	↔	↔	↔	↔	↓	↓	↔
72 h exposure	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓	↓	↔

Table 11: *Summary of the MTT test results for MoS_2 nanotubes.*

MoS_2 nanotubes												
Concentration (mg/L)	**1**			**10**			**100**			**1000**		
Dispersion protocol	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**	**DP1**	**DP2**	**DP3**
24 h exposure	↔	↑	↔	↔	↔	↑	↔	↔	↔	↔	↔	↔
48 h exposure	↔	↔	↔	↔	↔	↔	↓	↓	↓	↓	↓	↓
72 h exposure	↔	↔	↔	↓	↓	↔	↓	↓	↓	↓	↓	↓

MTT degradation absorbance values legend

- ↑ significantly ($p<0.05$) larger average metabolic activity than control
- ↓ significantly ($p<0.05$) lower average metabolic activity than control
- ↔ no significant difference ($p>0.05$) in average metabolic activity compared to control

Absorbance of Molybdenum compounds at wavelength 570 nm

Since molybdenum compounds used in our cytotoxicity assay (MoS_2 nanotubes and MoO_3 nanowires) are able to absorb light at wavelength 570 nm, there exists a possibility that residues of particle exposure will contribute to the measurements, therefore interfering with the MTT assay, more precisely, with formazan absorption measurement. The results of the absorbance levels at different tested concentrations of molybdenum NMs are presented on Figure 15, where it can be observed, that when testing higher concentration (1000 mg/L), the effect of absorbance is more pronounced.

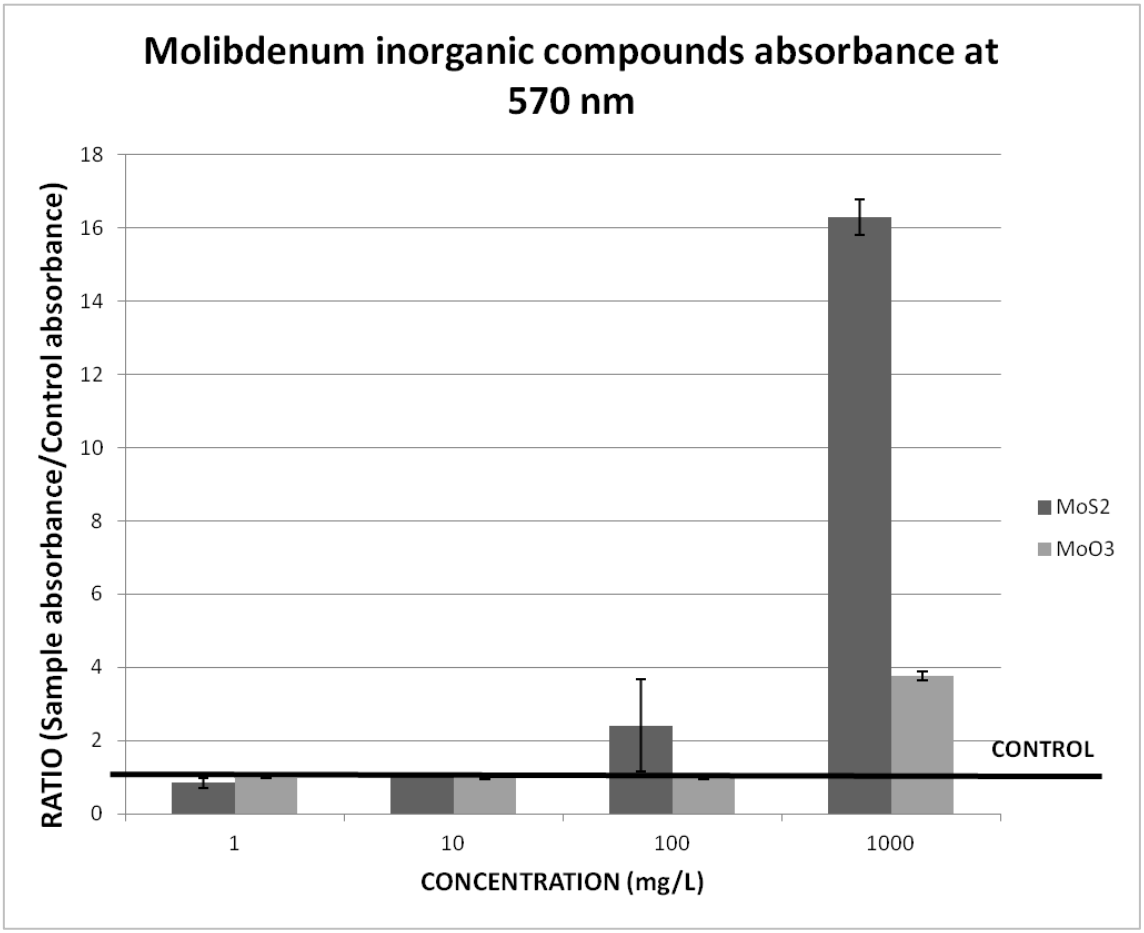

Figure 15: *Absorbance results of MoS_2 nanotubes and MoO_3 nanowires dispersions of different concentrations at 570 nm.*

4.1.3 Genotoxicity assay (H2A.X histone phospohorylation test)

There are molecular mechanisms that get activated after DNA damage in order to correct genomic instability; they are known as DNA damage response. One of the earliest sign of DNA damage responses is the activation of phosphorylated H2A.X, potentially occurring also as a result of double-strand breaks. This response occurs within minutes of the damage, thus making it a useful marker of DNA damage (Garcia-Canton et al., 2012). H2A.X could become phosphorylated at any point during the cell cycle, including during mitosis while other DNA damage response proteins are limited to interphase cells (Nakamura et al., 2010).

In our experiment we examined whether selected NMs (TiO_2 P25 and PC500, MoO_3 nanowires and MoS_2 nanotubes) could generate γH2A.X in a cultured cell line (HEK 293) at the concentration 1000 mg/L. Tested concentration is extremely high and it does not relate to realistic exposure or predicted environmental concentrations, but rather to worst case exposure scenario. There were two samples (n=2) of the same kind in one treatment. Furthermore we determined whether there is a difference in cell survival and γH2A.X formation when using different dispersion preparation methods.

The results on number of cell recovery in control and after NPs exposure are shown on Figure 16. It can be observed that in the case of MoS_2 prepared by DP2 (sonication) and TiO_2 PC500 prepared by DP2, there has been a statistically significant decrease (t-test, type 2, $p=0.038$ for PC500 and t-test, type 2, $p=0.002$ for MoS_2) in cell survival (DAPI cell recovery test). Interestingly, using metabolic MTT test, at the same concentration and preparation method (DP2), also MoO_3 compounds were able to reduce cell metabolism (MTT test).

Percentage of nuclei immunopositive for H2A.X when exposed to concentration 1000 mg/L of selected NMs prepared according to three different dispersion preparation methods can be seen on Figure 17. It can be observed from the data, that only cells exposed to TiO_2 P25 prepared according to DP2 and DP3 induced significant (t-test, type 3, $p=5*10^{-4}$ for DP2 and t-test, type 2, $p=0.007$ for DP3) H2A.X phosphorylation, a sign of activation of DNA repair system, suggesting long-term genotoxic action. Molybdenum compounds were not genotoxic in our experiments. There was no significant increase ($p>0.05$) in H2A.X comparing to the control. When comparing effect of different dispersion preparation methods, we can conclude, that in the case of TiO_2 P25, both DP2 and DP3 induced significant genotoxic effects, however, when using DP1 as dispersion protocol, none of the compounds seemed to induce significant ($p>0.05$) genotoxicity.

Intensity of H2A.X signal is proportional to the damage (see Figure 18) indicating statistically significant differences with respect to the control in case of dispersion protocols DP2 (t-test, type 2, $p=0.004$) and DP3 (t-test, type 2, $p=0.001$) for TiO_2 P25 and DP3 (t-test, type 2, $p=0.011$) in case of TiO_2 PC500.

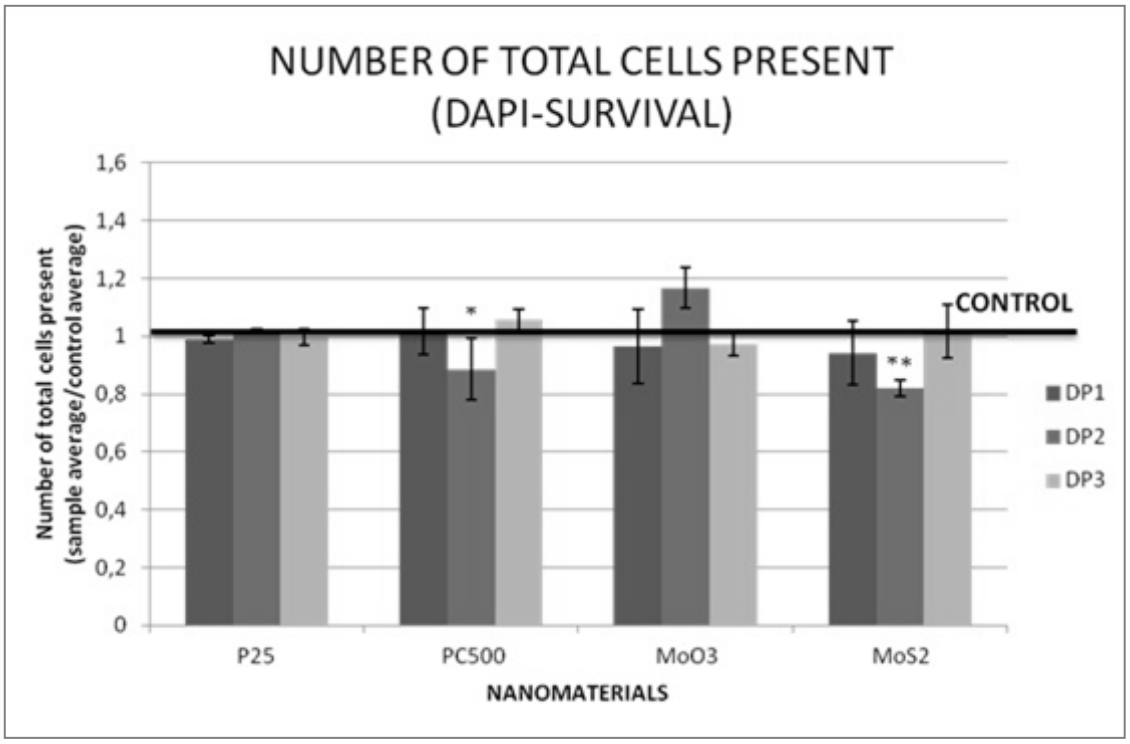

Figure 16: *Number of recovered cells after exposure to concentration 1000 mg/L of selected NMs prepared according to three different dispersion preparation methods. *p<0.05, **p<0.01 vs. control cells (n=2, regions of interest (ROIs)=9)*

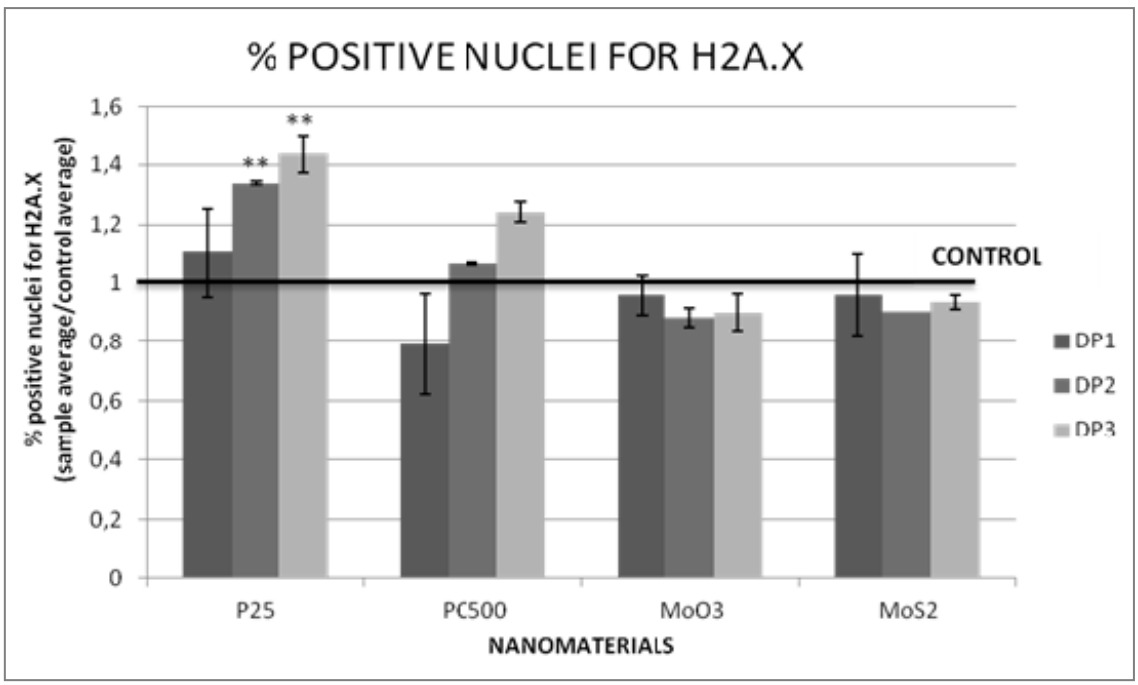

Figure 17: *Percentage of positive nuclei for phosphorylated H2A.X when exposed to concentration 1000 mg/L of selected NMs prepared according to three different dispersion preparation methods. *p<0.05, **p<0.01 vs. control cells (n=2, ROIs=9)*

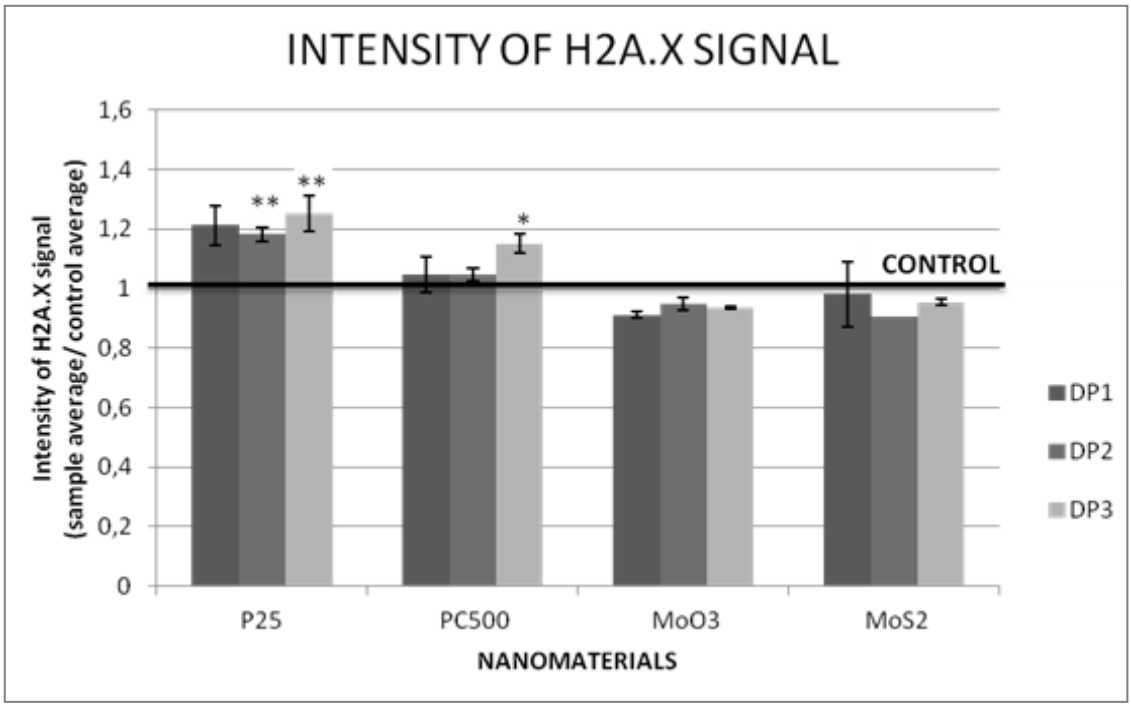

Figure 18: *Intensity of H2A.X signal when exposed to concentration 1000 mg/L of selected NMs prepared according to three different dispersion preparation methods. *p<0.05, **p<0.01 vs. control cells (n=2, ROIs=9)*

4.1.4 Ecotoxicity assay (*Vibrio fischeri* bioluminescence inhibition test)

Luminescence inhibition results on testing four different NMs (TiO_2 P25, TiO_2 PC500, MoS_2 and MoO_3) of a concentration 1000 mg/L using three different dispersion protocols (DP1, DP2, DP3) are presented in Figure 19. Tested concentration is high and simulates worst case high exposure, rather than realistic scenario. Data on predicted environmental concentrations for TiO_2 NPs are thus much lower.

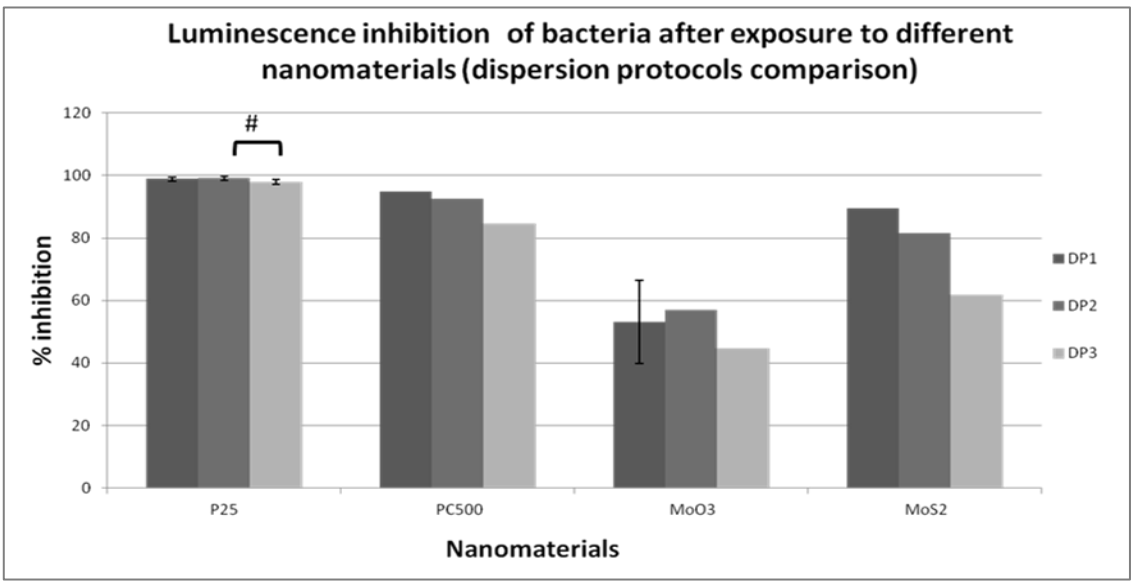

Figure 19: *Vibrio fischeri bioluminescence inhibition after exposure to different NMs prepared according to three different dispersion protocols.*

For TiO_2 P25, inhibition values were high (98.86% for DP1, 99.09% for DP2 and 97.85% for DP3) showing a high toxicity of P25 to *Vibrio fischeri* at high concentration 1000 mg/L tested. There was a significant difference (t-test, type 2, p=0.039, n=4) among dispersion protocol 2 (DP2-sonication without addition of protein stabilizer) and dispersion protocol 3 (DP3-no sonication or dispersion stabilizer addition, only stirring). Comparing DP1 to DP2 and DP1 to DP3, no significant difference was found (t-test, type 2, p=0.623), and (t-test, type 2, p=0.098), respectively.

TiO_2 PC500 indicated high toxic effects with inhibition values of 94.94% for DP1 (n=2), 92.67% for DP2 (n=2) and 84.60% for DP3 (n=1), however number of samples was too small to evaluate the results statistically.

Molybdenum NMs compounds showed certain ecotoxicity to bacteria *Vibrio fischeri,* where in the case of MoO_3 inhibition values obtained were 53.22% for DP1 (n=3), 57.02% for DP2 (n=2) and 44.72% for DP3 (n=2). For the MoS_2 the inhibition values were 89.55% for DP1 (n=2), 81.43% for DP2 (n=2) and 61.82% for DP3 (n=1). In both cases (MoO_3 nanowires and MoS_2 nanotubes) calculating statistical differences among different dispersion protocols was not feasible, since the number of parallels was too small.

4.2 Discussion

4.2.1 Particle size distribution analysis (Dynamic Light Scattering)

We confirmed accepted paradigm that dispersion of NMs in solution rarely leads to the distribution at the primary particle size. According to DLS results, all the NMs in the dispersion readily tend to agglomerate and their hydrodynamic diameter strongly differ from the primary particle diameter declared by the manufacturer.

When comparing size distributions among different dispersion protocols, we can conclude, that for all the tested NMs (considering number distribution data), optimized dispersion protocol (DP1) that includes sonication and use of protein serum albumin as dispersion stabilizer, is the most efficient protocol to maintain the particles dispersion at the minimum aggregation state. On the other hand, considering volume distribution data indicated that the smallest agglomerates were obtained using DP2 (sonication with no protein serum stabilizer). DP3 (only stirring) obtained the largest particles by number distribution as well as by volume distribution when comparing to DP1 and DP2.

When comparing secondary size distributions of TiO_2 PC500 (smaller primary size) with TiO_2 P25 (larger primary size), we can conclude, that P25 forms larger agglomerates in dispersion than PC500, when comparing the same dispersion preparation protocols. Our results are not in accordance with the published study of Nguyen et al. (2005), where both particles were also found aggregated, but with PC500 characterized by a larger dispersive particle size of 600-700 nm and P25 with smaller dispersive particle size of 200-215 nm.

There was no nano fraction (<100nm) present in our tested dispersions in all four tested NMs (see Table 7), which indicates, that all the particles were agglomerated and there was no particles of the primary sizes present in the dispersions. Consequently,

the toxicity observed is not related to nano sized particles, but rather to different sizes of their agglomerates.

However, according to the observed results of size distribution analysis, we hypothesized that dispersions containing smaller agglomerates were more likely to induce toxic effects. According to this, DP1 and DP2 dispersion protocols were expected to induce higher toxic effects in comparison to DP3 that contained much larger particles. Besides size, particles surface reactivity must also be taken into account when considering toxic potential. That is why it is possible, that dispersions prepared using DP1 would induce lower toxicity than dispersions prepared by DP2, as protein serum forms a coating around particles which can decrease surface reactivity of the particles and consequently, lowering toxicity levels.

4.2.2 Nanomaterials primary size (surface area) and crystal phase dependent cytotoxicity

Primary physical-chemical characteristics of NPs (such as size, chemical composition, crystalline structure, surface properties...) are proposed to be critical determinants of their toxic potential (Johnston et al., 2009).

TiO_2 nanoparticles

Comparing primary characteristics of TiO_2 P25 (↑primary crystalline size, ↓ surface area, rutile:anatase=20%:80% crystal phase) with the one of TiO_2 PC500 NPs (↓ primary crystalline size, ↑ surface area, 100% anatase crystal phase), our results demonstrated that PC500 particles, characterized by smaller primary size, larger surface area and higher percentage of anatase crystalline phase induced indeed significantly larger cytotoxic effects (a significant decrease in metabolic activity) than larger NPs having smaller surface area and less anatase in the crystalline phase (P25). P25 indeed induced no significant cytotoxic effect at all concentrations tested and at all three different exposure times (see Figures 12, 13 and 14). It has to be noted at this point, that observed toxicity might be also related to agglomerate size that was smaller in case of PC500, which could contribute to higher toxic effects of this compound.

Cytotoxicity of TiO_2 in our experiment seems to be crystalline size (surface area) and crystal phase dependent since smaller particles with larger surface area and more anatase crystal phase (PC500) induced cytotoxic effects while larger particles with smaller surface area and less anatase in crystal phase (P25) did not. It needs to be pointed out, that not all dispersion preparation methods (DP1, DP2 and DP3) of PC500 induced cytotoxicity at above mentioned concentrations and that toxicity could be related besides to primary size also to size of the agglomerates (secondary size). This indicates that cytotoxicity is not only size and crystal phase, but also depends on dispersion preparation method and exposure time. Below, dispersion preparation and exposure time dependency is discussed.

The crystal size and phase dependency of TiO_2 toxicity has been frequently demonstrated in previously published literature (see Table 4). Anatase crystal phase has been shown to be the most toxic form of TiO_2, with a number of studies supporting this conclusion (Dunford et al., 1997; Nakagawa et al., 1997; Wang et al., 2007; Warheit et al., 2007). Nakagawa et al. (1997) also illustrated that besides crystal phase also primary size (surface area) contributed to the response.

While most of the studies are confirming crystal size and phase dependent toxicity there are some that failed to demonstrate such a relationship (Lanone et al., 2009) and no correlation between cytotoxicity and equivalent spherical diameter, specific surface area and crystal phase was found.

MoO_3 nanowires and MoS_2 nanotubes

MoS_2 nanotubes and MoO_3 nanowires showed a significant cytotoxic effect on HEK 293 cell line. MoO_3 significantly decreased cell viability at the concentration above 100 mg/L. Except for 48 h MTT test, there was some significant toxicity using optimal dispersion protocol (DP1) already at the concentration 10 mg/L. MoS_2 induced no significant toxicity at 24 h, but significant decrease in metabolic activity was found at 48 h (above 10 mg/L) and at 72 h (above 1 mg/L) exposure.

This toxicity could be attributed to extremely non isotropic shape (an extremely large surface to weight ratio) with a layer type structure.

No studies on size- and phase-dependent toxicity of molybdenum inorganic nanotubes and nanowires were found reported to-date, however there were few studies (Braydich-Stolle et al., 2005; Hussain et al., 2005; Wu et al., 2011) assessing their toxicity. Our results are difficult to compare with different studies due to the difference in assay types, cell lines, exposure times and dispersion preparation methods details.

Higher toxicity of MoS_2 is not in accordance with our expectations, since MoS_2 is considered as very inert material in comparison to MoO_3 which is known for its toxic effects. These differences in observed toxicity cannot be a consequence of primary size effects, since both compounds have a similar primary sizes, as they are synthesized out of the same material.

Higher toxicity of MoS_2 might be attributed to a high density of surface defects on MoS_2 nanotubes, like sulphur vacancies and broken molecular layers. These defects represent possible oxidative cites due to presence of unsaturated bonds. Alternative explanation is presence of iodine impurities in 100 ppm in the sample (Viršek et al., 2012).

It needs to be pointed out, that the NMs intrinsic characteristics can determine material-derived interferences and artifacts in biological colorimetric assays. In particular, the known absorbance of molybdenum samples at 570 nm, prompted us to validate MTT measurements with specific control tests. The experiments showed a possible residual interference of molybdenum compounds absorbance at higher concentrations (1000 mg/L), which suggested potential artifacts in these measurements. The measured absorbance of the sample could therefore be a consequence of a molybdenum compound and formazan absorbance, so the results should be kept with caution. For these reasons, and to develop new biomarker-based tests to assess material cytotoxicity, we have optimized fluorescence DAPI /H2A.X assays, for a less problematic results interpretation.

According to published literature (Braydich-Stolle et al., 2005; Hussain et al., 2005), MoO_3 is reported to be non toxic until concentrations of 50 mg/L, while at higher doses significant toxicity was observed. This corresponds well with our results, where significant decrease in MTT activity was found above 100 mg/L for three dispersion protocols, except in the case of 48 h exposure time for optimal dispersion protocol (DP1), where significant toxicity was observed already at 10 mg/L.

In regards to MoS_2, our results showed no significant toxicity at 48 h exposure time at the concentrations up to 10 mg/L for all three different dispersion preparation methods. Our data are in accordance with the published study of Wu et al. (2011).

4.2.3 Nanomaterials exposure time dependent cytotoxicity

Comparing cytotoxicity data from 24, 48 and 72 h exposure time, a time-dependent toxicity was observed.

For TiO_2 PC500 and MoS_2, longer exposure (72 h) induced higher toxic effects than shorter one (24 h).

MoS_2 compounds showed no significant decrease in metabolic activity at 24 h exposure time. In case of 48 h exposure time, significant decrease in metabolic activity was detected at the concentrations of 100 mg/L, and in case of 72 h exposure at 10 mg/L, which indicated that longer time of exposure induced higher cytotoxicity. TiO_2 PC500 also followed this trend. For MoO_3 and TiO_2 P25, no time dependent cytotoxicity was observed.

Cherchi and Gu (2010) reported the exposure time to have a greater influence on TiO_2 toxicity than the TiO_2 NPs concentration.

Our results are in accordance with Dechsakulthorn et al. (2007), who reported exposure time-dependant TiO_2 toxicity using MTS ('one-step' MTT) assay. The results showed that 4 h exposure of TiO_2 NPs has only a mild adverse effect to human cell fibroblasts. However, when the exposure time increased up to 24 h, NPs had a substantial toxic impact to cells. We can say that chronic toxicity (longer exposure time) is higher than acute toxicity of TiO_2 NPs.

4.2.4 Nanomaterials dispersion protocol (secondary size) dependent cytotoxicity

Differences in particle dispersion/agglomeration have recently been shown to play an important role in NM toxicity. It has been found that the NPs with different surface properties and aggregation behavior can cause different responses to the culture medium, which in turn can lead to different degrees of toxicity (Somasundaran et al., 2010; Magdolenova et al. 2012; Laban et al., 2010; Malhi, 2012).

Different dispersion protocols tested for PC500 induced different cytotoxic responses; DP3 induced significantly lower toxicity when compared to DP1 and DP2. This was most probably due to smaller agglomeration state of dispersed particle prepared using DP1 and DP2 methods than DP3.

Similar size-related (agglomeration state) observations due to dispersion differences can be observed also in the case of MoO_3, becoming apparent only at very high concentrations (1000 mg/L) with the most explicit decrease confirmed at 48 h and 72 h exposure. For all different dispersion protocols, statistically significant difference was found.

Similar data were obtained for MoS_2 nanotubes where DP3 dispersion protocol induced significantly lower MoS_2 cytotoxicity than DP1 and DP2.

As described in the results (section 4.1.2 Cytotoxicity assay-MTT cell viability test), NMs dispersed to generate less agglomerates (DP1 and DP2 vs. DP3) were proven to induce higher cytotoxicity in our experiment (see Figures 12, 13 and 14).

Due to a fact that very few reports on effects of differently dispersed NMs on *in vitro* toxicity exist; we were limited in the comparisons, however our results are an important step for further studies. One of the few studies on this issue (Magdolenova et al., 2012) showed that the dispersion method used can influence the results of toxicity studies. Regarding cytotoxicity, TiO_2 NPs dispersion with large agglomerates (3 min sonication and no serum in stock solution) induced higher cytotoxic effects at higher concentrations of TiO_2 NPs than dispersion with smaller agglomerates (serum in stock solution and sonication 15 min). Most probably due to larger agglomerates being less stable and can thus be released into cells as individual NPs. In case where no serum was used in the dispersion protocol, the surface of NPs in agglomerates might not be covered by a protein corona and thus the effect can be more pronounced.

In our experiments, DP1 characterized by smaller agglomerates induced slightly higher cytotoxic levels than DP2 characterized by larger agglomerates. Although addition of serum to the dispersion medium was reported to be sufficient to decrease cytotoxicity (Magdolenova et al., 2012), in our study dispersion protocols using protein stabilizer induced higher toxicity than serum free dispersion protocols. The reasoning for increased toxicity when using protocol that includes addition of protein serum stabilizer is most probably due to the fact, that the presence of serum in NPs dispersion cause a reduction in the size of NP aggregates in the dispersion (Johnston et al., 2010). The size-related changes in the agglomeration state after introduced into the cell media do not occur (Sohaebuddin et al., 2010), so the size of agglomerates in media is the same as in dispersion.

In addition, smaller NPs are considered to be more toxic because they can more easily enter the cell. As reported by Semakov and Tsur (2006), adsorption of molecules onto the surface may increase the energy of repulsion (electrostatic or steric); a fact that imply that the attraction between the surfaces may be reduced, leading to weaker agglomeration. Weaker agglomeration and consequently smaller agglomerates were also shown in our experiment, and are key factors in causing toxicity since dispersion with larger agglomerates caused lower toxicity than dispersion with smaller agglomerates when comparing different dispersions of the same nanomaterial.

However, as differences in toxicity effects of DP1 compared to DP2 were relatively small, this was an indication that addition of protein stabilizer after sonication does not have a major effect on the cytotoxicity when compared to sonicated only dispersions. This is in accordance with Murdock et al. (2008) and Corradi et al. (2012), who reported that TiO_2 particles did not show any toxicity regardless the serum present in the dispersion media.

We also observed significant toxicity differences when comparing DP1 and DP2 dispersion preparation methods with DP3 (stirring). Our data confirmed the hypothesis that exposure to sonicated NPs exert higher toxicity than weakly dispersed solution obtained with stirring only. Sonication limits particle aggregations, causes particle agglomerates fragmentation, and increases particle dissolution, that all turn into increased toxicity (Laban et al., 2010; Malhi, 2012; Hund-Rinke et al., 2010).

4.2.5 Nanomaterials genotoxicity (H2A.X histone phosphorylation test)

Cells exposed to TiO_2 P25, prepared according to DP2 and DP3 protocols, showed significantly larger activation of the DNA repair system namely H2A.X phosphorylation than controls exposed with medium only. These data suggest long-term genotoxic action of TiO_2 P25 at high concentration of 1000 mg/L. TiO_2 PC500 did not show any significant difference respect to control, in terms of percentage of positive nuclei for H2A.X. However, significant difference in intensity of H2A.X signal with respect to control was observed for DP3. Molybdenum compounds did not activate DNA repair marker H2A.X phosphorylation in our experiments when testing high exposure concentration of 1000 mg/L.

A number of studies have shown that cells exposed to TiO_2 NPs have the potential to exhibit genotoxic activity in cultured cell lines (see Table 4 for references). On the other hand, some reports showed that TiO_2 NPs did not induce DNA damage and mutation using the Ames test, micronucleus assay, comet assay and other assays used to determine genotoxic effects.

Recently Toyooka et al. (2012) studied the TiO_2 NPs genotoxicity using H2A.X histone phosphorylation assay. This study showed that TiO_2 particles generated H2A.X phosphorylation (in the A549 cell line) that was positively correlated to the extent of TiO_2 particles uptake. On the other hand, H2A.X phosphorylation was attenuated by coating the TiO_2 surface particles with bovine serum albumin. The same observation was made by Magdolenova et al. (2012), using comet assay to determine genotoxicity, since TiO_2 NPs dispersion with large agglomerates (sonication and no serum) induced DNA damage in different cell lines, while TiO_2 NPs agglomerates with <200 nm diameter in serum sonicated solution had no effect on genotoxicity.

Among all the dispersion protocols tested in the presence of albumin as protein stabilizer we found no significant genotoxic effect, in accordance with results of Toyooka et al. (2012), who suggested that serum albumin coating decrease generation of γH2A.X and DNA damage.

Once cells are exposed to NP, spontaneous cell uptake occurs. The consequences of particle internalization induce oxidative, cytotoxic and genotoxic damage. The physical-chemical properties (size and surface charge) of the particles influence their internalization in the cells (Johnstone et al., 2009).

Despite we have not tested the ROS generation in our model, published literature suggest that TiO_2 NPs lead to oxidative stress followed by inflammation, cytotoxicity and genotoxicity (Dunford et al., 1997; Gurr et al., 2005; Wang et al., 2007). No published data are available for MoO_3 and MoS_2 toxicity, rendering our preliminary data original and highly interesting for further studies.

4.2.6 Nanomaterials ecotoxicity (*Vibrio fischeri* bioluminescence inhibition test)

Although some of the selected NMs showed bioluminescence inhibition of *Vibrio fisheri*, these measurements lack consistent replications of the experimental data. Nevertheless, we will present some rudimentary data, keeping in mind that these are only preliminary results.

For TiO_2 P25 and PC500, inhibition values indicated a high toxicity to *Vibrio fischeri,* with the luminescence inhibition values of 98.86% for DP1, 99.09% for DP2 and 97.85% for DP3 in case of TiO_2 P25; 94.94% for DP1, 92.67% for DP2 and 84.60% for DP3 in case of TiO_2 PC500; 53.22% for DP1, 57.02% for DP2 and 44.72% for DP3 in case of MoO_3; 89.55% for DP1, 81.43% for DP2 and 61.82% for DP3 in case of MoS_2. We need to point out that we tested very high concentration of 1000 mg/L, which is not a realistic predicted environmental concentration, but rater simulates worst case exposure scenario.

Positive toxic effect of TiO_2 to bacteria was obtained in study of Adams et al. (2006). However, the studies on toxicity of TiO_2 NPs have mainly reported no toxic effects (no inhibition of luminescence) to bacteria *Vibrio fischeri* (Pereira et al., op cit Lopes et al., 2012; Lopes et al., 2012; Heinlaan et al., 2008; Garcia et al., 2011; Velzeboer et al., 2008).

In addition, Lopes et al. (2012) reported no toxicity for TiO_2, as no inhibition in bioluminescence of *V. fischeri* was observed at any tested concentration. It was assumed that the reason for this lack of effects of TiO_2 is related to particle agglomeration resulting in much larger particles (the particle size of these NPs ranged from 2.5 to 6.8 μm diameter, and no sonication was used). This alteration of particles size may influence its effects, toxicity and availability (as it is not expected that particles with such sizes will cross the cells membrane, unless by processes such as endocytosis). However, in the study, no sonication was used (aiming for a higher ecological relevance in exposure conditions).

The results of the previous studies confirmed the importance of dispersion protocols on toxicity, keeping the size of NP at a particulate, rather than on the agglomerate level. As in the above mentioned studies, low luminescence inhibition was due to the larger particles, much higher toxicity levels are most probably due to the smaller particle sizes. In addition, there might be other reasons for higher values of luminescence inhibition, one of them being turbidity of the samples.

The measurement could be subjected to variations in the presence of turbidity, which interferes with luminous intensity measurements (Parvez et al., 2006). The samples in our experiment samples were strongly turbid since the high concentrations of 1000 mg/L were used to test the toxicity. As a consequence, turbid samples require centrifugation or filtration before contacting with the test culture suspension. Alternatively, luminometer equipped with dispenser, controller and mixer would be required (Parvez et al., 2006), where partial solution for this would be repeated measurements of toxicity after the filtration or centrifugation.

4.2.7 Behavior, occurrence and effects of nanomaterials in the environment, recommendations and future needs

NPs toxicity testing is an important step in assessing the risks of NPs in the environment. Besides toxicity testing, risk assessment also requires understanding of NPs mobility, persistency, reactivity and bioavailability (Nowack and Bucheli, 2007).

Release of NPs may occur from point sources (production facilities, landfills or wastewater treatment plants) or from nonpoint sources (wear from materials containing NPs). During production or transport accidental release is also possible. Humans can

be directly influenced by NPs, either through exposure to air, soil or water or indirectly by consuming plants or animals which have accumulated NPs (Nowack and Bucheli, 2007).

Size distribution analysis confirmed that NMs in our study were highly agglomerated. Agglomerated NPs will be less mobile and disperse and consequently less toxic, however the uptake by sediment-dwelling animals or filter feeders would still be possible (Nowack and Bucheli, 2007).

Our toxicity results show that tested NMs in our experiment have the effect on HEK 293 cell line and bacteria *Vibrio fischeri* at high concentrations used. The next step towards risk assessment of our selected NMs should therefore be an estimation of the predicted environmental concentrations of NMs and their exposure in the environment.

To date only few quantitative analytical techniques for measuring NPs in natural systems are available, which results in a serious lack of information about their occurrence in the environment (Nowack and Bucheli, 2007). Data on the production and predicted environmental concentrations of NMs are sparse and mostly based on models.

From the production point of view, Hendren et al. (2011) estimated production for TiO_2 NPs as a basis for exposure assessment. It was estimated that upper bound would be 38000 t and lower bound to be 7800 t per year for annual U.S. production. Study conducted by Müller (2007) predicted that 35 t (according to realistic scenario) and 400 t (according to high exposure scenario) of TiO_2 NPs used in Switzerland are distributed to the environmental compartments. Predicted environmental concentrations (PEC) were calculated for each of the compartments (see Table 3) ranged from $1.55*10^{-6}$ µg/L (realistic scenario) to 3933,33 µg/L (high exposure scenario). If we compare this concentrations with our results, we can conclude that none of TiO_2 NMs tested in our study was toxic at this concentration level.

No data on PEC for molybdenum NMs exists up to date; however our study indicated that threshold value in case of MoS_2 is set between 1 and 10 mg/L and in case of MoO_3 between 10 and 100 mg/L. We propose this concentration range to be tested in further studies assessing toxicity of such compounds.

Because there is no information so far on the validity of the use of current exposure models, their validity should be assessed and, if necessary, new models and methods should be developed for the prediction of the PEC in water, soil and air (SCENIHR, 2007b).

Furthermore, despite the wide range of potential exposure situations and therefore potential risks, specific regulation has been slow to emerge, this being partly due to the broad range of NM applications where different regulatory frameworks apply (Seaton et al., 2012).

One of the main problems when testing toxicity of NPs is the lack of appropriate standardized protocols, since data from different tests with different procedures and conditions are hard to compare. Although there exist some recommendation reviews for testing NMs (e.g. Preliminary Review of OECD Test Guidelines for Their Applicability to Manufactured Nanomaterials) (OECD, 2009b), there is a great need to establish standard protocols for testing NMs in order to ensure an efficient assessment of these materials. In addition, it is also urgent to define “reference” materials and

properties that should be characterized for NMs used in environmental and toxicological studies. When testing the toxicity of number of different NMs, testing protocol should be adopted to NMs properties that play a role in the particle behavior in the environment and in organisms (Stone et al., 2010).

Our study can serve as a basis for future development of standardized procedures for toxicity testing as dispersion preparation methods, exposure time and crystalline size were shown to have an impact on toxicity testing outcome.

Finally, European commission (2008) discussed whether EU law ensures all applications of nanoscience and nanotechnologies to meet a high level of public health, safety and environmental protection. It was concluded that there are main challenges lying ahead in its implementation which may require adaptations to the legislation as well as the development of new guidelines.

5 CONCLUSIONS

Dispersion of NMs does not lead to same distribution as reported at the primary particle size. According to results of secondary characterization, all the NMs in the dispersion were highly agglomerated, no nano fraction was obtained and their hydrodynamic diameter strongly differed from the primary particle diameter declared by the manufacturer. Using three different protocols, we established that there are significant differences in size distributions among them.

NMs-induced cytotoxicity varied based on particle composition, size (surface area), crystal phase (for TiO_2 samples), exposure time and dispersion preparation method used. Although all the parameters are inextricably connected, each of the parameter impacted the MTT reduction to each own extent.

Cells exposed to TiO_2 P25, showed significantly larger activation of the DNA repair system namely H2A.X phosphorylation than controls exposed with medium only. TiO_2 PC500 did not show any significant difference than control, in terms of percentage of positive nuclei for H2A.X. However, significant difference in intensity of H2A.X signal with respect to control was observed. Among all the dispersion protocols tested in the presence of albumin as protein stabilizer, we found no significant genotoxic effect. Molybdenum compounds did not activate DNA repair marker H2A.X phosphorylation in our experiments.

Using luminescence inhibition test high luminescence inhibition was found for both TiO_2 compounds and to a lower extent in inorganic molybdenum NMs compounds, however additional experiments are necessary to confirm the current results.

In conclusion, the dispersion preparation method used can only in some cases influence the results of toxicity studies. The impact of dispersion method on the results of toxicity testing therefore depends also on various parameters, such as the type of nanomaterial, concentration, exposure time and type of the assay being used.

Our work demonstrates the complexity of this issue and the difficulties to obtain reproducible data. Due to the little background knowledge on the physical/chemical and biological mechanisms supporting the NPs behavior in physiological conditions, more work is required to better dissect the effects of NPs in the environment and in biological models.

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ANNEX A

Dynamic light scattering particle size distribution analysis for selected NMs using three different dispersion protocols.

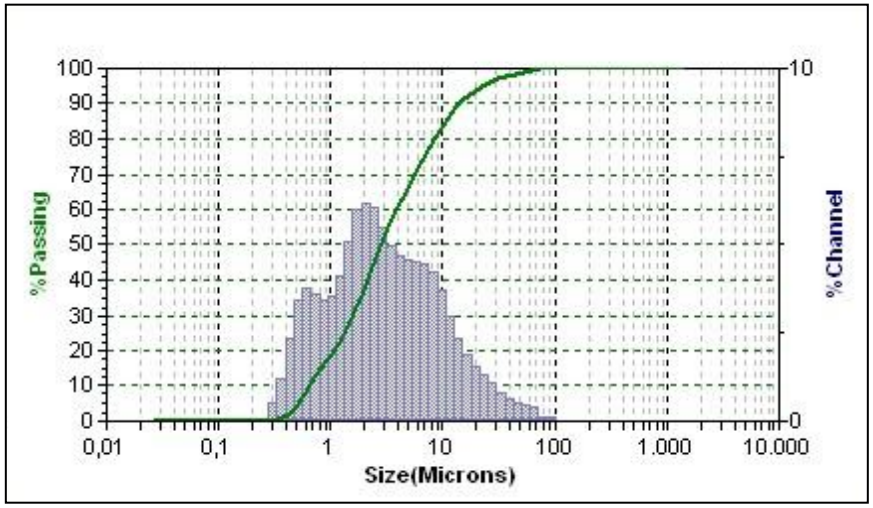

Figure 20: *DLS particle size distribution analysis for P25 DP1*

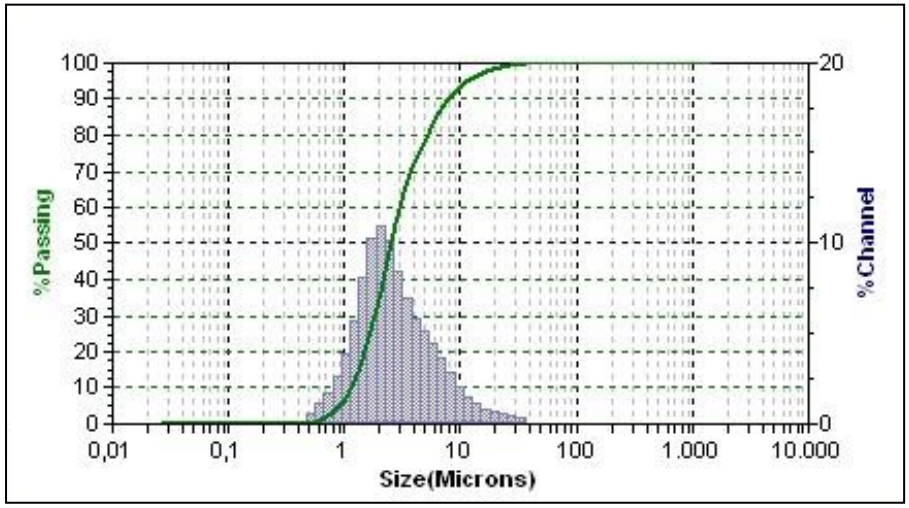

Figure 21: *DLS particle size distribution analysis for P25 DP2.*

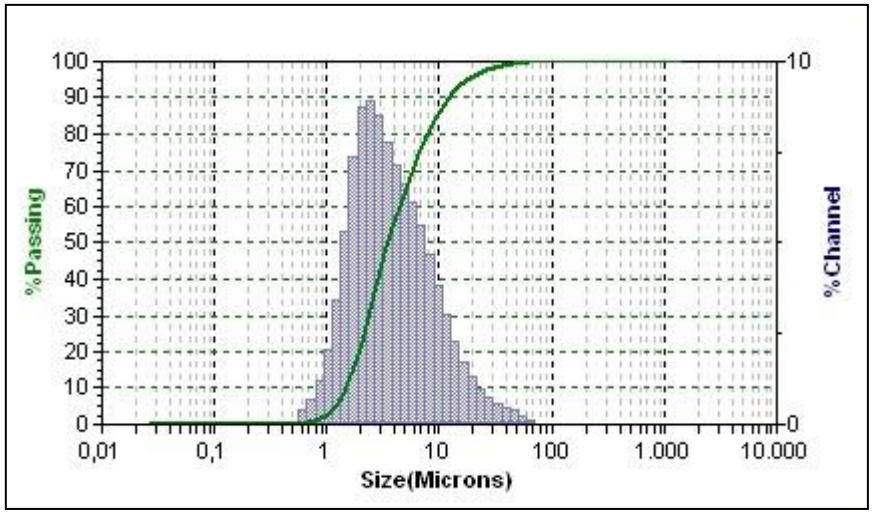

Figure 22: *DLS particle size distribution analysis for P25 DP3.*

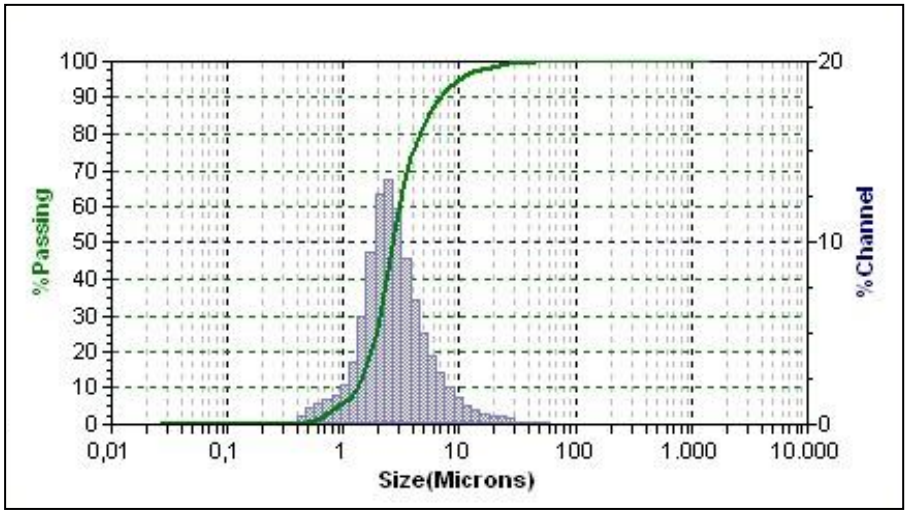

Figure 23: *DLS particle size distribution analysis for PC500 DP1.*

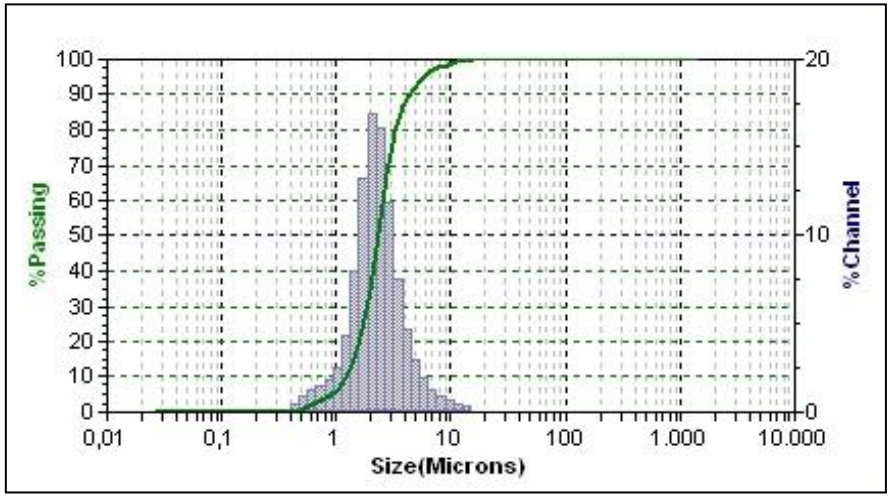

Figure 24: *DLS particle size distribution analysis for PC500 DP2.*

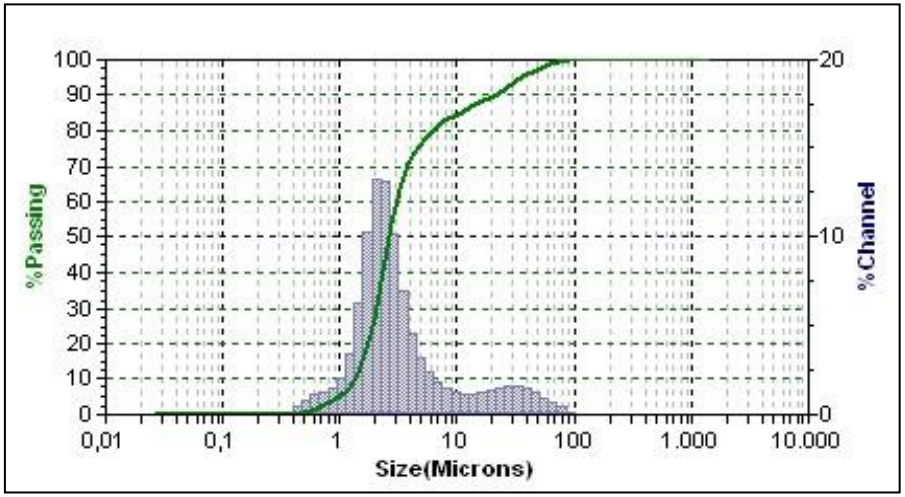

Figure 25: *DLS particle size distribution analysis for PC500 DP3.*

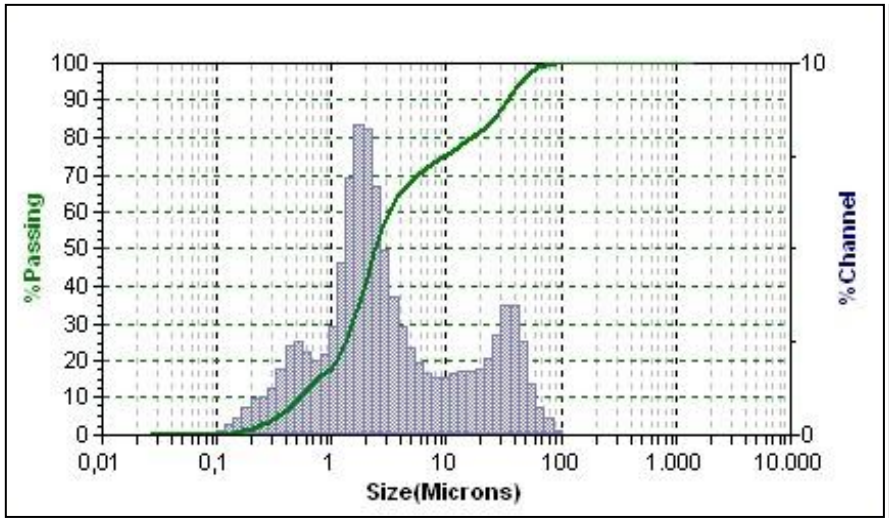

Figure 26: *DLS particle size distribution analysis for MoO_3 DP1.*

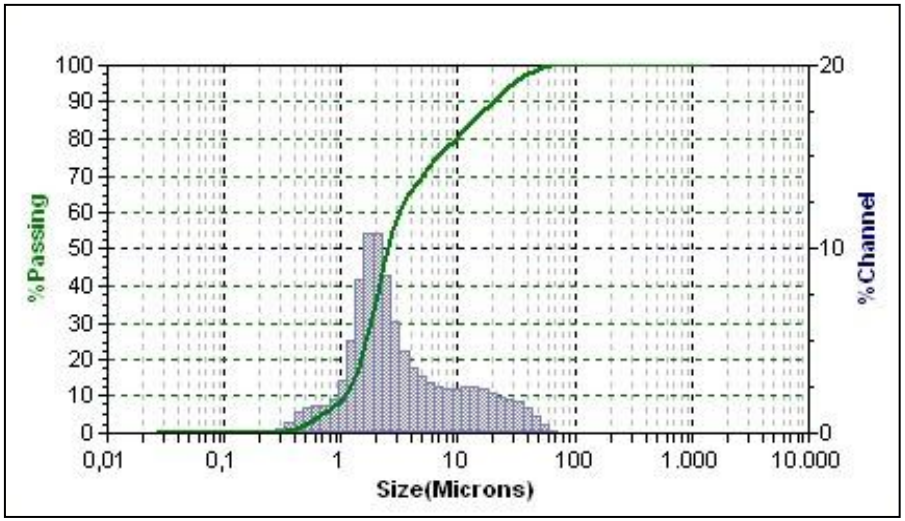

Figure 27: *DLS particle size distribution analysis for MoO_3 DP2.*

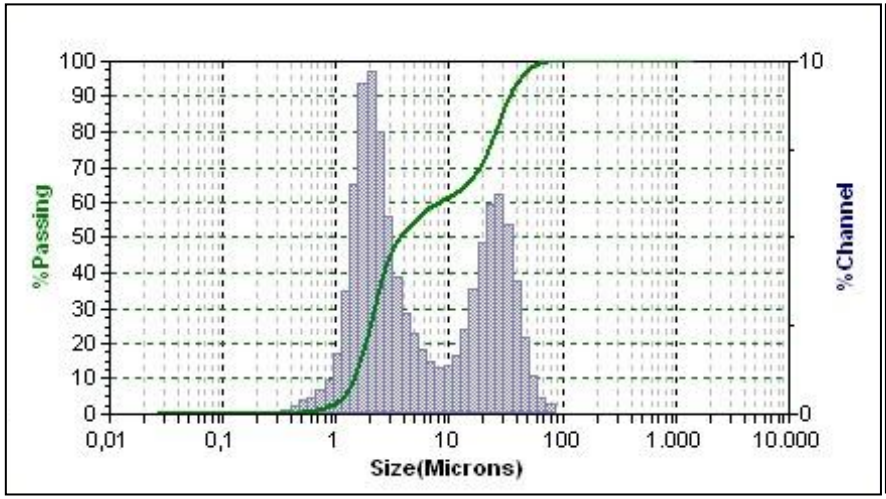

Figure 28: *DLS particle size distribution analysis for MoO_3 DP3.*

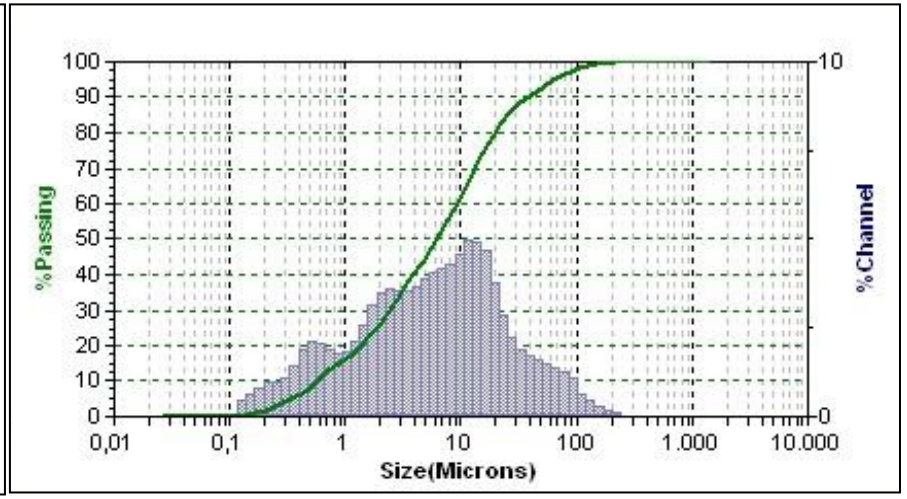

Figure 29: *DLS particle size distribution analysis for MoS_2 DP1.*

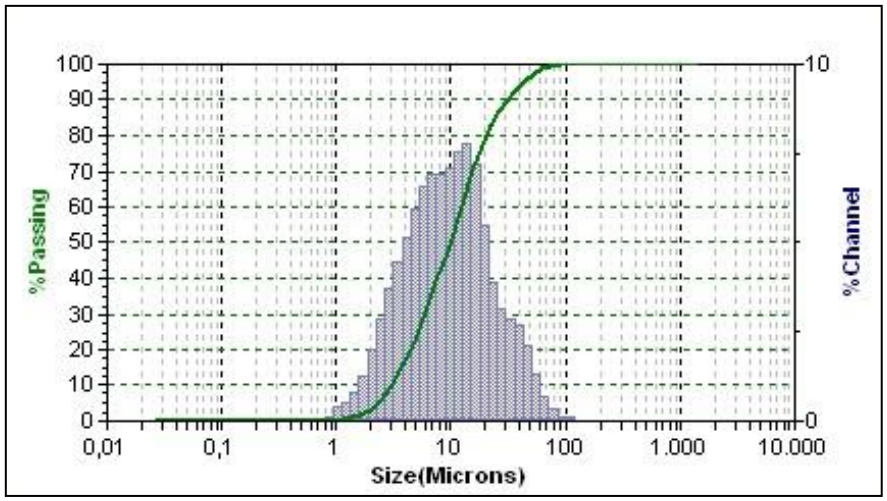

Figure 30: *DLS particle size distribution analysis for MoS_2 DP2.*

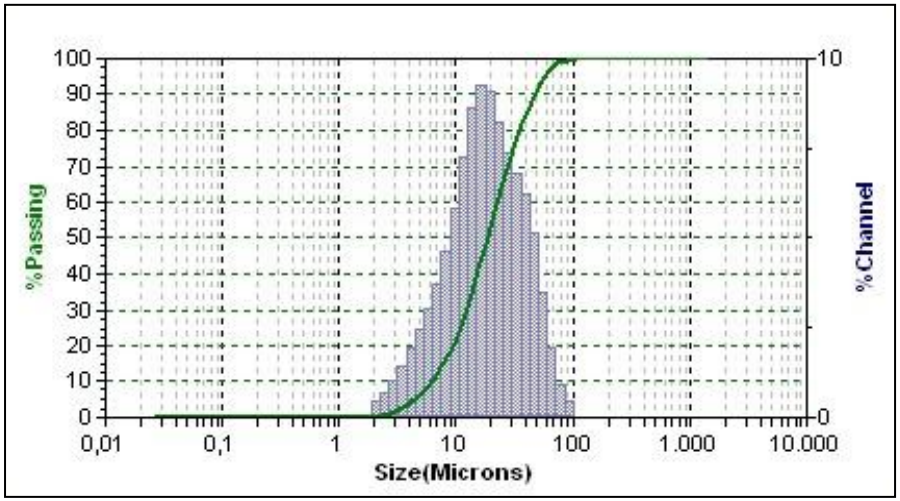

Figure 31: *DLS particle size distribution analysis for MoS_2 DP3.*

ANNEX B

Representative microphotographs, of cells exposed to different NP and stained with antibodies against the DNA repair marker phospho-H2AX. Each cell sample has been scanned in 9 ROIs (each ROI is 2x1 mm dimension) and was analyzed in duplicate (n=2).

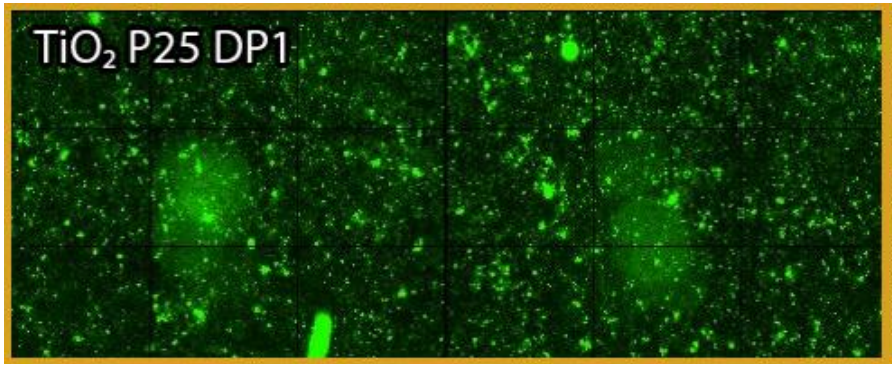

Figure 32: *Cells exposed to TiO_2 P25 NPs prepared by DP1.*

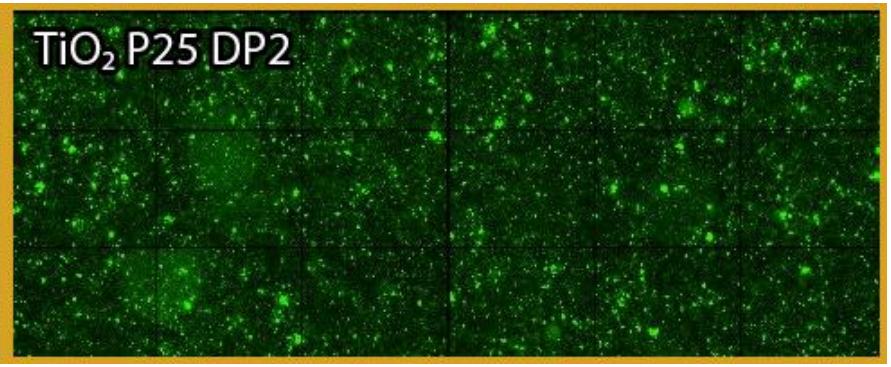

Figure 33: *Cells exposed to TiO_2 P25 NPs prepared by DP2.*

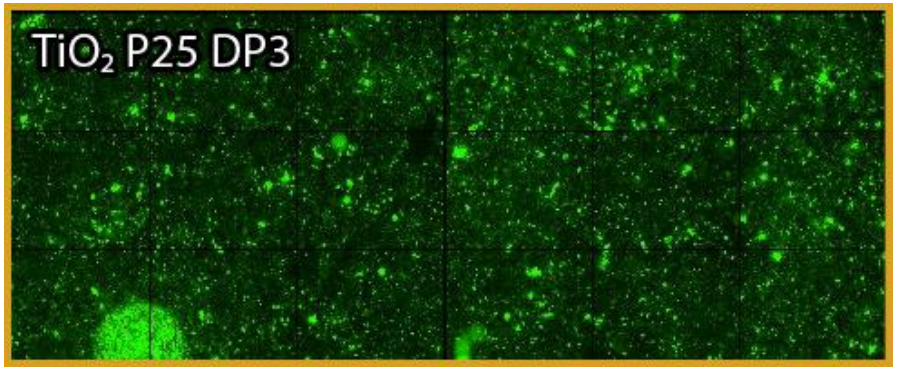

Figure 34: *Cells exposed to TiO_2 P25 NPs prepared by DP3.*

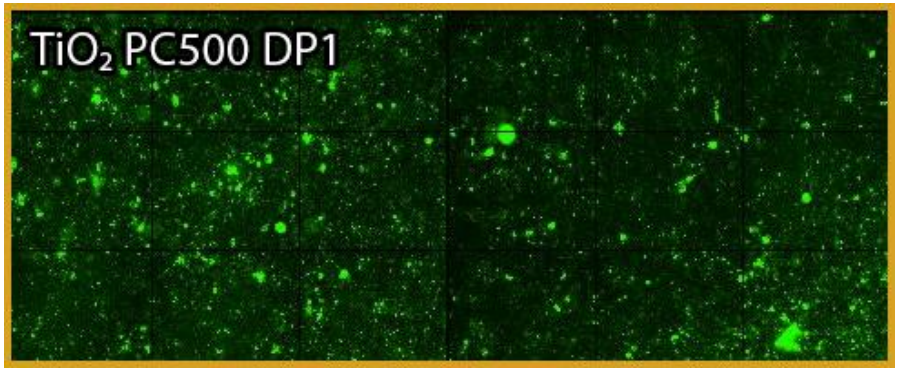

Figure 35: *Cells exposed to TiO_2 PC500 NPs prepared by DP1.*

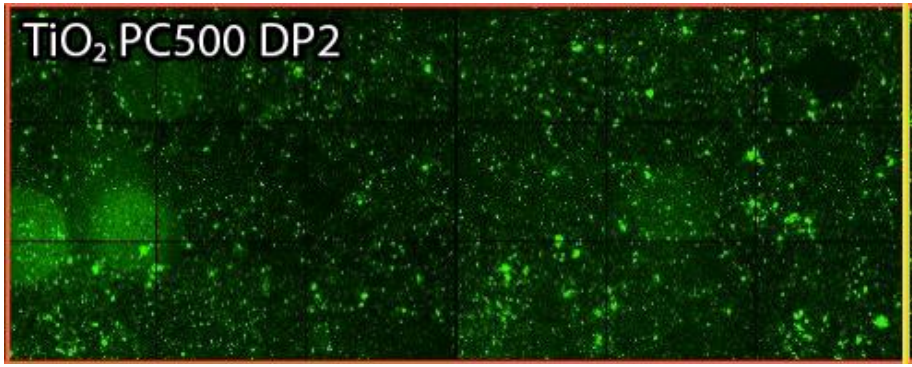

Figure 36: *Cells exposed to TiO_2 PC500 NPs prepared by DP2.*

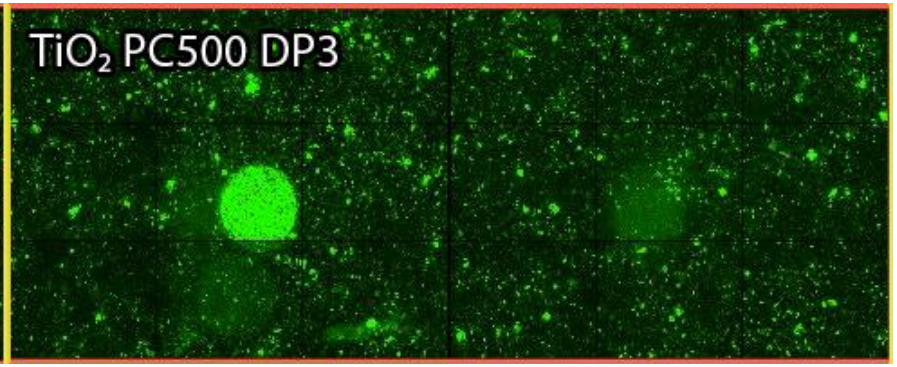

Figure 37: *Cells exposed to TiO_2 PC500 NPs prepared by DP3.*

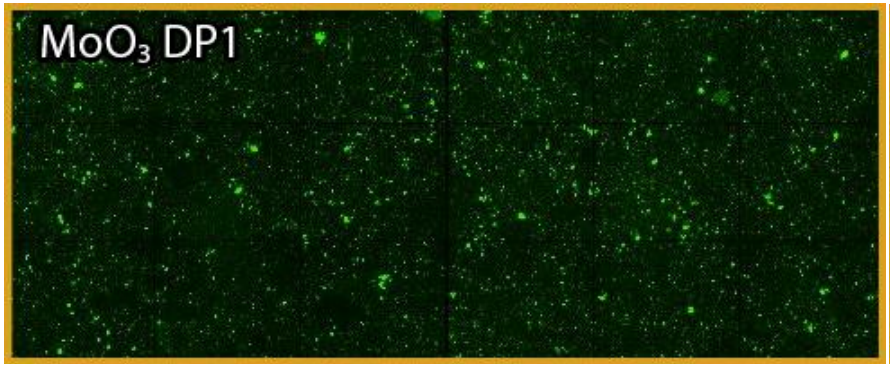

Figure 38: *Cells exposed to MoO_3 NMs prepared by DP1.*

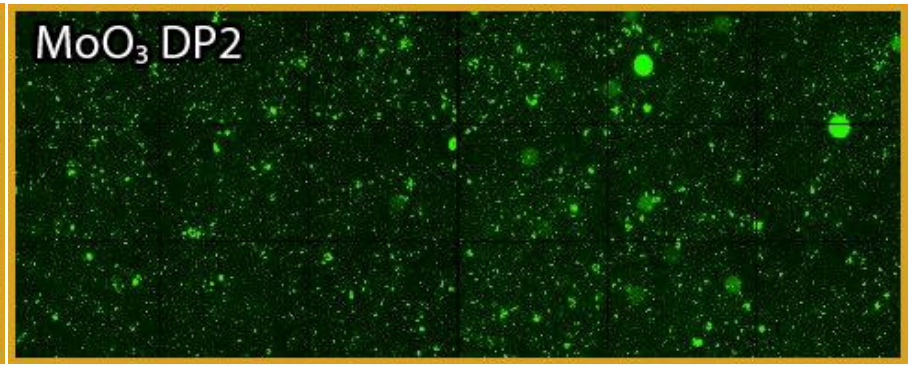

Figure 39: *Cells exposed to MoO_3 NMs prepared by DP2.*

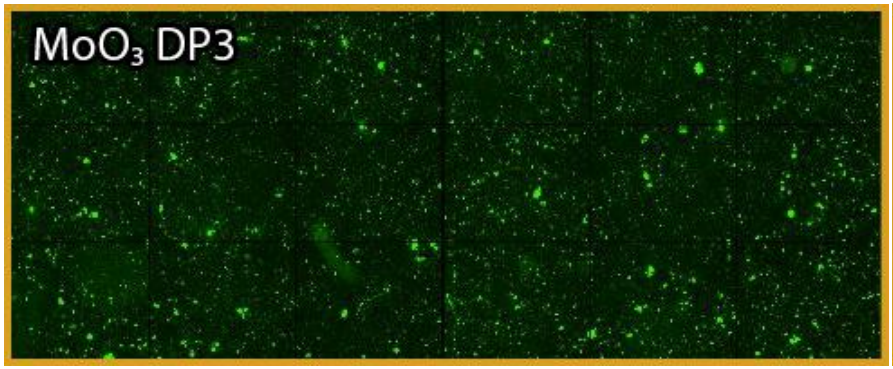

Figure 40: *Cells exposed to MoO_3 NMs prepared by DP3.*

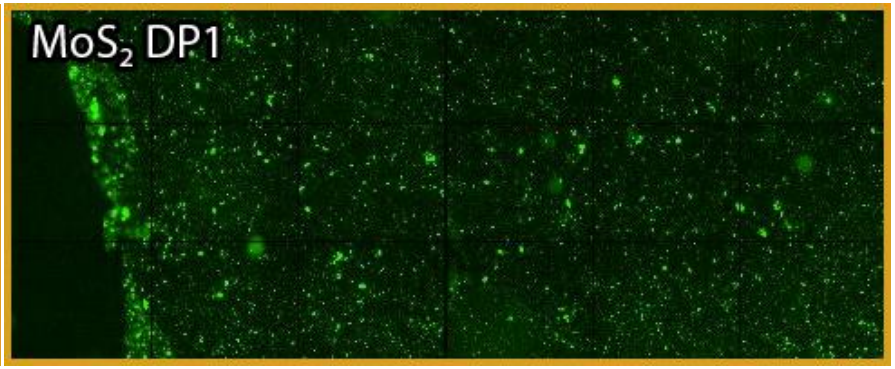

Figure 41: *Cells exposed to MoS_2 NMs prepared by DP1.*

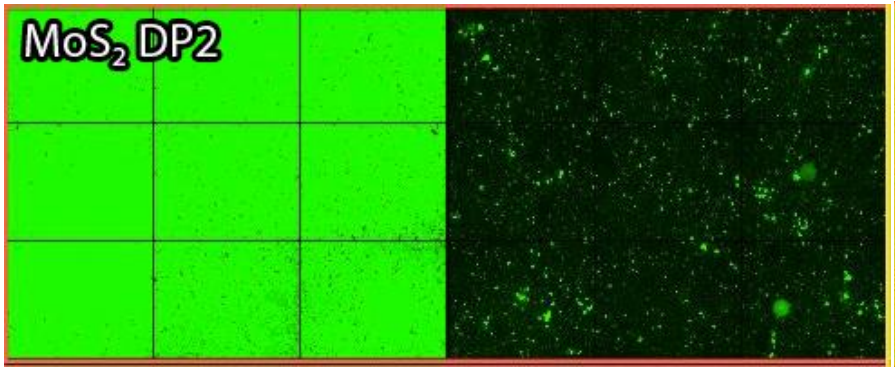

Figure 42: *Cells exposed to MoS_2 NMs prepared by DP2.*

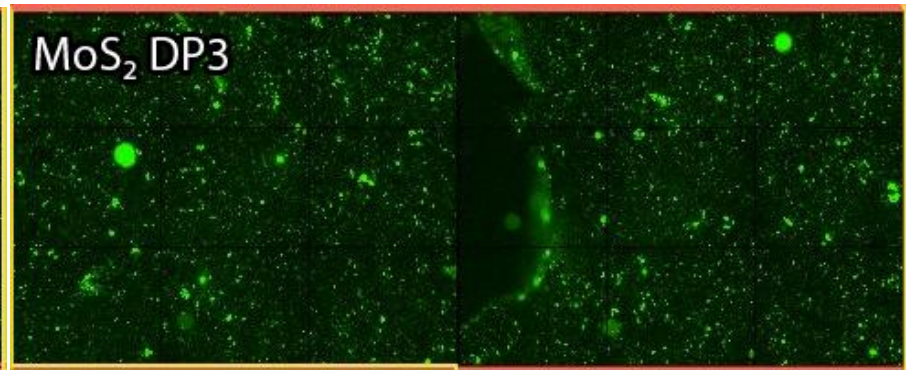

Figure 43: *Cells exposed to MoS_2 NMs prepared by DP3.*

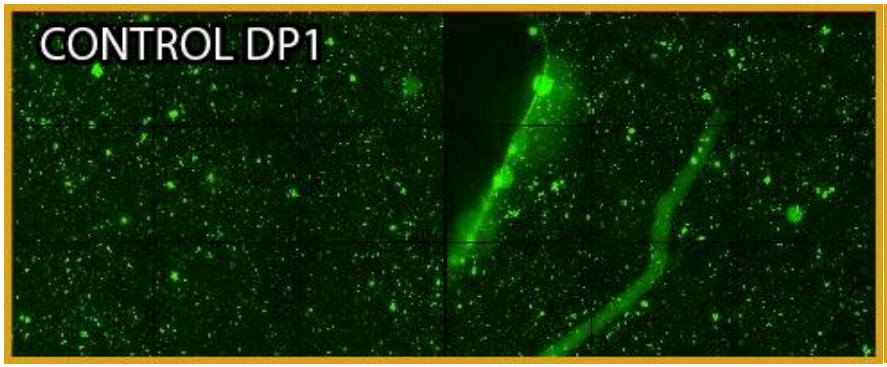

Figure 44: *Cells exposed to vehicle only (no NPs).*

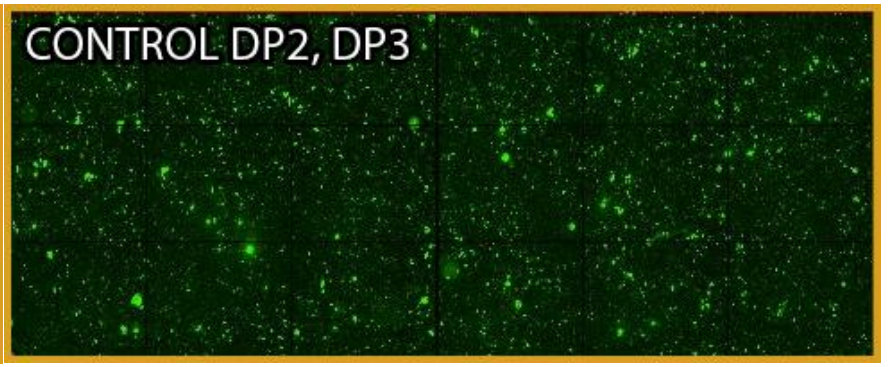

Figure 45: *Cells exposed to vehicle only (no NPs).*

ANNEX C

Representative example of High content analysis microphotographs, of the same cells as in B, exposed to different NPs. DNA was stained with Hoechst 33342, to visualize nuclei. Each cell sample has been scanned in 9 ROIs (each ROI is 2x1 mm dimension) and was analyzed in duplicate (n=2).

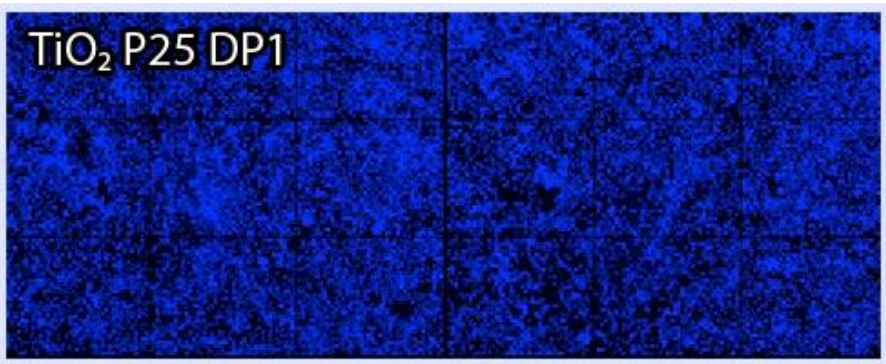

Figure 46: *Cells exposed to TiO_2 P25 NPs prepared by DP1.*

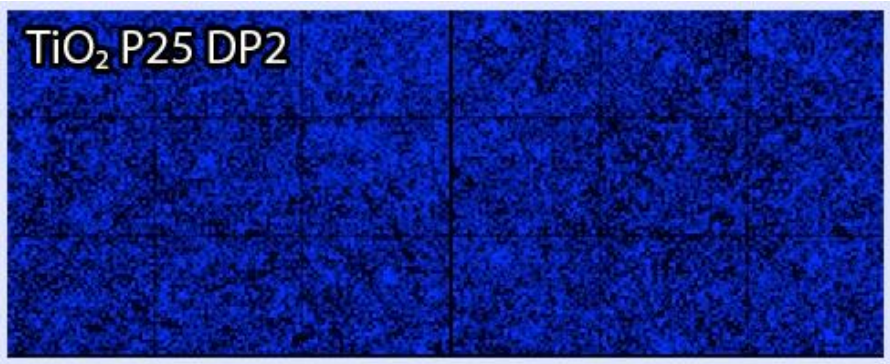

Figure 47: *Cells exposed to TiO_2 P25 NPs prepared by DP2.*

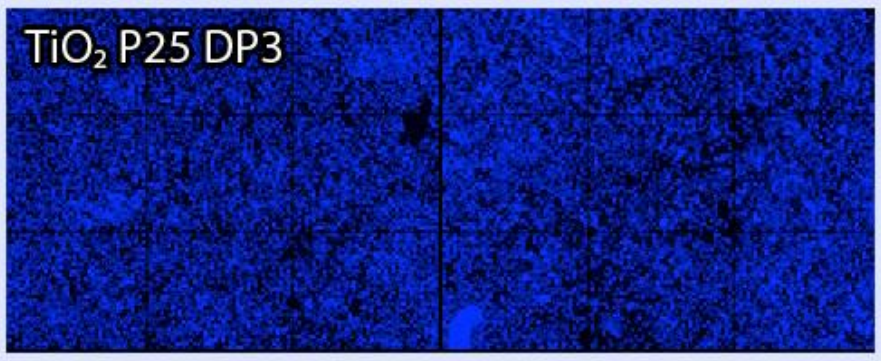

Figure 48: *Cells exposed to TiO_2 P25 NPs prepared by DP3.*

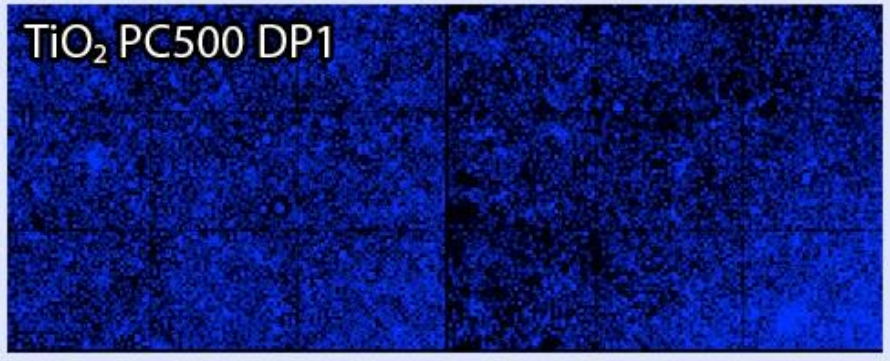

Figure 49: *Cells exposed to TiO_2 PC500 NPs prepared by DP1.*

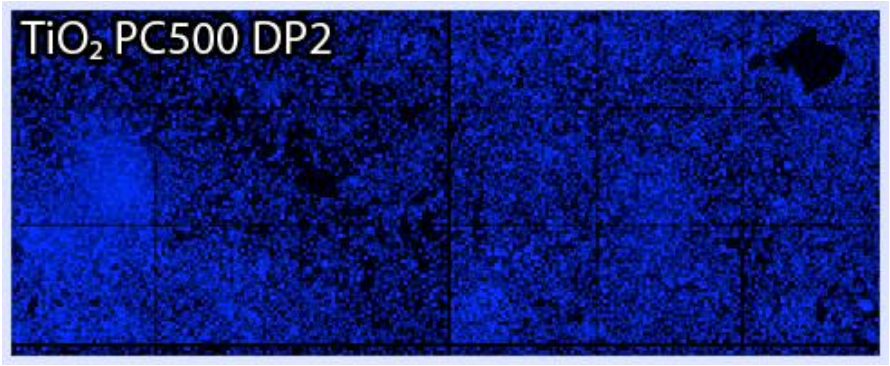

Figure 50: *Cells exposed to TiO_2 PC500 NPs prepared by DP2.*

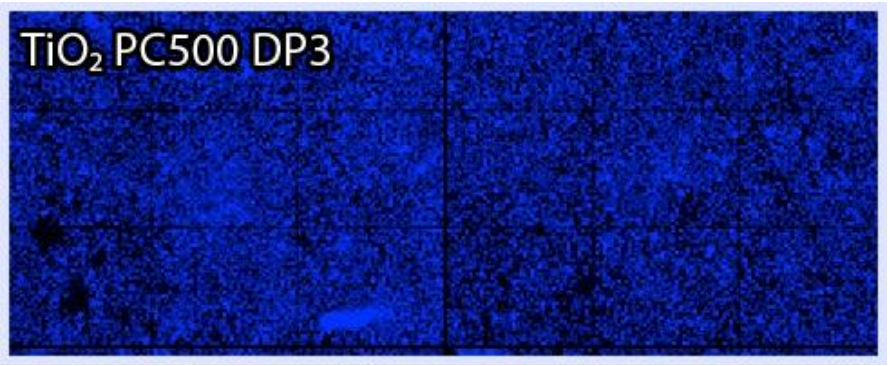

Figure 51: *Cells exposed to TiO_2 PC500 NPs prepared by DP3.*

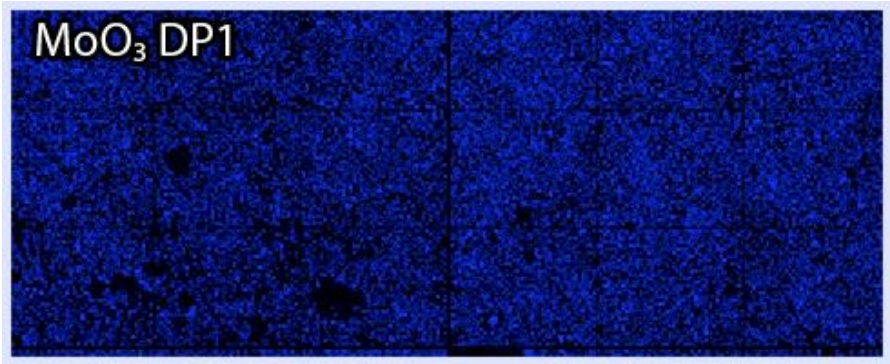

Figure 52: *Cells exposed to MoO_3 NMs prepared by DP1.*

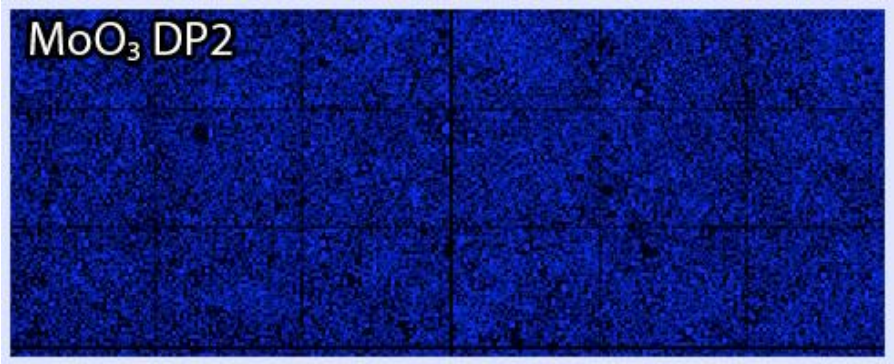

Figure 53: *Cells exposed to MoO_3 NMs prepared by DP2.*

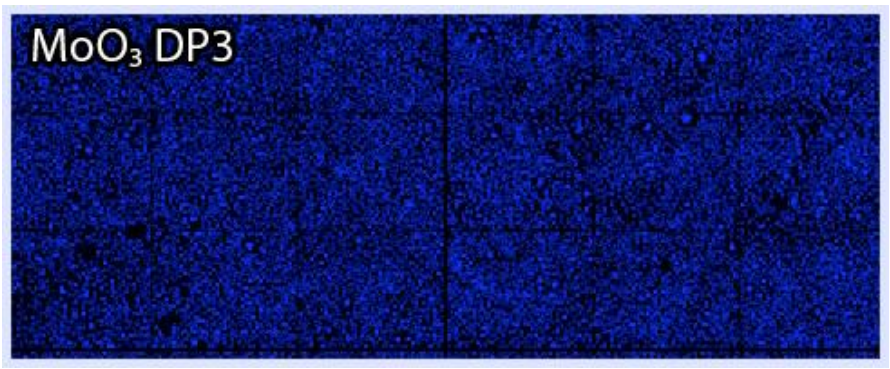

Figure 54: *Cells exposed to MoO_3 NMs prepared by DP3.*

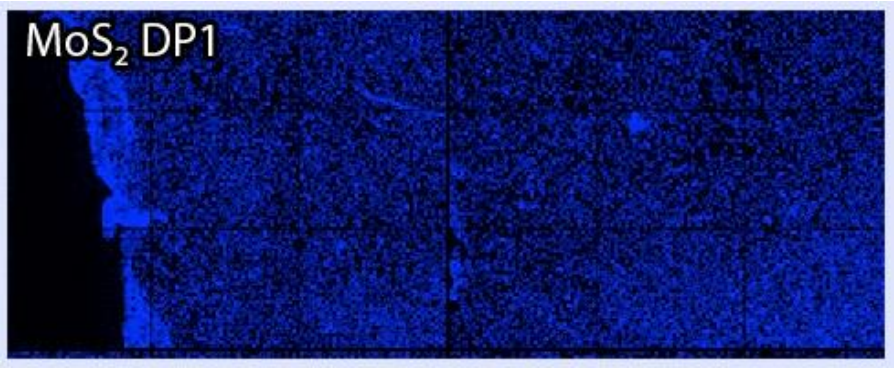

Figure 55: *Cells exposed to MoS_2 NMs prepared by DP1.*

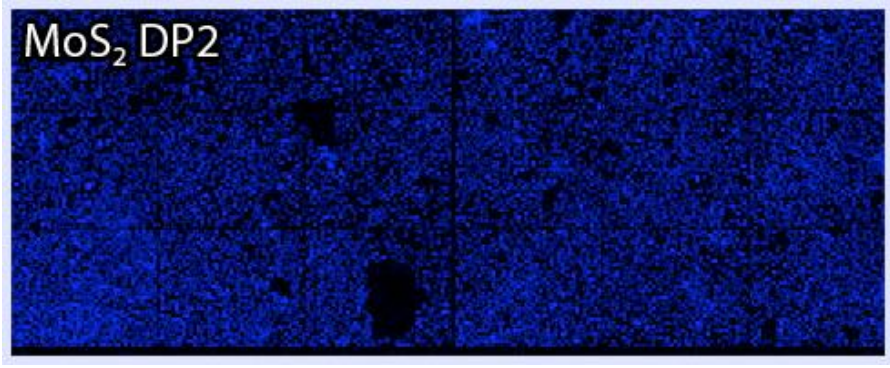

Figure 56: *Cells exposed to MoS_2 NMs prepared by DP2.*

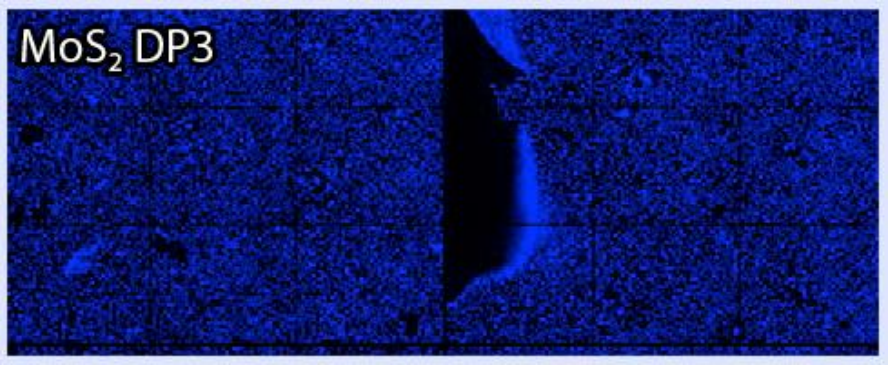

Figure 57: *Cells exposed to MoS_2 NMs prepared by DP3.*

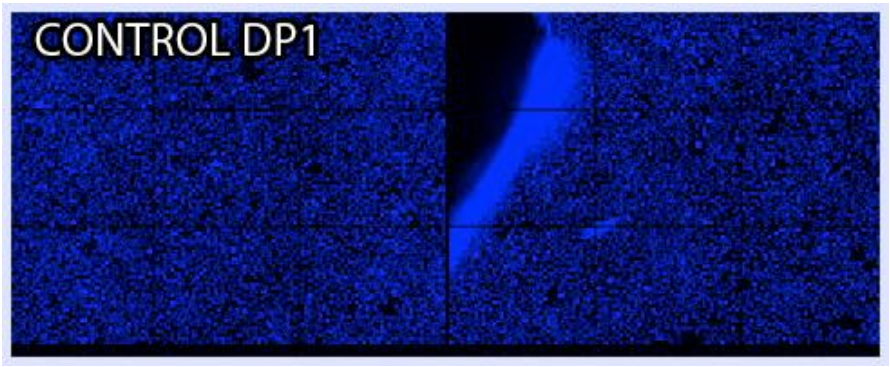

Figure 58: *Cells exposed to vehicle only (no NPs).*

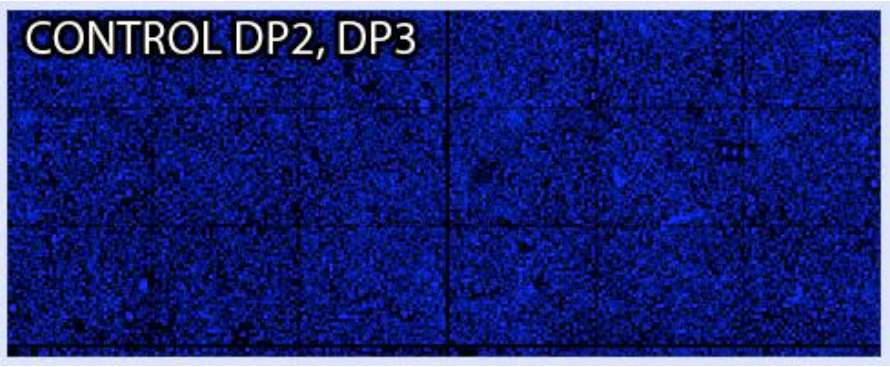

Figure 59: *Cells exposed to vehicle only (no NPs).*