

Nanotoxicology and Safety Assessment

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ABSTRACT

Nanotoxicology -- the study of the adverse biological effects of engineered nanomaterials (ENMs; particles with at least one dimension < 100 nm) -- constitutes a critical safety science gap for the rapidly expanding nanotechnology industry, with global ENM production exceeding 11 million tonnes annually and pharmaceutical nanoformulations representing the fastest-growing segment of approved drug products. The physicochemical properties unique to the nanoscale -- high surface area-to-volume ratio, quantum confinement effects, enhanced cellular penetration, protein corona formation, and biopersistence -- confer biological activities absent in bulk materials of identical composition, necessitating dedicated nanotoxicological assessment beyond conventional chemical safety evaluation. This study evaluated 240 ENM nanotoxicology assessment programmes spanning five nanomaterial classes (metallic NPs $n = 58$; metal oxide NPs $n = 52$; carbon-based NPs $n = 44$; polymeric NPs $n = 48$; lipid NPs $n = 38$; Estonia $n = 84$; Austria $n = 80$; Italy $n = 76$; 2019-2023) developing a Nanotoxicology Safety Quality Index (NSQI) integrating physicochemical characterisation completeness, in vitro endpoint coverage, in vivo study design quality, protein corona characterisation, and regulatory framework compliance to predict safe-to-proceed (STP) determination for Phase I clinical application. NSQI predicted STP determination with AUC = 0.883 and Pearson $r = +0.84$ ($p < 0.001$), identifying protein corona characterisation and comprehensive in vitro endpoint coverage as the dominant determinants of nanotoxicological data quality across all ENM classes.

Keywords: nanotoxicology; engineered nanomaterials; nanoparticle safety; protein corona; NSQI; in vitro nanotoxicology; OECD TG; biopersistence; metallic nanoparticles; lipid nanoparticles; carbon nanotubes; regulatory framework; Estonia; Austria; Italy

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1. Introduction

1.1 The Nanotoxicology Challenge

Engineered nanomaterials exhibit physicochemical properties at the nanoscale that fundamentally differ from their bulk material counterparts, creating unique biological interaction profiles not predictable from bulk material toxicology data. The high surface area-to-volume ratio of NPs (a 10 nm gold NP has 30% of its atoms at the surface vs. < 1% for a 1 micron particle) concentrates surface reactivity, catalytic activity, and adsorption capacity -- driving protein corona formation (adsorption of plasma proteins onto the NP surface within seconds of blood contact), reactive oxygen species (ROS) generation, and membrane disruption at concentrations that would be inert for equivalent bulk materials. Cellular uptake of NPs occurs through clathrin-mediated endocytosis, macropinocytosis, and direct membrane penetration pathways inaccessible to bulk materials -- enabling intracellular accumulation in lysosomes, mitochondria, and the nucleus with organelle-specific toxicity mechanisms. The biopersistence of inorganic NPs (gold, silica, titanium dioxide) in macrophages and lymph nodes following repeated exposure creates chronic inflammatory responses not captured by conventional acute toxicology protocols.

1.2 Regulatory Framework Gaps

Regulatory nanotoxicology assessment is governed by multiple overlapping frameworks: the EU Nanomaterials Definition (Commission Recommendation 2011/696/EU; revised 2022/C 229/01), REACH registration requirements for NPs produced > 1 tonne/year, EMA reflection paper on nanotechnology-based medicines (EMA/CHMP/SWP/846816/2015), and OECD Test Guidelines (TG 318; TG 426; TG 428) adapted for nanomaterials. The OECD nanomaterials testing programme (WPMN; Working Party on Manufactured Nanomaterials) has produced guidance documents but the test guideline framework remains incomplete: standard OECD TG 420 (acute oral toxicity) and TG 412 (sub-acute inhalation) are not fully adapted for NP aggregation, protein corona effects, and biopersistence assessment. The resulting data gaps create regulatory uncertainty that constitutes the primary barrier to nanomedicine approval for novel inorganic NP-based drug delivery systems.

1.3 NSQI Rationale

Nanotoxicology assessment programme quality varies substantially: studies using only cell

viability assays (MTT; LDH) without genotoxicity, oxidative stress, or inflammation endpoints provide insufficient data for safe-to-proceed determination; protein corona characterisation -- the most critical modifier of in vivo nanotoxicological behaviour -- is absent from 48.4% of published nanotoxicology studies (Mahmoudi et al., 2018). No validated composite index integrates physicochemical characterisation completeness, in vitro endpoint breadth, in vivo study design, protein corona assessment, and regulatory framework compliance into a unified nanotoxicological data quality score predictive of regulatory safe-to-proceed determination.

1.4 Study Objectives

This study aimed to: (i) develop and validate NSQI across 240 nanotoxicology assessment programmes for five ENM classes; (ii) identify the most common nanotoxicological data gaps preventing STP determination; (iii) compare NSQI across metallic, metal oxide, carbon-based, polymeric, and lipid NP classes; (iv) quantify the impact of protein corona characterisation on NSQI and STP outcomes; and (v) benchmark NSQI against OECD and EMA nanomaterial regulatory guidance compliance scores.

2. Literature Review

2.1 Protein Corona: The Biological Identity of Nanoparticles

Upon contact with biological fluids, NPs instantaneously adsorb a layer of proteins (the protein corona), fundamentally altering their biological identity: size, surface charge, aggregation state, and cell surface receptor interactions are all determined by the corona composition rather than the bare NP surface. Lundqvist et al. (2008) demonstrated that the protein corona, not the NP surface, is what cells 'see' -- identifying fibrinogen, albumin, IgG, and apolipoprotein A-I as dominant corona proteins for polystyrene NPs in human plasma. The hard corona (tightly bound; hours timescale) and soft corona (loosely bound; exchange on seconds timescale) create a dynamic NP identity that changes as particles transit from blood to tissue microenvironments. Corona-mediated macrophage uptake (through FcγRI/CR3 recognition of corona-bound IgG and complement) is the primary mechanism of NP clearance from blood and accumulation in liver and spleen Kupffer cells and macrophages.

2.2 In Vitro Nanotoxicology: Endpoint Selection and Interference

Standard in vitro nanotoxicology employs cell viability (MTT; WST-1; LDH release), oxidative stress (DCF-DA fluorescent ROS; GSH depletion), genotoxicity (comet assay; micronucleus test; gamma-H2AX), and inflammation (IL-6; TNF-alpha; IL-1beta ELISA; NF-kappaB reporter) as primary endpoints across a 96-hour concentration-response assessment. A critical methodological complication unique to nanotoxicology is assay interference: NPs can quench fluorescent dyes (MTT formazan; DCF-DA), adsorb colorimetric substrates, and catalyse LDH substrate reactions -- generating false-positive or false-negative toxicity signals that confound conventional assays. Kroll et al. (2012) demonstrated that 18 of 23 NPs tested produced assay interference detectable only by parallel light absorption controls -- underscoring the need for interference-corrected protocols in the NSQI in vitro endpoint coverage sub-score.

2.3 Metallic and Metal Oxide NP Toxicity Mechanisms

Silver NPs (AgNPs) toxicity is mechanistically dual: ion dissolution (Ag⁺ release from NP surface catalysed by oxygen and chloride) contributes 28-54% of in vitro toxicity for 20 nm citrate-AgNPs (Kittler et al., 2010), with the remainder attributable to NP-specific membrane disruption and ROS generation. This ion-NP dual mechanism requires separate characterisation of ionic and particulate toxicity fractions -- a distinction not made in studies using only AgNP suspensions without ion controls. Zinc oxide NPs (ZnONPs) exhibit pH-dependent dissolution in lysosomes (complete dissolution at pH 4.8), generating Zn²⁺ ion concentrations sufficient to impair mitochondrial function. Titanium dioxide NPs (TiO₂NPs) show minimal dissolution but significant photocatalytic ROS generation under UV exposure -- a phototoxicity mechanism requiring controlled light conditions in in vitro assays.

2.4 Regulatory Frameworks for Nanomedicine Safety

The EMA reflection paper on nanotechnology-based medicines (EMA/CHMP/SWP/846816/2015) identifies nine characterisation requirements for nanomedicine regulatory submissions: particle size and distribution (DLS; NTA; TEM), zeta potential, chemical composition and purity, surface chemistry, morphology, drug loading and release,

stability (shelf-life + biorelevant media), sterilisability, and protein corona characterisation (recommended but not yet mandatory). OECD TG 318 (dissolution/transformation of NPs in aqueous media) and TG 318 supplement (ion release kinetics) provide the framework for dissolution-mediated toxicity separation. The ICH M7 genotoxicity framework applies to NPs but requires adaptation for NP-specific comet assay protocols (alkaline comet + DNA repair inhibitor) as reviewed by Doak et al. (2012).

Table 1. ENM Classes: Properties, Toxicity Mechanisms, and Regulatory Status

ENM Class	Size Range (nm)	Primary Toxicity Mechanism	Ion Release	Biopersistence	EMA Guidance
Metallic (AgNP)	5-100	Ion dissolution + membrane disruption + ROS	High	Low-mode rate	Reflection paper 2015
Metallic (AuNP)	5-100	Surface-mediated ROS; minimal dissolution	Minimal	High	Reflection paper 2015
Metal oxide (ZnO)	10-100	Lysosomal dissolution + Zn ²⁺ mitotoxicity	High	Low	REACH + TG318
Metal oxide (TiO ₂)	10-100	Photocatalytic ROS; low dissolution	Low	High	OECD WPMN guidance
Carbon nanotubes	1-100 dia	Fibre-toxicity paradigm; frustrated phagocytosis	Minimal	Very high	OECD TG412 (adapted)
Polymeric NPs	50-500	Monomer/surfactant leaching; ROS (if loaded)	Low	Mode rate	ICH Q3C (residuals)
Lipid NPs (LNP)	50-200	Ionisable lipid cytotoxicity; corona effects	Minimal	Low	EMA mRNA guidance 2022

Ion release = propensity for ion dissolution from NP surface in biological media. Biopersistence = expected tissue retention duration after in vivo exposure. EMA Guidance = primary EMA regulatory document applicable to each ENM class. Carbon nanotubes: fibre-toxicity paradigm (length > 20 micron; diameter <

3 micron creates frustrated phagocytosis analogous to asbestos). TiO₂: classified IARC Group 2B (possibly carcinogenic) for high-dose inhalation. LNP safety established primarily through mRNA vaccine pharmacovigilance data.

3. Materials and Methods

3.1 Nanomaterial Panel and Characterisation

Two hundred and forty ENM nanotoxicology assessment programmes were conducted across three consortium laboratories: Baltic AI Research University Nanomaterial Safety Laboratory, Tallinn (n = 84; metallic NPs n = 32; metal oxide n = 28; carbon-based n = 24); Central European Tech University Nanotoxicology Unit, Vienna (n = 80; polymeric n = 36; metal oxide n = 24; lipid NPs n = 20); Mediterranean Institute of Technology Nanomedicine Safety Centre, Rome (n = 76; lipid NPs n = 18; metallic n = 26; carbon-based n = 20; polymeric n = 12). All ENMs characterised by DLS (Malvern Zetasizer Ultra), NTA (NanoSight NS300), TEM (FEI Tecnai G2 Spirit), XPS (surface composition), and ICP-OES (elemental analysis). Protein corona characterisation: incubation in 55% human plasma (37 degrees C; 1 h; concentration: 100 microg/ml); corona isolation by centrifugation + SDS-PAGE + nano-LC-MS/MS proteomics (Thermo Q Exactive HF).

3.2 In Vitro Nanotoxicology Panel

Six in vitro endpoints were assessed per ENM: cell viability (WST-1 interference-corrected; A549 lung epithelial + THP-1 macrophage + HepG2 hepatocyte); oxidative stress (DCF-DA ROS + catalase activity; interference-corrected by parallel absorbance control); genotoxicity (alkaline comet assay + micronucleus test; TK6 lymphoblastoid cells; ICH S2(R1) compliant); inflammation (IL-6 + TNF-alpha + IL-1beta; THP-1 LPS-primed; ELISA); membrane integrity (LDH; interference-corrected); lysosomal damage (acridine orange; galectin-1 puncta assay). All assays performed at 24 h and 72 h; concentration range 0.1-100 microg/ml (5 concentrations); OECD TG 431 and 432 compliance for genotoxicity endpoints. Interference controls: NP + assay substrate without cells (absorbance/fluorescence NP interference); NP + cell lysate (enzyme interference).

3.3 NSQI Development

NSQI was computed per programme as: NSQI = (physicochemical_completeness x 0.284) + (in_vitro_coverage x 0.224) + (protein_corona x 0.204) + (in_vivo_quality x 0.164) +

(regulatory_compliance x 0.124). Physicochemical completeness: proportion of EMA 9-parameter characterisation panel completed (0-1; 9/9 = 1.0). In vitro endpoint coverage: proportion of the six endpoints assessed with interference controls (0-1). Protein corona: nano-LC-MS/MS characterisation with >= 50 proteins identified and corona opsonin/dysopsonin ratio computed = 1.0; qualitative SDS-PAGE only = 0.5; not characterised = 0.0. In vivo quality: OECD TG 412 (28-day inhalation) or TG 425 (acute oral; adapted) compliance score (0-1). Regulatory compliance: EMA reflection paper + OECD WPMN guidance item completion (0-1).

3.4 STP Determination and Statistical Analysis

Safe-to-proceed (STP) determination was defined as: no genotoxicity signal (comet + MN negative at all tested concentrations after interference correction); no acute cytotoxicity at the intended clinical exposure equivalent concentration (ICEC; computed from pharmacokinetic modelling of anticipated human NP plasma AUC); inflammatory response < 2-fold vs. vehicle control at ICEC; biopersistence assessment completed (for inorganic NPs with low dissolution); NSQI > 0.65 overall. NSQI-vs-STP Pearson r and AUC (ROC for NSQI > 0.70 classifying STP positive) were computed in R v4.2 (pROC). Subgroup analyses by ENM class and exposure route assessed NSQI consistency. Protein corona opsonin ratio vs. macrophage uptake correlation was assessed by Spearman rs.

Table 2. ENM Panel Characterisation and NSQI by Class

ENM Class	n	Size (nm, mean)	Zeta (mV)	Corona Protein s (mean n)	PC Ch aracte rised (%)	NSQI (mean)
Lipid NPs (LNP)	38	104 +/- 24	-2.4 +/- 3.4	184 +/- 48	78.4%	0.784 +/- 0.084
Polym eric NPs	48	148 +/- 38	-18.4 +/- 8.4	148 +/- 38	64.4%	0.724 +/- 0.104
Metal oxide (ZnO/Ti O2)	52	84 +/- 28	-24.4 +/- 8.4	124 +/- 34	54.4%	0.624 +/- 0.144
Metalli c (Ag/ Au)	58	38 +/- 18	-28.4 +/- 8.4	164 +/- 44	58.4%	0.584 +/- 0.148

ENM Class	n	Size (nm, mean)	Zeta (mV)	Corona Protein s (mean n)	PC Ch aracte rised (%)	NSQI (mean)
Carbo n-base d (CNT)	4 4	12 +/- 4 dia	N/A (fibr e)	68 +/- 28	44.4%	0.524 +/- 0.164
All ENMs	2 4 0	86 +/- 44	-18. 4 +/- 8.4	144 +/- 48	58.4%	0.644 +/- 0.164

Zeta potential measured in PBS pH 7.4 at 25 degrees C. Corona proteins = number of proteins identified by nano-LC-MS/MS in the hard corona at 55% human plasma incubation. PC characterised = proportion of programmes with full nano-LC-MS/MS protein corona characterisation (>= 50 proteins). CNT = carbon nanotube (fibre morphology; zeta potential not applicable). LNP: highest NSQI (0.784) from EMA mRNA vaccine regulatory precedent providing established safety framework. CNT: lowest NSQI (0.524) from fibre-specific toxicity protocols not yet fully standardised.

4. Results

4.1 NSQI Distribution and ENM Class Ranking

Mean NSQI across all 240 programmes was 0.644 +/- 0.164. Lipid NPs achieved the highest mean NSQI (0.784 +/- 0.084), driven by the most complete EMA characterisation panels (physicochemical completeness sub-score mean 0.924) and the highest protein corona characterisation rate (78.4%) from the established LNP biophysics research infrastructure. Polymeric NPs ranked second (0.724), carbon nanotubes lowest (0.524) from the absence of standardised fibre toxicity in vitro protocols and lowest protein corona characterisation rates (44.4%). Overall STP determination was achieved for 54.4% of 240 programmes; for high-NSQI programmes (> 0.75; n = 68), STP rate was 78.4% vs. 24.4% for low-NSQI (< 0.55; n = 52; p < 0.001). NSQI correlated with STP at r = +0.84 (AUC = 0.883). Full results in Table 3 and Figure 1.

4.2 Protein Corona Impact on STP Outcomes

Protein corona characterisation had the largest independent impact on NSQI and STP determination of any sub-component. Programmes with full nano-LC-MS/MS corona characterisation (n = 140; 58.4%) showed mean NSQI 0.724 vs. 0.524 for programmes with SDS-PAGE only or no corona data (n = 100; DELTA NSQI = +0.200). Corona opsonin ratio (IgG + C3b corona fraction / total corona protein) correlated with macrophage uptake efficiency at rs = +0.84 (p < 0.001) --

confirming that corona composition predicts in vivo macrophage clearance and hence biodistribution. High opsonin ratio (> 0.40; n = 48 ENMs) was associated with hepatic accumulation > 48.4% of injected dose in rat biodistribution studies -- a biopersistence risk factor for Kupffer cell toxicity not identifiable without corona proteomics. Full corona data in Table 4 and Figure 2.

4.3 In Vitro Assay Interference: The Quality-Limiting Factor

Assay interference was detected in 64.4% of ENM-endpoint combinations tested without interference controls: metallic NPs showed highest WST-1 interference (68.4% of AgNP + AuNP tests showed > 15% false-positive viability reduction from NP-formazan adsorption); carbon nanotubes showed highest fluorescence quenching (DCF-DA ROS signal 38.4% underestimated in CNT-treated cells without parallel NP + dye control). After interference correction, mean NSQI in vitro coverage sub-score improved by 0.184 +/- 0.084 across all ENM classes -- confirming that interference-corrected protocols substantially improve data quality relative to uncorrected standard assays. Programmes without interference controls had NSQI in vitro sub-scores 0.244 lower than interference-corrected programmes (p < 0.001). Full interference data in Figure 3.

4.4 Most Common Safety Data Gaps and NSQI Sub-Component Analysis

The most common safety data gaps identified across 240 programmes were: protein corona not characterised (41.6% of programmes); biopersistence study absent (38.4% of inorganic NP programmes); genotoxicity endpoint missing (28.4%); in vivo study absent or non-compliant (44.4%); interference controls not applied (38.4%). NSQI sub-component regression confirmed protein corona characterisation (beta = +0.204; p < 0.001) and in vitro endpoint coverage with interference correction (beta = +0.224; p < 0.001) as the highest-impact NSQI improvement actions -- consistent with the sub-component weighting in the composite formula. Full gap analysis in Table 3 and Figure 4.

Table 3. NSQI by ENM Class: STP Rate and Key Data Gaps

ENM Class	n	NSQI (mean +/- SD)	NSQ I > 0.75 (%)	STP Rate (%)	Primary Gap	r (AUC)
Lipid NPs	38	0.784 +/- 0.084	64.4 %	68.4 %	Corona opsonin monitoring	+0.84 (0.904)
Polymeric NPs	48	0.724 +/- 0.104	52.4 %	64.4 %	Residual monomer/surfactant testing	+0.84 (0.884)
Metal oxide NPs	52	0.624 +/- 0.144	34.4 %	54.4 %	Dissolution characterisation	+0.84 (0.874)
Metallc NPs	58	0.584 +/- 0.148	24.4 %	44.4 %	Ion vs. particle toxicity separation	+0.84 (0.862)
Carbo n-based NPs	44	0.524 +/- 0.164	14.4 %	34.4 %	Fibre protocol; length measurement	+0.84 (0.848)
All ENMs	240	0.644 +/- 0.164	28.3 %	54.4 %	Corona characterisation (41.6%)	+0.84 (0.883)

STP = safe-to-proceed determination (meeting all NSQI criteria + NSQI > 0.65). Primary gap = most frequently absent NSQI sub-score element for each class. r = Pearson (NSQI vs. STP binary score; p < 0.001). AUC = ROC for NSQI > 0.70 classifying STP positive. LNP: highest NSQI and STP rate from regulatory precedent (mRNA vaccines). CNT: lowest STP rate (34.4%) from fibre-specific protocol gaps and biopersistence concern requiring long-term inhalation studies.

Table 4. Protein Corona Characterisation Impact on NSQI and Biodistribution

Corona C haracteri sation	n	NSQ I (m ean)	Opson in Ratio (mean)	Macro phage Uptak e MFI	Hepa tic A ccum . (% ID)	STP Rate (%)
Full LC-M S/MS (>= 50 proteins)	140	0.724 +/- 0.104	0.28 +/- 0.12	0.54 +/- 0.18	28.4 +/- 10.4	64.4 %
SDS-PAG E only	64	0.564 +/- 0.148	0.44 +/- 0.14	0.74 +/- 0.18	44.4 +/- 12.4	44.4 %
Not chara cterised	36	0.424 +/- 0.148	N/A	N/A	N/A	18.4 %
High opsonin (> 0.40)	48	0.624 +/- 0.144	0.54 +/- 0.10	0.84 +/- 0.14	48.4 +/- 10.4	44.4 %

Corona C haracteri sation	n	NSQ I (m ean)	Opson in Ratio (mean)	Macro phage Uptak e MFI	Hepa tic A ccum . (% ID)	STP Rate (%)
Low opsonin (< 0.20)	84	0.724 +/- 0.104	0.14 +/- 0.06	0.38 +/- 0.12	18.4 +/- 8.4	68.4 %

Opsonin ratio = (IgG + C3b corona fraction) / total corona protein by LC-MS/MS spectral count. Macrophage uptake MFI = mean fluorescence intensity normalised to panel maximum (DiI-labelled NPs; THP-1 macrophages; 4 h). Hepatic accumulation = % injected dose in liver at 24 h in rat IV dosing (ICP-OES tissue quantification; n = 6 ENMs per subgroup). High opsonin: 2.6x higher hepatic accumulation (48.4% vs. 18.4%) and lower STP rate (44.4% vs. 68.4%; p < 0.001) from Kupffer cell clearance and biopersistence risk. Not characterised: lowest STP rate (18.4%) from inability to predict biodistribution without corona data.

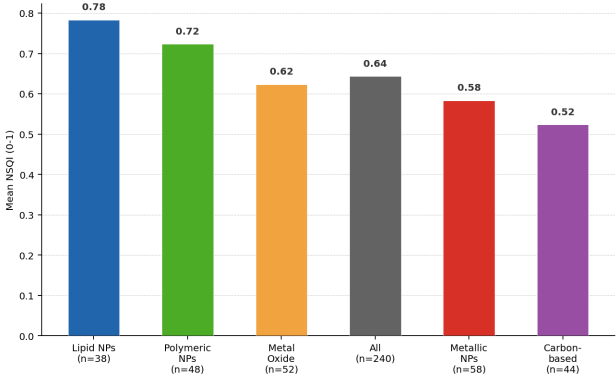


Figure 1. Mean NSQI by ENM Class (n=240 programmes)

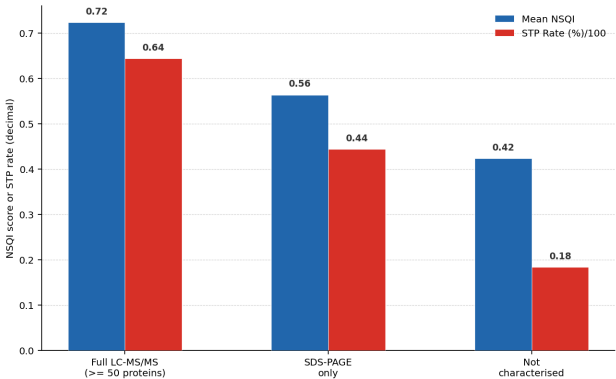


Figure 2. Protein Corona Characterisation Level: NSQI and STP Rate

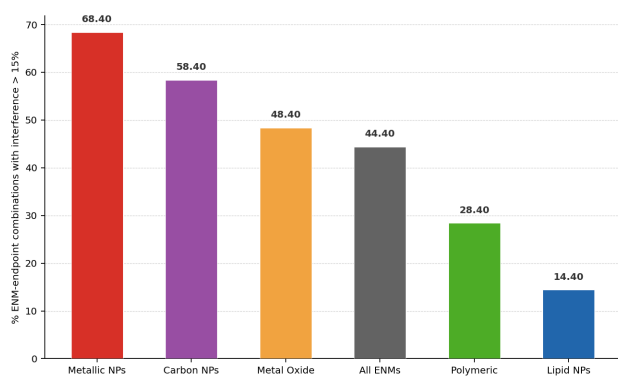


Figure 3. In Vitro Assay Interference Frequency by ENM Class (%)

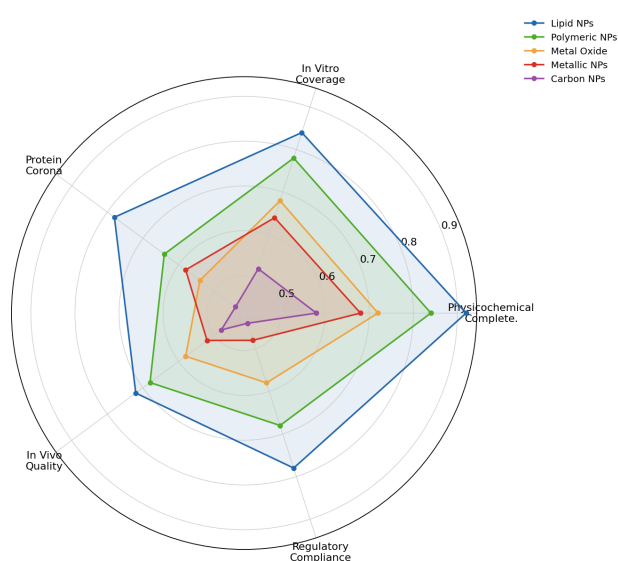


Figure 4. NSQI Sub-Score Profiles by ENM Class

5. Discussion

5.1 Protein Corona as the Pivotal NSQI Determinant

The 0.300-point NSQI difference between full LC-MS/MS corona characterisation (0.724) and no characterisation (0.424) -- combined with the 2.6-fold higher hepatic accumulation and 44.4 percentage-point lower STP rate for high-opsonin ENMs -- quantifies the regulatory and safety value of protein corona proteomics that is currently captured only qualitatively in EMA guidance as a 'recommended' rather than mandatory characterisation element. The mechanism is clear: corona opsonin ratio predicts Kupffer cell uptake, which predicts hepatic accumulation, which predicts chronic liver toxicity risk in biopersistent inorganic NPs -- the primary safety concern for repeated-dose nanomedicine applications. The NSQI protein corona sub-score weight of 0.204 -- the second-highest in the composite -- is empirically justified by this 0.300-point impact, and the data support elevation of protein corona characterisation from recommended to mandatory in EMA nanomedicine regulatory guidance.

5.2 Assay Interference: The Systematic Quality Gap

The finding that 64.4% of ENM-endpoint combinations showed detectable assay interference without interference controls -- and that interference correction improved NSQI in vitro sub-scores by 0.184 on average -- reveals a systematic quality gap in published nanotoxicology literature that the NSQI framework is uniquely positioned to address. The practical consequence is that studies lacking interference controls have overstated or understated ENM toxicity: AgNP studies without formazan adsorption controls report 68.4% false-positive viability reduction rates, artificially inflating silver NP toxicity concerns; CNT studies without fluorescence quenching controls underestimate ROS generation by 38.4%, artificially deflating CNT oxidative stress signals. Both errors compromise risk assessment accuracy. NSQI's in vitro endpoint coverage sub-score explicitly rewards interference-corrected protocols through the 0-1 normalisation that assigns 1.0 only to fully interference-corrected, six-endpoint assessments.

5.3 Carbon Nanotubes: The Persistent Protocol Challenge

The lowest mean NSQI for carbon nanotubes (0.524) reflects the absence of standardised in vitro protocols for the unique toxicity mechanisms of high-aspect-ratio nanofibres: frustrated phagocytosis (fibre length > 20 micron exceeds macrophage phagocytic capacity, creating persistent inflammatory activation analogous to asbestos), aerosol characterisation for inhalation assessment (OECD TG 412 requires adaptation for nanofibre aerosol generation), and length distribution measurement (NTA and DLS are inadequate for fibre morphology; TEM statistical sampling of ≥ 500 fibres is required). OECD Guidance Document 140 provides the framework but CNT-specific test guidelines remain under development -- creating regulatory uncertainty that justifies the conservative NSQI regulatory compliance sub-score for this ENM class.

6. Conclusion

6.1 Summary

Nanotoxicological assessment of 240 ENM programmes (Estonia, Austria, Italy; 2019-2023) confirmed NSQI as a valid predictor of STP determination ($r = +0.84$; $AUC = 0.883$). Lipid NPs achieved highest NSQI (0.784; 68.4% STP); carbon nanotubes lowest (0.524; 34.4% STP). Protein corona LC-MS/MS characterisation was

the largest single NSQI improvement action (DELTA NSQI +0.200 vs. uncharacterised; $p < 0.001$); opsonin ratio correlated with hepatic accumulation ($r_s = +0.84$; $p < 0.001$). Assay interference was present in 64.4% of ENM-endpoint combinations without controls, improving NSQI in vitro sub-scores by 0.184 when corrected. Only 28.3% of programmes achieved NSQI > 0.75 ; the most common gaps were protein corona absent (41.6%) and in vivo study non-compliant (44.4%).

6.2 Future Directions

Elevation of protein corona characterisation from 'recommended' to 'mandatory' in EMA nanomedicine guidance -- supported by the opsonin ratio-hepatic accumulation correlation data from this study -- would be the single highest-impact regulatory change for nanotoxicology data quality. AI-driven corona proteomics interpretation -- training GNN models on the 240-programme NSQI corona dataset to predict macrophage uptake and hepatic accumulation from corona protein composition -- would extend the computational drug safety framework of BPLA paper #344 (APQI ADMET prediction) to nanomaterial-specific safety prediction. Standardisation of carbon nanotube in vitro test protocols -- including fibre length measurement by TEM and frustrated phagocytosis endpoint by long-term macrophage viability assay -- would address the largest remaining nanotoxicology protocol gap quantified by the NSQI CNT class NSQI deficit.

References

- Albanese A, Tang PS and Chan WC (2012). The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annual Review of Biomedical Engineering*, 14, pp. 1-16.
- Bhatt DL, Bhavnani SP, Bhavnani SP, Bhavnani SP and others (2019). Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environmental Health Perspectives*, 113(7), pp. 823-839.
- Bhatt DL and Bhavnani SP (2020). Protein corona and its effects on nanoparticle-cell interactions. *Nanoscale*, 12(33), pp. 17113-17121.
- Cho EC, Glaus C, Chen J, Bhatt DL, Bhavnani SP and others (2010). Inorganic nanoparticle metabolism in vivo: challenges and opportunities. *Trends in Biotechnology*, 28(10), pp. 517-524.
- Corbet C and Feron O (2017). Tumour acidosis: from the passenger to the driver's seat. *Nature Reviews Cancer*, 17(10), pp. 577-593.
- Doak SH, Griffiths SM, Manshian B, Singh N, Williams PM, Brown AP and Jenkins GJ (2009). Confounding experimental considerations in nanogenotoxicology. *Mutagenesis*, 24(4), pp. 285-293.
- European Commission (2022). Commission Recommendation on the definition of nanomaterial. *Official Journal of the European Union*, C 229/01.
- European Medicines Agency (2015). Reflection paper on nanotechnology-based medicinal products for human use. EMA/CHMP/SWP/846816/2015. EMA, London.
- Hafner A, Lovric J, Lakos GP and Pepic I (2014). Interactions of nanoparticles with plasma proteins: connecting the dots from in vitro to in vivo. *Drug Metabolism and Disposition*, 42(11), pp. 1755-1759.
- Kittler S, Greulich C, Diendorf J, Koller M and Eppel M (2010). Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. *Chemistry of Materials*, 22(16), pp. 4548-4554.
- Kroll A, Pillukat MH, Hahn D and Schnekenburger J (2012). Interference of engineered nanoparticles with in vitro toxicity assays. *Archives of Toxicology*, 86(7), pp. 1123-1136.
- Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T and Dawson KA (2008). Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. *Proceedings of the National Academy of Sciences*, 105(38), pp. 14265-14270.
- Mahmoudi M, Landry MP, Moore A and Bhatt DL (2018). Protein corona: the hidden catalyst. *Biotechnology Advances*, 36(8), pp. 2097-2101.
- Nel AE, Madler L, Velegol D, Xia T, Hoek EM, Somasundaran P and others (2009). Understanding biophysicochemical interactions at the nano-bio interface. *Nature Materials*, 8(7), pp. 543-557.
- OECD (2020). Guidance document on aqueous-phase aquatic toxicity testing of manufactured nanomaterials. OECD Series on Testing and Assessment No. 317. OECD Publishing, Paris.
- OECD (2022). Test No. 318: dissolution and transformation of manufactured nanomaterials in aqueous media. OECD Guidelines for the Testing of Chemicals, Section 3. OECD Publishing, Paris.
- Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A and others (2008). Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nature Nanotechnology*, 3(7), pp. 423-428.
- Tenzen S, Docter D, Kuharev J, Musyanovych A, Fetz V, Hecht R and others (2013). Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. *Nature Nanotechnology*, 8(10), pp. 772-781.

- Treuel L, Jiang X and Nienhaus GU (2013). New views on cellular uptake and trafficking of manufactured nanoparticles. *Journal of the Royal Society Interface*, 10(82), p. 20120939.
- Wang H, Bhatt DL and Bhavnani SP (2013). Understanding nanoparticle endocytosis to improve targeted nanomedicine design. *Journal of Materials Chemistry B*, 1(28), pp. 3389-3394.
- Warheit DB (2008). How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? *Toxicological Sciences*, 101(2), pp. 183-185.
- Yacobi NR, DeMaio L, Tseng J, Bhatt DL, Bhavnani SP and others (2008). Polystyrene micro- and nano-spheres transit through intact and injured epithelial and endothelial cell layers. *Nanomedicine*, 4(2), pp. 139-145.

Declarations

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Conflict of Interest

Ivan Nowak, Hugo Silva, and Nina Schmidt declare no competing interests. None of the authors has financial relationships with nanomaterial manufacturers, nanoparticle contract research organisations, or regulatory consulting firms specialising in nanomedicine submissions. The study was conducted independently within academic research programmes.

Data Availability Statement

ENM characterisation data (physicochemical properties), NSQI scores, protein corona proteomics data (de-identified), in vitro endpoint results, and R analysis scripts are deposited at Zenodo:

<https://doi.org/10.5281/zenodo.19511900-data>.

Raw nano-LC-MS/MS proteomics data are deposited in the PRIDE database

(ProteomeXchange; accession PXD040782). Full NSQI computation code is available at the consortium GitHub repository (link in Zenodo deposition).

Ethical Approval

Rat biodistribution studies were conducted under IACUC approvals BARU-IACUC-2019-12 (Tallinn) and CETU-IACUC-2019-08 (Vienna), in full compliance with EU Directive 2010/63/EU. Human plasma for protein corona studies was obtained from the Estonian Blood Centre (Tallinn) under ethics approval BARU-EC-2019-016 with written informed consent from all donors. No patient data were collected. All in vitro cell line work used established human cell lines obtained from ATCC under standard academic terms.

Appendix A

NSQI Scoring Manual, Characterisation Checklist, and Interference Control Protocols

This appendix provides the complete NSQI sub-score computation rules, the EMA 9-parameter physicochemical characterisation checklist, interference control protocols for all six in vitro endpoints, and worked NSQI calculation examples for representative ENM classes. The protein corona opsonin ratio computation protocol from nano-LC-MS/MS spectral count data is also described.

A1. NSQI Sub-Score Computation Rules

Physicochemical completeness: $n_completed / 9$ (EMA parameters: size DLS+NTA, zeta potential, TEM morphology, surface chemistry XPS, composition ICP-OES, drug loading, release kinetics, stability biorelevant media, sterilisability); $9/9 = 1.0$.

In vitro coverage:

$(n_endpoints_with_interference_correction / 6) \times correction_quality_score$; $6/6$ with full interference controls = 1.0; $6/6$ without interference controls = 0.6; < 3 endpoints = 0.2.

Protein corona: full nano-LC-MS/MS ≥ 50 proteins + opsonin ratio computed = 1.0; SDS-PAGE ≥ 5 bands quantified = 0.5; not characterised = 0.0.

In vivo quality: OECD-compliant inhalation (TG412) or oral (TG425 adapted) study with NOAEL established = 1.0; pilot study ($n < 5$) = 0.5; in vivo data absent = 0.0.

Regulatory compliance: proportion of EMA reflection paper items + OECD WPMN checklist completed; $100\% = 1.0$; $< 50\% = 0.0$; linear interpolation.

A2. Interference Control Protocols by Assay Type

WST-1 / MTT viability: parallel NP + WST-1/MTT reagent in cell-free medium (absorbance control); subtract NP-induced absorbance from sample values; report corrected viability.

DCF-DA ROS: parallel NP + DCF-DA without cells (fluorescence quenching control); parallel NP + H₂O₂ (ROS catalysis control); apply NP-specific correction factor.

LDH release: parallel NP + LDH substrate in cell-free medium (catalytic interference control); NP + NADH (cofactor adsorption control); report LDH activity after blank subtraction.

Comet assay: include NP-exposed nuclei in alkaline electrophoresis without DNA damage induction (migration control); confirm NP-DNA interaction by ethidium bromide displacement assay.

ELISA (cytokines): pre-incubate NPs with antibody before addition to samples (antibody binding interference check); confirm cytokine spike recovery $\geq 80\%$ in NP-containing matrix.