Università
Ca'Foscari
Venezia

Corso di Dottorato di ricerca

in Scienze Ambientali

ciclo 34°

Development and application of a Risk Management Framework for nano-biomaterials used in medical devices and medicinal products

SSD: CIM/12

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To my aunt Patrizia

Summary

The convergence between nanotechnology and biotechnology has offered great improvements thanks to the use of nano-biomaterials (NBMs) in diagnostic, therapeutic, and regenerative medicine with several applications for drug delivery, bioimaging, as biosensors, contrast agents or as important components in medical implants.

Along with the increasing need to effectively evaluate the safety for patients intentionally exposed to NBMs for treatment purpose (as a prerequisite for marketing approval), there are still substantial gaps in understanding the occupational and environmental risks resulting from unintentional exposure to NBMs along the life cycle of nano-enabled biomedical products.

To this purpose, within the present PhD thesis and in the context of the H2020 BIORIMA project, a Risk Management Framework (RMF) was developed for the assessment and management of NBMs used in medical devices and medicinal products, which complements the preclinical benefit-risk analysis of these technologies with a complete assessment of their risks for the environment as well as for medical professionals and workers during production (e.g., powder/liquid handling), use (e.g., abrasion, leaching), and/or end-of-life treatment (e.g., disposal, incineration). The proposed RMF is based on two main pillars: occupational and environmental risk assessment, and benefit-risk analysis for patients.

The RMF was applied to case studies identified in the BIORIMA project to verify and evaluate the applicability of the proposed approaches to risk assessment and management of NBMs. A probabilistic occupational risks of magnetite nanoparticle (NPs) used as contrast agent was performed with the support of the BIORIMA Decision Support System. A Safe-by-Design procedure was developed and tested to support the selection of the best alternative among a set of Ag NPs wound dressings. Finally, the potential of System Thinking approach as a basis of benefit-risk analysis of nanomedicines was explored through an application to magnetite NPs used for theranostic purposes. The case studies supported the identification and critical evaluation of potentials and limitations of the methodological approaches included in the RMF and provided suggestions for future research developments.

Sommario

La convergenza tra nanotecnologia e biotecnologia ha offerto grandi miglioramenti grazie all'utilizzo di nano-biomateriali (NBM) nella medicina diagnostica, terapeutica e rigenerativa con diverse applicazioni per la somministrazione di farmaci, per il bioimaging, come biosensori, agenti di contrasto o come componenti importanti negli impianti biomedici.

Insieme alla crescente necessità di valutare efficacemente la sicurezza per i pazienti intenzionalmente esposti a NBM a scopo terapeutico (come prerequisito per l'approvazione all'immissione in commercio), esistono ancora notevoli lacune nella comprensione dei rischi professionali e ambientali derivanti dall'esposizione involontaria ai NBM lungo il ciclo di vita di prodotti biomedici contenenti nanoparticelle (NP).

A tal fine, nell'ambito della presente tesi di dottorato e nell'ambito del progetto H2020 BIORIMA, è stato sviluppato un Risk Management Framework (RMF) per la valutazione e la gestione dei NBM utilizzati nei dispositivi medici e nei medicinali, che integra l'analisi preclinica dei rischi-benefici di queste tecnologie con una valutazione completa dei loro rischi per l'ambiente, nonché per i professionisti e gli operatori sanitari durante la produzione (ad es. manipolazione di polvere/liquidi), l'uso (ad es. abrasione, lisciviazione) e/o il fine del ciclo di vita del prodotto (es. smaltimento, incenerimento). Il framework proposto si basa su due pilastri principali: valutazione del rischio occupazionale e ambientale e analisi rischi-benefici per i pazienti.

Il framework è stato successivamente applicato ai casi di studio identificati nel progetto BIORIMA per verificare e valutare l'applicabilità degli approcci proposti alla valutazione del rischio e alla gestione di NBM. A tal proposito, è stata eseguita un'analisi di rischio occupazionale probabilistica di nanoparticelle di magnetite, utilizzate come agente di contrasto, con il supporto del Sistema di Supporto alle Decisioni BIORIMA. Inoltre, è stata sviluppata e testata una procedura Safe-by-Design per supportare la selezione della migliore alternativa tra una serie di garze per ferite contenenti nanoparticelle di argento. Infine, l'approccio System Thinking è stato esplorato come base per l'analisi rischi-benefici di nano farmaci attraverso la sua applicazione a nanoparticelle di magnetite utilizzate per scopi teranostici.

I casi di studio hanno supportato l'identificazione e la valutazione critica delle potenzialità e dei limiti degli approcci metodologici inclusi nel Framework e hanno fornito suggerimenti per futuri sviluppi della ricerca.

List of contributions

Published articles

Cazzagon V., Giubilato E. *, Pizzol L., Ravagli C., Doumett S., Baldi G., Blosi M., Brunelli A., Fito C., Huertas F., Marcomini A., Semenzin E., Zabeo A., Zanoni I., Hristozov D.* (2022). *Occupational risk of nano-biomaterials: assessment of nano-enabled magnetite contrast agent using the BIORIMA Decision Support System*. NanoImpact, 25, 100373. https://doi.org/10.1016/j.impact.2021.100373

Cazzagon V.*, Romano A., Gonella F. (2021). *Using Stock-Flow Diagrams to visualize theranostic approaches to solid tumors in personalized nanomedicine*. Frontiers in Bioengineering and Biotechnology, 9, 709727. https://doi.org/10.3389/fbioe.2021.709727

Giubilato E., **Cazzagon V.**, Amorim M. J. B., Blosi M., Bouillard J., Bouwmeester H., Costa A. L., Fadeel B., Fernandes T. F., Carlos Fito C., Hauser M., Marcomini A., Nowack B., Pizzol L., Powell L., Prina-Mello A., Sarimveis H., Scott-Fordsmand J. J., Semenzin E., Stahlmecke B., Stone V., Vignes A., Wilkins T., Zabeo A., Tran L., Hristozov D.* (2020). *Risk Management Framework for Nano-Biomaterials Used in Medical Devices and Advanced Therapy Medicinal Products*. Materials, 13, 4532. https://doi.org/10.3390/ma13204532

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Submitted article

Powell L. G. and Gillies S., Fernandes T. F., Murphy F., Giubilato E., **Cazzagon V.**, Hristozov D., Pizzol L., Blosi M., Costa A. L., Prina-Mello A., Bouwmeester H., Sarimveis H., Janer G., Stone V.* (submitted). *Developing Integrated Approaches for Testing and Assessment (IATAs) in order* to support nanobiomaterial safety. Nanotoxicology.

Article in preparation

Cazzagon V., Giubilato E., Blosi M., Zanoni I., Bonetto A., Marcomini A., Vineis C., Varesano A., Hristozov D., Semenzin E., Badetti E. (in preparation). *Identification of the Safe(r) By Design alternatives of nanoSilver- enabled wound dressings.*

Abstracts

Cazzagon V., Giubilato E., Pizzol L., Ravagli C., Fito C., Huertas F., Baldi G., Campagnolo L., Doumett S., Marcomini A., Semenzin E., Zabeo A., Hristozov D. *Occupational risk assessment of nano-biomaterials used in medical devices and advanced therapy medicinal products and its application to a case study using the BIORIMA Decision Support System.* Platform presentation at the conference NanoTox 2021, 20-22 April 2021, Online conference.

Giubilato E., **Cazzagon V.**, Amorim M. J. B., Blosi M., Bouillard J., Bouwmeester H., Costa A. L., Fadeel B., Fernandes T. F., Carlos Fito C., Hauser M., Marcomini A., Nowack B., Pizzol L., Powell L., Prina-Mello A., Sarimveis H., Scott-Fordsmand J. J., Semenzin E., Stahlmecke B., Stone V., Vignes A., Wilkins T., Zabeo A., Tran L., Hristozov D. BIORIMA *Risk Management Framework for*

Nano-Biomaterials Used in Medical Devices and Advanced Therapy Medicinal Products. Poster presentation at the NanoTox conference 2021, 20-22 April 2021, Online conference.

Giubilato E., **Cazzagon V.**, Marcomini A., Pizzol L., Powell L., Zabeo A., Hristozov D. *The Risk Management Framework for nano-biomaterials used in medical devices and advanced therapy medicinal products*. Poster presentation at the conference NanoSafe 2020, 16-23 November 2020.

Blosi M., Zanoni I., Faccani L., Ortelli S., Schwingenschuh S., Bonetto A., **Cazzagon V.**, Badetti E., Marcomini A., Costa A. L. *Safe-by-design alternatives for the production of Ag NPs based wound dressings*. Poster presentation at the conference NanoSafe 2020, 16-23 November 2020.

Zabeo A., **Cazzagon V.**, Giubilato E., Pizzol L., Hristozov D. *Decision Support System for risk assessment and management of nano(bio)materials used in medical devices and advanced therapy medicinal products.* Poster presentation at the conference NanoSafe 2020, 16-23 November 2020.

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Vignes A., Wang X., Wilkins T., Zabeo A. *Development and application of a risk management framework for nanobiomaterials used in medical devices and ATMPs*. Platform presentation at the Nanosafety Cluster Annual Meeting, 7-10 October 2019, Copenhangen.

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Acronyms

AFW, Artificial Fresh Water

AMW, Artificial Marine Water

AOP, Adverse Outcome Pathway

ATMP, Advanced Therapy Medicinal Product

BAL, Bronchoalveolar Lavage

BMD, Benchmark Dose

BRA, Benefit Risk Analysis

BS, Bloodstream

BSI, The British Standards Institution

CES, Contributing Exposure Scenario

CFU, Colony Forming Unit

CPC, Condensation Particle Counter

CSA, Centrifugal Separation Analysis

dART, Dermal Advanced REACH Tool

D, anticancer drug

DIN, German Institute for Standardisation

DLS, Dynamic Light Scattering

DNEL, Derived No-Effect Level

DSS, Decision Support System

EC, European Commission

ECEL, Exposure Control Efficacy Library

ECHA, European Chemicals Agency

EDX, Energy Dispersive X-ray

EF, Extrapolation Factor

EFM, Environmental Fate Models

ELS, Electrophoretic Light Scattering

EMA, European Medicines Agency

ERA, Environmental Risk Assessment

ES, Exposure Scenario

EV, Exposure Value

EUON, European Union Observatory for Nanomaterials

FDA, Food and Drug Administration

FESEM, Field Emission Scanning Electron Microscope

GM, Geometric Mean

HD, Hazard Dose

HEC, hydroxyethyl cellulose

IATA, Integrated Approaches to Testing and Assessment

ICP-MS, Inductively Coupled-Plasma Mass Spectrometry

IS, Immune System

ISO, International Organization for Standardization

LCL, Lower Confidence Limit

LOAEL, Lowest Observed Adverse Effect Level

LoD, Limit of Detection

MCDA, Multi Criteria Decision Analysis

MD, Medical Device

MF, Magnetic Field

MFA, Material Flow Analysis

MRI, Magnetic Resonance Imaging

NBM, nano-biomaterial

NM, nanomaterial

NOAEL, No-Observable Adverse Effect Level

NP, nanoparticle

OECD, Organisation for Economic Co-operation and Development

OEL, Occupational Exposure Limit

PBPK, Physiologically-Based Pharmaco-Kinetic

PEC, Predicted Environmental Concentrations

PEG, Polyethylene glycol

PLGA, Poly (lactic-co-Glycolic Acid)

PLGA-*b*-PEG-COOH, Poly (lactic-co-Glycolic Acid) (PLGA)-*block*- Polyethylene glycol (PEG)-carboxylic acid

PLLA, poly-L-lactide

PNEC, Predicted No-Effect Concentrations

PoD, Point of Departure

PVA, polyvinyl alcohol

RCR, Risk Characterisation Ratio

REACH, Regulation, Evaluation, Authorization and Restriction of Chemicals

RMF, Risk Management Framework

RMM, Risk Management Measure

RMP, Risk Management Plan

ROS, Reactive Oxygen Species

SbD, Safe-By-Design

SbMD, Safe-By-Material-Design

SbPD, Safety-By-Process-Design

SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks

SEM, Scanning Electron Microscopy

SME, Small-Medium Enterprise

SPION, superparamagnetic iron oxide nanoparticle

ST, System Thinking

SUN, Sustainable Nanotechnologies

SUNDS, SUN Decision Support System

TARMM, Technological Alternatives and Risk Management Measures

TC, Tumour Cells

TEM, Transmission Electron Microscopy

UCL, Upper Confidence Limit

UMBRA, Universal Methodology for Benefit Risk Assessment

WD, Wound Dressing

WHO, World Health Organization

WHO/IPCS, World Health Organization International Programme on Chemical Safety Workgroup

Chapter 1

Introduction

1.1 Motivations and objectives

The convergence of nanotechnology and biotechnology has created huge potential for advancements in medical diagnosis, therapy, and regenerative medicine (Wang et al. 2018) which has fostered large investments in developing novel nano-biomaterials (NBMs) for use in medical devices (MD) and in medicinal products, including advanced therapy medicinal products (ATMP). Due to their physicochemical and functional properties, NBMs can mimic the native tissues, and their components can be identified, handled, and mediated by researchers because of their comparable size to bio-microstructures (Wang et al. 2018). Besides, advanced techniques permit to modify NBM surface properties in order to drive and optimise the interaction with the biological system, providing a better biocompatibility, improving efficacy and reducing adverse side effects (Singh et al. 2016; Genchi et al. 2017; Balasubramanian et al. 2017; Li et al. 2015). As NBMs exhibit distinctive mechanical, electrical, and optical properties compared to other microscopic structures, they have been used for drug delivery, bioimaging, as biosensors, contrast agents or as important components in implants (Pelaz et al. 2017; Sitharaman 2011). For example, biocompatible lipidic materials are used as lipid-based vesicles and liposomes to attenuate side effects of cytotoxic antitumour medicines (e.g., doxorubicin, daunorubicin, paclitaxel, vincristine, irinotecan) (van der Meel et al. 2019), while superparamagnetic iron oxide nanoparticles (SPIONs) with biocompatible polymers can enhance contrast in Magnetic Resonance Imaging (MRI), heat capacity in alternating magnetic fields, and enable magnetic targeting (Janko et al. 2019).

However, the precise interactions of NBMs with biological system is not yet fully understood. Because of the complex nature of NBMs and the variety of nanoforms, standardised and

validated protocols on physico-chemical characterization as well as specific procedures to conduct (eco)toxicological tests for NBMs are still incomplete (Amorim et al. 2020; Gao and Lowry 2018). In the last years, several H2020 project (such as NANoREG, Marina, ITS-Nano, SUN) aimed to fill the data gaps in nanomaterial safety, developing frameworks for risk assessment and management and proposing nano-specific considerations in standard testing strategies on the physico-chemical characterization as well as (eco)toxicological tests of nanomaterials (NMs).

For NBMs, standardised tests, protocols and tools are only partially investigated, and there is still the need to verify if Standard Operating Procedures and guidelines for NMs are also suitable for NBMs. The lack of ad-hoc methods for NBMs could lead to misleading results on the behaviour of NBMs in biological systems and their effects on human health and environment. Therefore, there is the need to properly investigate effects of NBMs in organisms, identifying target potentially exposed to NBMs along the entire life cycle of the nano-enabled product.

To investigate safety aspects of NBMs, medical devices/medicinal products developers need to submit a marketing authorization application to the European Medicines Agency (EMA) which contains information on the safety use and administration of the product before marketing authorisation. In this context, a benefit-risk analysis (BRA) is required by EMA throughout the whole R&D phases of medicinal products, including the non-clinical discovery phase, the clinical phases (phase I, II, III) and the post-marketing pharmacovigilance (Cavero 2009; Curtin and Schulz 2011), where benefits and risks are identified, weighted and compared in order to evaluate if the benefits outweigh risks. For medical devices, clinical investigations are needed to assess the safety or performance of a device involving one or more human subjects, including outcome(s) related to diagnosis, or a positive impact on patient management or public health (European Commission, 2021).

However, as the identification and assessment of occupational and environmental risks are not strictly required by EMA or other national authorities and could therefore be overlooked during the R&D stages, a comprehensive assessment of risks derived from the administration/use of nano-enabled medical device/medicinal product as well as risks for human health and the environment exposed to NBMs is needed through the adoption of a life cycle perspective.

To address this issue, the development of a Risk Management Framework (RMF) of NBMs used in MD and ATMP is the main objective of this PhD thesis. This framework has been designed in the frame of the H2020 BIORIMA project (described in Paragraph 1.3) through an interdisciplinary collaboration between project partners and stakeholders. The RMF complements the preclinical benefit-risk analysis of NBMs for patients with a complete assessment of their risks for the environment as well as for workers (e.g., producers, physicians, technicians, nurses and healthcare assistants, healthcare waste personnel) exposed to NBMs during production (e.g., powder/liquid handling), use (e.g., abrasion, leaching) and/or end-of-life treatment (e.g., disposal, incineration).

Furthermore, the PhD thesis has focused on testing the applicability of the RMF to real NBMs used in medical devices and medicinal products, to investigate strengths and limitations of the approaches proposed for the two main pillars of the framework (i.e., risk assessment and benefit-risk analysis) and identify future research needs.

In conclusion, the specific objectives of the work presented in this PhD thesis are:

- To present a background on NBM classifications and their regulatory context.
- To develop a Risk Management Framework of NBMs used in MDs and ATMPs, with the aim to integrate benefit-risk analysis and risk assessment procedures for NBMs.
- To apply the developed framework in a real case study by conducting an occupational risk assessment of a nano-based contrast agent.
- To develop a Safe-By-Design procedure for wound dressings containing silver nanoparticles and apply it in real case studies.
- To use the System Thinking approach to visualise the complexity of the administration of a nano-based theranostic agent in solid tumours as a promising strategy for benefit-risk analysis.

1.2 Thesis structure

The thesis is structured in seven chapters, where a background of NBMs and their regulations, the development of the Risk Management Framework and its application in three case studies are presented. More specifically, chapters are briefly depicted here:

Chapter 2- "Classifications, applications, and regulatory context of nano-biomaterials" includes the definition and classifications of 'nano-biomaterials', their applications in medical devices and medicinal products and an overview of European regulations concerning the use of nanotechnologies in medical applications.

Chapter 3- "**Risk Management Framework for nano-biomaterials used in Medical Devices and Advanced Therapy Medicinal Products"** presents the development of a Risk Management Framework for NBMs used in MD and ATMP based on regulatory requirements, standards, and protocols for NMs and their adaptation to NBMs.

Chapter 4- "Occupational risk assessment of nano-enabled magnetite contrast agent"

describes the development of an occupational risk assessment methodology for NBMs used in MD and ATMP and its application in a real case study using the BIORIMA Decision Support System.

Chapter 5- "Identification of the Safe(r) By Design alternatives of Ag NPs- enabled wound dressings"

presents a Safe-By-Design procedure developed for nano-enabled wound dressings (WDs) and its application to five nano-Ag based WDs and two commercial nano-based WDs considering specific human health and environmental criteria for the investigated medical devices.

Chapter 6- "Systemic stock-flow diagrams to visualize theranostic approaches to solid tumours in personalized nanomedicine as a basis of benefit-risk analysis"
illustrates the complexity of theranostic approaches to solid tumours through the development of a systemic stock-flow diagram as a basis for benefit-risk analysis of nanomedicines.

Chapter 7- "Conclusions"
includes considerations on the main findings of the developed activities, pointing up potential and critical issues and possible improvements of the proposed framework and its further applications.

1.3 BIORIMA project

The work presented in this PhD thesis has been developed within the European Project BIORIMA (BIOmaterials Risk Management), a Horizon 2020 project (G.A. No 760928) started in November 2017 and coming to its conclusion in January 2022. The project was funded by the European Commission within the Thematic Priority "Development of a reliable methodology for better risk management of engineered biomaterials in Advanced Therapeutic Medicinal Products and/or Medical Devices".
BIORIMA project involved 39 partners from 11 EU Countries and 2 non-EU Countries and was coordinated by Professor Lang Tran of the Institute of Occupational Medicine (IOM) in Edinburgh (UK).
The first objective of the BIORIMA project is to develop a Risk Management Framework (RMF) for NBMs used in ATMPs and MDs. The BIORIMA RMF is a structure upon which the validated tools and methods for materials, exposure, hazard and risk identification/assessment and management are allocated plus a rationale for selecting and using them to manage and reduce the risk for specific NBMs used in ATMP and MD. Specifically, the RMF consists of: (i) Risk Management strategies and systems, based on validated methodologies, tools, and guidance, for monitoring and reducing the risks together with methods for evaluating them; (ii) Validated

methodologies and tools to identify the potential exposure and hazard posed by NBMs to humans and the environment; (iii) A strategy for Intelligent Testing (ITS) and Tiered Risk Assessment for NBM used in ATMP and MD.

BIORIMA workplan consists of 7 work packages (WPs), where WP1 coordinates the project, ensuring the correct development of Deliverables and Milestones of each WPs, WP2 selects and performs a physico-chemical characterization of relevant materials developed by industrial partners of the Consortium, WP3 performs measurements of the release of NBMs from ATMP and MD over the entire life cycle to address workers exposure in all life cycle stages, WP4 adapt and validate current test methods for NMs and/or develop new test methods for NBMs, including in vitro and in vivo methods, for the detection of adverse effects of NBMs on human health and the environment, WP6 assess the performance of the proposed RMF by testing, evaluating, and validating the methodologies, strategies and tools developed under representative "real-life" situations over the entire life cycle of selected NBMs, WP7 support the controlled release of all results through the continuous development of a Plan for Exploitation & Dissemination of Results using communication channels and transfer of the pre-normative research results to regulatory bodies.

The work presented in this PhD thesis was mainly developed within the WP5 activities. Indeed, the RMF for NBMs used in MD and ATMP has been developed in collaboration with project partners as well as stakeholders. Then, the application of the RMF in case studies has been exploited involving experimental data of materials (in collaboration with WP2), modelling tools and measurements of NBMs exposure (in collaboration with WP3) and hazard data (performed in WP4). Moreover, this thesis contributes to the development of a web-based Decision Support System (DSS) (as one of the objectives of the WP5) developed for the assessment of risks for workers and environment exposed to NBMs through the selection of hazard and exposure models.

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Chapter 2

Classifications, applications, and regulations of nano-biomaterials

2.1 Classifications and application of nano-biomaterials

According to the definition of the American National Institute of Health, a biomaterial can be defined as any substance or combination of substances, other than drugs, synthetic or natural in origin, usable for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual (NIH, 1982), and if it has a constituent or a surface dimension in the nano range (i.e., 1–100 nm) can be classified as NBM (Yang, Zhang, and Webster 2011).

The major issue associated with NBMs is the potential toxicity of reactive nanoparticles (NPs), which depends on the specific characteristics of the material (Razavi et Thakor 2017). Therefore, there is the need to categorise NBMs to direct the application of the most appropriate methodologies for risk assessment and management, taking into account the specific properties of the nano form (e.g., composition, physical form, reactivity) as well as the regulatory classification based on the purpose of the biomedical application and the type of actions. For this reason, within the PhD thesis, a NBM classification scheme was proposed in collaboration with BIORIMA partners (Figure 1) based on the intended use and regulatory classification of the entire biomedical product (in red boxes) and specific physico-chemical properties of NBMs (in blue boxes). As the focus of the BIORIMA project is to investigate NBMs used in MD and ATMP, the classification scheme proposed involves these two categories of products.

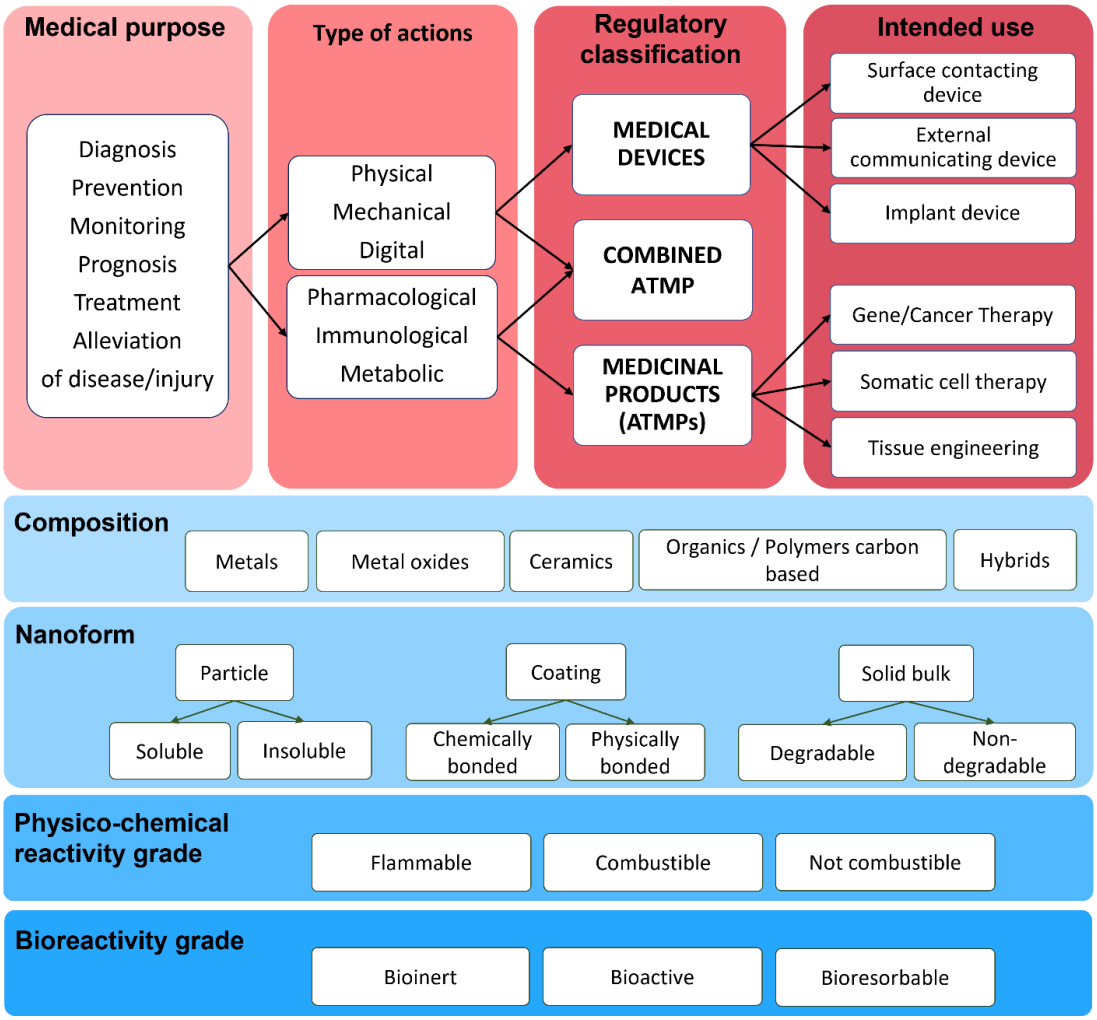

Figure 1. BIORIMA NBMs classification scheme

The ***medical purposes*** covered by the biomaterials can be grouped as follows:

- *diagnosis,*
- *prevention,*
- *monitoring,*
- *prognosis,*
- *treatment, and*
- *alleviation of disease or injury*.

Depending on the ***type of action*** used to reach such purposes (i.e., physical, digital, mechanical or pharmacological, immunological, metabolic type of action), the EU regulatory classification

established different types of products, namely Medical Devices (MD), Medicinal Products, including Advanced Therapy Medicinal Products (ATMP) and combined ATMP.

A **Medical Device (MD)** is defined, according to Regulation 2017/745/EC as any instrument, apparatus, appliance, software, implant, reagent, material or other article intended to be used on humans for the diagnosis, prevention, monitoring, prediction, prognosis, treatment, alleviation of disease or compensation for, an injury or disability, investigation, replacement or modification of the anatomy; conception control by mechanical or physical means; examination of specimens derived from the human body and products specifically intended for the cleaning, disinfection or sterilization of devices. A medical device should not achieve its principal intended action by pharmacological, immunological or metabolic means, in or on the human body, but may be assisted in its function by such means.

A MD can have one or more of the following intended uses (ISO 10993-1:2009):

- Surface contacting devices: MD that comes into contact with skin. Examples are wound dressings containing nano-sized silver particles and metal oxide particles used for improved antibacterial and anti-fungal activity.
- External communicating devices: MD that comes into contact with the blood path, either indirectly or with circulating blood, and devices in contact with tissue/bone/dentin. Examples include catheters with a nanosilver coating, polymer-based dental composite filler materials and dental cements containing nanoparticles, surgical and dental instruments with nano-coatings structures used to enhance the wear resistance or to create non-sticky surfaces.
- Implant devices: MDs which are intended to be totally introduced into the human body, are in contact with tissues, bone, or blood, and are intended to remain in place after the procedure. Examples include nanocoated bare metal stents, implants for joint replacement (arthroplasties) and for fracture repair, surface nano-coatings on implants used to improve the biocompatibility or for antibacterial purposes, carrier material ('scaffold') for tissue engineering products with a nanoporous structure and surface properties that facilitate the growth of living cells and enabling the tissue of replace,

repair or regenerate tissues (e.g., bone fillers with hydroxyapatite and tricalcium phosphate nanoparticles, carbon nanotubes in bone cements).

A Medicinal Product (MP) is defined by Directive 2001/83/EC as "a substance or combination of substances that is intended to treat, prevent or diagnose a disease, or to restore, correct or modify physiological functions by exerting a pharmacological, immunological or metabolic action" and Advanced Therapy Medical Products (ATMPs) constitute a class of innovative pharmaceuticals based on emerging cellular and molecular biotechnologies, encompassing the following typologies:

- Gene therapy medicinal products (as defined in Part IV of Annex I to Directive 2001/83/EC, as amended): Medicines that contain genes that lead to a therapeutic, prophylactic or diagnostic effect. They work by inserting 'recombinant' genes into the body, usually to treat a variety of diseases, including genetic disorders, cancer, or long-term diseases. A recombinant gene is a stretch of DNA that is created in the laboratory, bringing together DNA from different sources.
- Somatic cell therapy medicinal products (as defined in Part IV of Annex I to Directive 2001/83/EC, as amended): Medicines containing cells or tissues that have been manipulated to change their biological characteristics or cells or tissues not intended to be used for the same essential functions in the body. They can be used to cure, diagnose, or prevent diseases.
- Tissue engineered products (as defined in Article 2(1)(b) of Regulation (EC) No. 1394/2007): Medicines that contain cells or tissues that have been modified so they can be used to repair, regenerate, or replace human tissues. Moreover, tissue or cell can be associated to a medical device as an integral part of the product and in this case, we refer to combined ATMPs (e.g., cells embedded in a biodegradable matrix or scaffold), which can fulfil any of the above-mentioned intended uses.

The **combined ATMPs** are based on the combination of tissues or cells with a medical device and are able to fulfil all the above identified intended uses.

Regulation (EC) No 1394/2007 and Directive 2001/83/EC provide detailed definitions of the different types of ATMP.

For some types of biomedical applications of NBMs of recent development, the classification as MD or ATMP is still under debate. For example, injectable nanomaterials generating heat upon electromagnetic stimulation for cancer therapy (i.e., heat therapy with iron oxide nanoparticles, heat ablation with gold nanoparticles) are classified as **specific types of medical devices**, because of their immediate mechanical action (the tumour cells burst). On the other hand, the legislation on medicines might be applicable because the burst cells are later metabolised.

Another example stems from *theranostic agents*, namely the product which exert a therapeutic and diagnostic action. This is the case of iron oxide nanoparticles, whose super paramagnetic properties are exploited both for diagnostics and heat therapy. In fact, it has not yet been determined if their use falls under the legislation on medicines or under the legislation on medicinal devices; however, it is likely that these products will be considered as ATMPs.

NBMs can be further classified on the basis of their **composition** and **reactivity grade** (Kiaie, Aavani, and Razavi 2017).

According to their composition, NBMs can be classified as:

- *Metal oxides*: they represent together with ceramics the main class of inorganic biomaterials. In addition to their use as scaffolding material, they are widely investigated as inorganic nanotherapeutics. Metal oxide nanoparticles such as Fe_3O_4, CuO, TiO_2, ZnO, MgO, CaO, CeO_2, Al_2O_3, Y_2O_3 or metal nanoparticles such as Ag and Au are studied with interest due their antimicrobial properties and their potential application for cancer applications, including diagnosis, therapy, and imaging.
- *Metals:* they are frequently used in biomedical applications to substitute hard tissue. For instance, metals such as titanium-alloys based make them interesting candidates for various applications (e.g., bone plates and screws, hip and knee artificial joints, dental implants, vascular stents).
- *Ceramics*: these are biocompatible ceramics (calcium silicates, carbonates, phosphates, hydroxyapatite), having high toughness, elastic modulus, heat- and corrosion-resistance.

They have many applications in biomedicine, can be used for orthopaedic purposes in tissue engineering scaffolds and graft substitution, have many applications in dentistry as dental implants, gold porcelain crowns, glass-filled cement, and dentures.

- *Organics/polymers*: include organic materials having some properties such as biocompatibility and that can replace or restore function to a body tissue and replace hard or soft tissues; some of the biopolymers fall into this category and cover the widest range of applications in biomedical field. Beside biopolymers, there is a small group of organic materials that are based on lipids (liposomes and niosomes are two subtypes of these materials).
- *Carbon based*: carbon-based nanomaterials (e.g., fullerenes, carbon nanotubes, graphene, carbon nanoparticles) serve as multipurpose innovative materials for biomedical applications. Their ability to hybridize with a wide range of organic and inorganic materials make them the ideal candidate for the development of novel and efficient composite materials for various biomedical applications: drug-delivery, photo-thermal therapy, bio-sensors, nano-probes for biomedical imaging.
- *Hybrids:* nanocomposites at the molecular scale, having at a minimum one component, either the organic or the inorganic constituting part, with a characteristic length on the nanometer size.

Moreover, three different generations of biomaterials have been defined based on their ***bioreactivity grade***:

- *Bioinert*: they are characterized by a minimum interaction with the human body. In the field of cardiovascular or orthopaedics, bioinert materials are developed to serve mainly mechanical and physical purposes and in many cases, they are intended to be used for long term applications.
- *Bioactive*: defined by their ability to interact with the biological environment, to enhance the biological response and the tissue/surface bonding, as well as by the development of bioresorbable materials ability to undergo a progressive degradation while new tissue regenerates and heals.

- *Bioresorbable/able to simulate specific cellular responses at molecular level*: new materials that can stimulate specific cellular responses at molecular level. For these biomaterials, the bioactivity and biodegradability concepts are combined and bioresorbable materials become bioactive and vice versa. Examples include temporary three-dimensional porous structures that stimulate cells' invasion, attachment, and proliferation, as well as functionalized surfaces with peptide sequences to trigger specific cell responses.

In a complementary manner, it can also be useful to classify NBMs based on their ***physico-chemical reactivity grade*** (or oxygen reactivity grade):

- *Not combustible*: materials that are not able to oxidize with oxygen.
- *Combustible*: materials that can undergo an exothermic oxidation reaction with oxygen.
- *Flammable*: materials which ignite and burn, under effective ignition sources.

This complementary classification is of interest to identify potential safety issues at industrial scale that will need to be managed (e.g., fire, explosion), but also to consider the reactivity of the materials in a biologic oxidative environment. For instance, some metals can be highly combustible and one concern in a biologic medium is also their corrosion and degradation.

Finally, even the **form of nanomaterials** employed into NBMs can vary considerably. Basically, they can be:

- *Free particles*: free nanomaterials administered to the patient as such (e.g., iron oxide or gold nanomaterials for heat therapy against cancer, liposomal composition for drug delivery), free nanomaterials in a paste-like formulation (e.g., dental filling composites), free nanomaterials added to a medical device (e.g., nanosilver as antibacterial agent in wound dressings).
- *Coatings*: nanomaterials forming a coating on implants to increase biocompatibility (e.g., nano-hydroxyapatite) or to prevent infection (e.g., nano-silver).
- *Fixed in a bulk structure*: embedded nanomaterials to strengthen biomaterials (e.g., carbon nanotubes in a catheter wall) or forming bulk structure (e.g., nanoporous and nanostructured scaffolds for bone regeneration).

2.2 Regulatory context of nano-biomaterials

In order to investigate the state-of-art of regulatory context related to risk assessment and benefit-risk analysis of NBMs, a literature search on standards, regulations and guidelines on environmental, occupational risks and patient-safety assessment of nano products was conducted. Moreover, documents related to the evaluation of risks for non-nano products were considered to understand if the material has similar properties to NBMs (e.g., occupational exposure assessment of powder not in nano form). Documents proposed by European Commission (EC), European Chemicals Agency (ECHA), European Medicines Agency (EMA), Organisation for Economic Co-operation and Development (OECD), International Organization for Standardization (ISO), The British Standards Institution (BSI), the German Institute for Standardisation (DIN), the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and the World Health Organization (WHO) were considered for both chemical substances and nanomaterials.

In Table 1, all the documents found in literature are presented and classified according to the type of document (i.e., regulation, guideline, standard), the investigated product (i.e., nano, non-nano), its application (i.e., medical, or non-medical) and the target (i.e., environment, consumer/patient, worker).

Table 1. Regulations, standards and guidelines for nano and not nano-based products, applied in medical applications or in other sectors suitable for the assessment of environmental and occupational risks or for the safety assessment of patients. In bold the acronym of the standard (if available), in italics the title, and underlined the name of the institution/author of the document.

Regulation	Guideline	Standard	Nano product	Non-nano product	Medical application	Non- medical application	Acronym, title, author of the document	Environment	Consumer/ patient	Worker
		X	X			X	ASTM E2535–07 (2018) ASTM International *Standard Guide for Handling Unbound Engineered Nanoscale Particles in Occupational Settings*			X
X				X		X	**CLP Regulation (EC) No 1272/2008** European Parliament and the Council *Classification, Labelling and Packaging of substances and mixtures*	X		X
X				X	X		**Commission Directive 2009/120/EC** European Parliament of the Council on the Community *Medicinal products for human use as regard advanced therapy medicinal products*		X	
	X		X			X	**Current Intelligence bulletin 63, 2011** Department of health and human services, Centers for Disease Control and Prevention. National Institute for Occupational Safety and Health (NIOSH) *Occupational exposure to titanium dioxide*			X
	X		X			X	**Current Intelligence bulletin 65** Department of health and human services, Centers for Disease Control and Prevention. National Institute for Occupational Safety and Health (NIOSH) 2013 *Occupational exposure to carbon nano tubes*			X

Regulation	Guideline	Standard	Nano product	Non-nano product	Medical application	Non- medical application	Acronym, title, author of the document	Environment	Consumer/ patient	Worker
		X	X			X	**DIN EN 16897: 2015-09** Deutsches Institut für Normung e. V. *Workplace exposure - Characterization of ultrafine aerosols/nanoaerosols - Determination of number concentration using condensation particle counters*			X
X				X	X		**Directive 2001/83/EC** European Parliament and the Council *Community code relating to medicinal products for human use*		X	
X				X		X	**Directive 2004/37/EC** European Parliament and the Council *Protection of workers from the risks related to exposure to carcinogens or mutagens at work*			X
	X			X	X		European Medicines Agency (EMA), 2005 *Guideline on risk management systems for medicinal products for human use*		X	
	X		X		X		European Medicines Agency (EMA), 2006 *Reflection Paper on Nanotechnology-Based Medicinal Products for Human Use*		X	
	X			X	X		European Medicines Agency (EMA), 2007 *Report of the CHMP working group on benefit-risk assessment models and methods*		X	
	X			X	X		European Medicines Agency (EMA), 2008 *Guideline on Safety and Efficacy Follow-Up - Risk Management of Advanced Therapy Medicinal Products*		X	

Regulation	Guideline	Standard	Nano product	Non-nano product	Medical application	Non- medical application	Acronym, title, author of the document	Environment	Consumer/ patient	Worker
	X			X	X		European Medicines Agency (EMA), 2011 *Procedural advice on the evaluation of combined advanced therapy medicinal products and the consultation of Notified Bodies in accordance with Article 9 of Regulation (EC) No. 1394/2007*		X	
	X			X	X		European Medicines Agency (EMA), 2015 *Reflection paper on classification of advanced therapy medicinal products*		X	
	X		X		X		European Medicines Agency (EMA), 2015 *Reflection paper on the data requirements for intravenous iron-based nano-colloidal products developed with reference to an innovator medicinal product*		X	
	X			X	X		European Medicines Agency (EMA), 2017 *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products*		X	
	X			X	X		European Medicines Agency (EMA) and European Commission (EC), 2017 *European Commission-DG Health and Food Safety and European Medicines Agency Action Plan on ATMPs*		X	
	X			X		X	**ENV/JM/MONO (2003) 16** Organisation for Economic Co-operation and Development (OECD) *Guidance document on reporting summary information on environmental, occupational and consumer exposure*	X	X	X
	X		X			X	**ENV/JM/MONO (2009) 16** Organisation for Economic Co-operation and Development (OECD) *Emission assessment for identification of sources and release of airborne manufactured nanomaterials in the workplace*			X

Regulation	Guideline	Standard	Nano product	Non-nano product	Medical application	Non- medical application	Acronym, title, author of the document	Environment	Consumer/ patient	Worker
	X		X			X	**ENV/JM/MONO (2015) 19** Organisation for Economic Co-operation and Development (OECD) *Harmonized tiered approach to measure and assess the potential exposure to airborne emissions of engineered nano-objects and their agglomerates and aggregates at workplaces*			X
	X		X			X	**ENV/JM/MONO (2015) 30** Organisation for Economic Co-operation and Development (OECD) *Guidance manual towards the integration or risk assessment into life cycle assessment of nano-enabled applications*	X		X
	X		X			X	**ENV/JM/MONO (2017) 32** Organisation for Economic Co-operation and Development (OECD) *Consumer and environmental exposure to manufactured nanomaterials*	X	X	
	X		X			X	European Chemicals Agency (ECHA), Guidance 2016 *Guidance on information requirements and chemical safety assessment. Chapter R.14 Occupational exposure estimation*			X
	X		X			X	European Commission. Employment, Social Affairs & Inclusion, Guidance 2014 *Guidance on the protection of the health and safety of workers from the potential risks related to nanomaterials at work*			X
	X		X			X	Health and Safety Executive (HSE), UK, Guidance 2013 *Using nanomaterials at work. Including carbon nanotubes (CNTs) and other bio-persistent high aspect ratio nanomaterials (HARNs)*			X

Regulation	Guideline	Standard	Nano product	Non-nano product	Medical application	Non- medical application	Acronym, title, author of the document	Environment	Consumer/ patient	Worker
		X	X			X	**ISO 10801:2010** International Organization for Standardization (ISO) *Nanotechnologies -- Generation of metal nanoparticles for inhalation toxicity testing using the evaporation/condensation method*			X
		X	X			X	**ISO 10808:2010** International Organization for Standardization (ISO) *Nanotechnologies- Characterization of nanoparticles in inhalation exposure chamber for inhalation toxicity testing*			X
		X	X			X	**ISO/TS 12025:2012** International Organization for Standardization (ISO) *Nanomaterials. Quantification of nano-object release from powders by generation of aerosols*			X
		X	X			X	**ISO/TS 12885:2008** International Organization for Standardization (ISO) *Nanotechnologies- Health and safety practices in occupational settings relevant to nanotechnologies*			X
		X	X			X	**ISO/TS 12901:2014** International Organization for Standardization (ISO) *Nanotechnologies- Occupational risk management applied to engineered nanomaterials*			X
		X		X		X	**ISO 14040:2006** International Organization for Standardization (ISO) *Environmental management - Life cycle assessment - Principles and framework*	X		

Regulation	Guideline	Standard	Nano product	Non-nano product	Medical application	Non- medical application	Acronym, title, author of the document	Environment	Consumer/ patient	Worker
		X		X		X	**ISO 14044:2006** International Organization for Standardization (ISO) *Environmental management - Life cycle assessment - Requirements and guidelines*	X		
		X		X	X		**ISO 14791:2007** International Organization for Standardization (ISO) *Medical devices- Application of risk management to medical devices*		X	
		X	X			X	**ISO/TR 18637:2016** International Organization for Standardization (ISO) *Nanotechnologies - Overview of available frameworks for the development of occupational exposure limits and bands for nano-objects and their aggregates and agglomerates (NOAAs)*			X
		X	X			X	**ISO/TR 19601:2017** International Organization for Standardization (ISO) *Nanotechnologies -- Aerosol generation for air exposure studies of nano-objects and their aggregates and agglomerates (NOAA)*			X
		X	X			X	**ISO/TS 21623:2017** International Organization for Standardization (ISO) *Workplace exposure -- Assessment of dermal exposure to nano-objects and their aggregates and agglomerates (NOAA)*			X
		X	X			X	**ISO/TR 27628:2007** International Organization for Standardization (ISO) *Workplace atmospheres - Ultrafine, nanoparticle and nano-structured aerosols - Inhalation exposure characterization and assessment*			X

Regulation	Guideline	Standard	Nano product	Non-nano product	Medical application	Non- medical application	Acronym, title, author of the document	Environment	Consumer/ patient	Worker
		X	X			X	**ISO 28439:2011** International Organization for Standardization (ISO) *Workplace atmospheres -- Characterization of ultrafine aerosols/nanoaerosols -- Determination of the size distribution and number concentration using differential electrical mobility analysing systems*			X
		X	X			X	**PAS 138:2012** The British Standards Institution (BSI) *Disposal of manufacturing process waste containing manufactured nano-objects*	X		
	X		X			X	**PD CEN/TS 17276:2018** The British Standards Institution (BSI) *Nanotechnologies - Guidelines for Life Cycle Assessment - Application of EN ISO 14044:2006 to Manufactured Nanomaterials*	X		
X				X		X	**Regulation (EC) No 1907/2006** European Parliament and the Council *Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)*	X		X
X				X	X		**Regulation (EU) 2017/745** European Parliament and the Council *Medical devices, amending Directive 2001/83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 and repealing Council Directives 90/385/EEC and 93/42/EEC*		X	

Regulation	Guideline	Standard	Nano product	Non-nano product	Medical application	Non- medical application	Acronym, title, author of the document	Environment	Consumer/ patient	Worker
X				X	X		**Regulation (EU) 2017/746** European Parliament and the Council *In vitro diagnostic medical devices and relating Directive 89/79/EC and Commission Decision 2010/227/EU*		X	
	X		X			X	Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2009 *Risk Assessment of Products of Nanotechnologies*	X	X	X
	X		X			X	Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2015 *Guidance on the Determination of Potential Health Effects of Nanomaterials Used in Medical Devices. Final Opinion*		X	X
	X			X	X		Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2018 *Guideline on the environmental risk assessment of medicinal products for human use*	X		
	X			X		X	World Health Organization (WHO) 2017 *International minimum requirements for health protection in the workplace*			X
	X		X			X	World Health Organization (WHO) 2017 *WHO Guidelines on protecting workers from potential risks of manufactured nanomaterials*			X

As can be seen from the table, no standards, protocols, or guidelines are available to assess both environmental/occupational and patients risks derived from exposure to nano-enabled biomedical products.

Two pie charts were developed to clearly visualize the % of standard, guidelines and regulations found in literature on exposure of patients and/or, workers and/or environment for not nano (Figure 2a) and nano (Figure 2b) products. From the results, workers are the most considered target exposure in nano-products, patients are the most studied for not nano products, while environment is assessed only by a small minority of documents both for nano and not nano products.

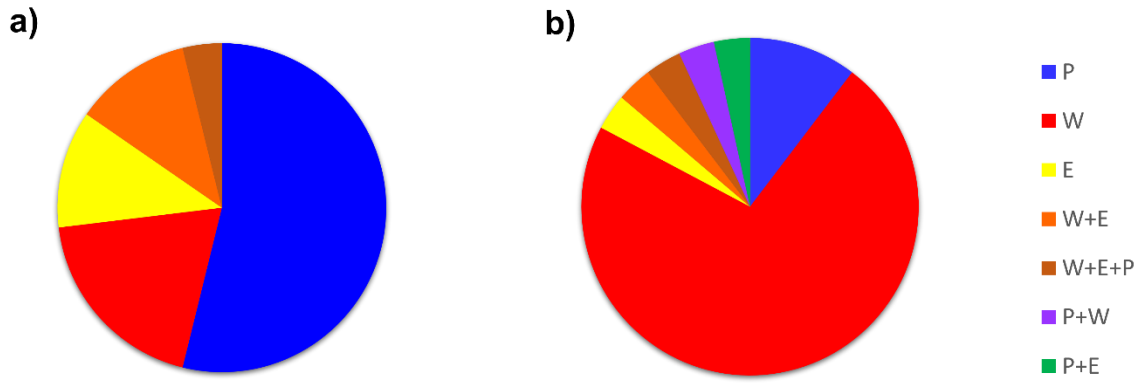

Figure 2. Regulations, standards, and guidelines on risk assessment and benefit risk analysis of a) non nano and b) nano products. Abbreviations: P: Patients, W: Workers, E: Environment, W+E: Workers and Environment, W+E+P: Workers and Environment and Patients, P+W: Patients and Workers, P+E: Patients and Environment.

As can be seen from figure 2, the safety assessment of patients (required in benefit-risk analysis), and the process of assessing occupational and environmental risks are assessed by distinct regulations. In benefit-risk analysis, risks for patient posed by medical applications are always compared to their clinical benefits and can be accepted if the benefits significantly outweigh the safety concerns, while in risk assessment the risks for workers and the environment are determined by unintentional exposure to the materials and any risks above the exposure/hazard risk ratio are considered unacceptable. For this reason, these fundamental differences have determined different obligations in the respective regulations

that require different testing strategies. However, some of the approaches can be used across these domains.

For this reason, the implementation of the RMF (proposed in chapter 3) can facilitate the integration of approaches between risk assessment and benefit-risk analysis by identifying areas of cross-fertilisation and promoting the communication and collaboration between scientists and regulators from different fields.

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Chapter 3

Risk Management Framework for nano-biomaterials used in Medical Devices and Advanced Therapy Medicinal Products

Contents included in:

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Specific contribution of the PhD candidate:

The work performed within the PhD thesis included the active contribution to the first conceptual design of the framework, the support to the periodic coordination of the group of experts, the review, harmonization and integration of their contributions, the support to the organization and implementation of the stakeholder workshop, the development of the strategy for occupational risk assessment, the writing and editing of the final description of the framework.

3.1 Introduction

As shown in chapter 2, no directives, regulations, or guidelines for the assessment of risks of NBMs for both patients and workers/environment are available in literature. This could be explained by the fact that the safety assessment of a new medical device/medicinal product (MD/MP) is investigated during the pre-market authorization process by EMA, without obligations for the manufacturers/producers on the evaluation of occupational and environmental risks of NBMs of the product.

On the other hand, as NMs fall under the existing REACH and CLP definition of substance, REACH regulation requires a Chemical Safety Assessment of a new substance produced or imported in quantities above 10 tonn/year where human health and environmental concerns are carefully assessed along the substance lifecycle.

Because of the lack of guidelines for the assessment of risks for both patients and environment/workers, a Risk Management Framework (RMF) has been developed to assess risks derived from the administration/use of nano-enabled MD/MP as well as risks for human health and the environment exposed to NBMs through the adoption of a life cycle perspective.

The RMF is the outcome of the interdisciplinary, collaborative effort of a team of experts from different European universities, research institutes and companies involved as partners in the BIORIMA project. This work is based on a comprehensive review of regulatory requirements and research trends related to the risk management of nano(bio)medical technologies, which was performed with the aim of collecting information and data to inform a subsequent expert discussion. Selected experts from the BIORIMA consortium formed three working groups, namely (a) human health and ecological risk assessment, (b) benefit-risk analysis, (c) risk prevention, control, and monitoring, depending on their field of expertise, and each group worked on developing/describing the corresponding parts of the framework. The draft framework was presented to stakeholders during the 1st BIORIMA Stakeholder Workshop held in Valencia in November 2018 that brought together more than 40 participants from industry, research, and regulatory institutions. Workshop participants were asked to comment on the proposed framework through guided discussions during three break-out sessions and a final plenary discussion and this resulted in constructive feedback by the stakeholders, which was incorporated in the current version of the RMF, presented here. For example, a risk control and management section was added in the benefit-risk analysis pillar, according to comments and suggestions received from stakeholders. Participants also suggested to avoid using generic terms like “holistic” and “integrated” in the framework description and to make the framework more focused and “operational”, by including description of target product categories, recommendations of guidelines and standards applicable at different stages, and providing examples of real case-study applications.

The goal has been to develop a RMF that is applicable to past and current generation of NBMs but at the same time is able to integrate new scientific outcomes to address the need of future generations of NBMs and to support the standardization and regulation of these materials.

3.2 The Risk Management Framework for NBMs used in MD and ATMP

The RMF is outlined in Figure 3. It is designed to facilitate benefit-risk analysis of NBMs applied in MD and ATMP for patients, as well as assessment of their occupational and environmental risks from a life cycle perspective. In cases of unacceptable risks, the RMF supports the identification of adequate risk control measures. The life cycle stages are not listed in the typical order (i.e., synthesis -> formulation -> use -> end-of-life treatment), but rather according to different exposure assessment targets: on the left side the stages where unintentional exposure of workers or environmental targets can occur, including unintentional exposure of medical professionals; on the right side the use stage where patients are intentionally exposed for therapeutic or diagnostic purposes.

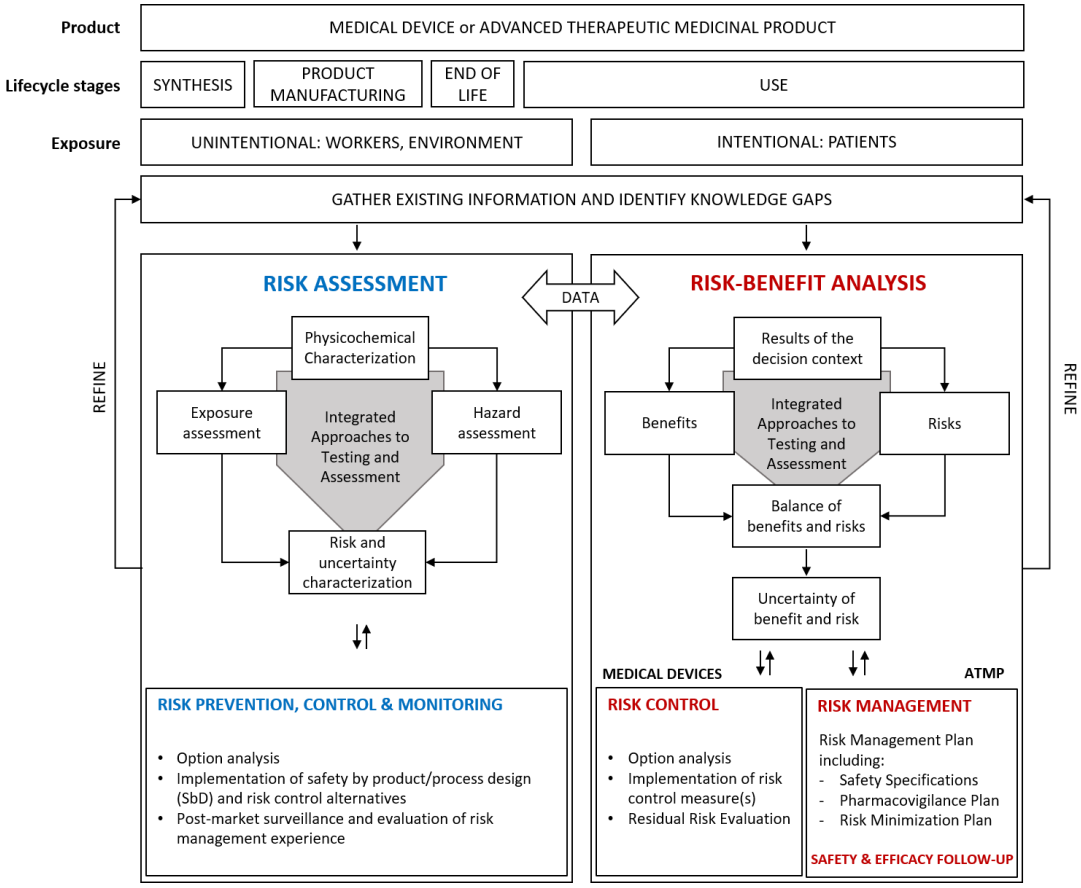

Figure 3. BIORIMA Risk Management Framework for NBMs used in MD and ATMP.

The framework starts with the choice of the type of product, i.e., a MD or an ATMP. The framework inputs include information on relevant life cycle stages of such products. Early life cycle stages such as synthesis and product manufacturing are most relevant in an occupational risk assessment, while during the use stage the risk assessment is relevant to both patients and workers (e.g., doctors, nurses, dentists). Following use, end-of life is most relevant to environmental risk assessment. The RMF provides two pillars to structure the risk assessment and management process: one relevant to occupational and environmental risks associated with unintentional exposure (left side), one relevant to the benefit-risk analysis of patients (right side).

The regulatory framework to address occupational health and safety and environmental risks of NBMs incorporated in MD and ATMP is built upon the provisions of REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) regulation, Environmental Health & Safety regulations and the associated guidance documents (e.g., REACH 2006 (European Commission 2006), documents supporting ATEX Directive 1999/92/CE (European Commission (EC) 2000)). For workers, both accidental and chronic risks need to be considered. In an

accident risk scenario, a potential hazard (e.g., explosion, fire, massive release) can impact a worker over a short period of time (an accident). This is different from the exposure frequency and duration paradigm used to estimate chronic risks and therefore requires a different approach. Once all health and environmental risks are quantified and evaluated, the outputs of the RMF include identification of appropriate approaches for risk prevention and control.

The right-side pillar of the RMF focuses on the use of a NBM-based MD or ATMP for diagnostic and/or therapeutic purposes, where the efficacy and the safety of these materials/products for the patients constitute the main concern and are assessed and weighed by means of benefit-risk analysis (European Medicines Agency (EMA) 2007). In order to obtain market authorization, benefits and risks are investigated through the execution of pre-clinical and clinical assessments, according to the current EU regulations for the commercial approval of MD and ATMP (e.g., Regulation (EU) 1394/2007 (European Commission 2007), Regulation (EU) 2017/74 (European Commission (EC) 2017), Directive 2009/120/EC (European Commission 2009)). Once on the market, adequate risk control measures must be considered for medical devices (following ISO 14971 (ISO 14971: 2019) provisions), while for ATMP current EU legislation asks for the implementation of a Risk Management Plan and a Safety and Efficacy Follow-up.

The two pillars are not disconnected. Instead, a flux of information and data is recommended in order to avoid duplication of efforts that can incur additional costs. For example, data on the intrinsic physicochemical properties of NBMs are relevant to any kind of appraisal of adverse effects or health benefits and, as such, once collected or generated they should be shared and exploited within both assessment processes regardless of the regulatory regime. The same holds true for the pre-clinical toxicological studies that are required for the hazard identification and the hazard characterisation of the NBMs.

In the paragraphs above, the RMF has been presented from a life cycle perspective, however it is worth considering that the strategies composing the framework can play different roles and can be applied at different levels of detail across the stages of the innovation process. The R&D phases of a medicinal product include the non-clinical discovery phase (identification of target and preliminary candidates, pre-clinical studies for the optimisation of candidates and selection of a drug candidate for clinical testing), the clinical phases (phase I, II, III) and the post-marketing pharmacovigilance (Cavero 2009; Curtin and Schulz 2011; European Commission 2017). In the early non-clinical discovery phase, industries typically rely on

existing data and/or less expensive screening-level assessments by means, for example, of *in silico* modelling or *in vitro* testing approaches. For instance, read-across data from similar materials or applications can be used to identify suitable candidates for further testing, or to remove potentially toxic materials from a candidate shortlist. Later, benefit-risk analysis methods are applied to support regulatory decisions about authorization of new MD or ATMP, when they are used to integrate data gathered through pre-clinical tests and clinical trials. In the post-marketing stage, benefit-risk analysis may be required again to weigh and integrate evidence provided by pharmacovigilance of new products. In general, approaches and methods used for risk assessment and management and benefit-risk analysis could be used in an iterative way along the innovation processes, providing at each stage new information which can support further development and assessment of the product and its production processes.

The conceptual strategies for the assessment and management of risks associated with NBMs used in MD and ATMP proposed in the BIORIMA RMF are described in detail in the following paragraphs.

3.2.1 Risk assessment strategy

Strategies for human health and environmental risk assessment of the NBMs from a life cycle perspective were adopted, considering the provisions of EU regulations, and using state-of-the-art scientific approaches for safety assessment of nanomaterials. These strategies are intended to support stakeholders (e.g., regulators, industries, consultants) in identifying and applying the most appropriate methods and tools (e.g., standards, testing protocols, predictive models) to assess potential risks associated with unintentional exposure of workers involved in manufacturing, use and disposal of nano-enabled MDs and ATMPs, as well as healthcare professionals exposed to NBMs while using the products. In addition, the strategy is aimed at guiding the identification of releases of NBMs into environmental compartments (i.e., air, soil, water, sediment) and the implementation of the most appropriate experimental and modelling tools to enable the assessment of their behaviour and fate (e.g., bio-persistence, bio-transformation) as well as their short and long-term toxicity effects for aquatic and terrestrial biota.

However, at several levels there is high complexity associated with the hazard assessment of NBMs, both for human risks as well as for the environment (Amorim et al. 2020; Jesus et al. 2019). Common issues are dealing with the physicochemical identity of the NBMs, the existence of different nanoforms and their transformations in physiological and environmental media (e.g., biocorona formation, weathering/ageing) (Krpetić et al. 2014; Ortelli et al. 2017; Soddu et al. 2020). In addition, it can be questioned which are the most relevant biomarkers for NBM hazard testing and whether they are currently adequately addressed in the OECD guidelines used for regulatory purposes.

Moreover, there are many possible human and environmental exposure scenarios that should be investigated. Therefore, a strategic approach is required to streamline, optimise and properly target the available resources using either an intelligent testing strategy (Stone et al. 2014), also known as an Integrated Approaches To Testing and Assessment (IATA) (OECD, 2016) specifically developed for NBMs used in MDs and ATMPs. Indeed, in order to optimise the selection of the most suitable methods and guide the identification and characterisation of human and ecological risks, the risk assessment strategy proposed in the BIORIMA RMF includes a set of IATA.

The IATA are also strongly influenced by the type of NBM to be investigated as well as the exposure route. The exposure/administration route and frequency determine important parameters such as relevant timepoints, dispersant/matrix and controls to be used in experimental testing. Each IATA is therefore tailored to address the physicochemical characteristics of the NBM, the likely route(s) of exposure or environmental compartments, the frequency of exposure and the needs of the risk assessor (e.g., regulator or developer during early innovation).

The BIORIMA IATAs follow the structure first recommended by the OECD (OECD, 2016). The IATAs take the form of decision trees, structured to collect the information and data needed for human health and ecological risk assessment of NBMs. Each question (or decision node) within the decision tree prompts the user to strategically select the most appropriate testing or alternative methods (or a combination) for hazard assessment. These include *in silico* (e.g., physiologically-based pharmaco-kinetic (PBPK) models), *in vitro*, *ex vivo*, and *in vivo* approaches. The IATAs have been developed to utilise, as far as possible, existing data (by utilising data in open-access databases), non-testing approaches (e.g., *in silico*), the use of abiotic chemical reactivity methods (i.e., *in chemico*) and other alternatives to animal testing

(e.g., *in vitro*). This is achieved by employing a tiered testing strategy to address each decision node, in which the complexity of the model employed increases from tier 1 to 3.

Before initiating any testing by means of the IATA, a review of the available data is required to identify areas of immediate concerns regarding the NBMs toxic potency and/or exposure potential. This includes information from clinical studies (preferably), then human relevant *in vitro* and *in silico* data, and lastly animal *in vivo* data. This analysis also allows a gap analysis of the hazard data relevant to that specific NBM. The missing data is then generated by employment of the IATA. For human hazard assessment, tier 1 focuses on *in silico*, *in chemico* and simple (one cell type monoculture) *in vitro* models, tier 2 includes more complex alternative approaches such as multi-cell lineage three dimensional co-cultures (e.g., organoids, organ-on-chip) and tier 3 is largely based on animal models including hazard and biodistribution testing. The *in vivo* data currently remains vital for the NBMs to progress to clinical trials, and also for their full occupational risk assessment. However, the results of tiers 1 and 2 are used to refine the animal studies in terms of the most relevant concentrations, timepoints and endpoints to assess, thereby reducing the number of animals used. The IATAs therefore optimise the cost of obtaining relevant information and data, while reducing the use of experimental animals in accordance with the 3Rs (Replacement, Reduction and Refinement) principles (Graham and Prescott 2015).

The tiers are useful since the nature and level of information needed to support product development decisions is different from the data needed for regulatory approval. Therefore, the type and extent of testing can be varied according to the actual purpose of the assessment.

3.2.1.1. Occupational risk assessment

In the European Union the occupational safety of NBMs is regulated by the Commission Regulation 2018/1881 (European Comission (EC) 2018b) which modified Annexes I, III, and VI-XII of the REACH regulation, introducing specific requirements and guidelines to cover nanoforms (The European Parliament and the Council 2006). These requirements are of course also applicable to the NBMs used in medicine. REACH requires Chemical Safety Assessment for each substance produced or imported in quantities above 10 tons per year, which is based on the traditional human health risk assessment paradigm. This involves a

series of assessment steps, namely detailed physicochemical characterization, exposure and hazard assessment, risk characterization and uncertainty analysis (van Leeuwen and Vermeire 2007).

The detailed characterisation of intrinsic physicochemical and extrinsic properties of the NBMs and medicinal products made thereof is essential to understand and predict their emissions/release and exposure, and to interpret the available toxicological data (Meißner, Potthoff, and Richter 2009). The European Chemicals Agency (ECHA) provided a recommended list of nanomaterial properties that should be measured as part of a Chemical Safety Assessment for nanomaterials (ECHA) 2016c and the characterization methods to measure physicochemical properties of manufactured nanomaterials (Gao and Lowry 2018) are generally applicable also to NBMs used in medicine.

Occupational exposure assessment starts with the identification of the possible sources of emissions/release for the activities and tasks performed by the workers or healthcare professionals, and the formulation of respective exposure scenarios that should include information on the substance, activities, route(s) of exposure, operational conditions, and risk management measures (RMMs). Exposure scenarios should be defined and assessed for the synthesis phase and downstream use when NBMs are incorporated into a MD or ATMP, during the use stage when the product is applied/administered to patients by medical professionals, as well as during waste recycling, incineration and/or disposal. Occupational exposure during manufacturing processes has been already investigated for a variety of nanomaterials and can be mostly negligible for medical NBMs if appropriate RMMs are implemented. However, for the use phase of NBM-based MD and ATMP realistic occupational exposure scenarios have been formulated in the BIORIMA project. Some relevant examples include dental and surgical procedures involving the milling, drilling, grinding and polishing of materials or implants, where composites may be a source of nanoparticle inhalation exposure for dentists and surgeons (Besinis et al. 2015; Van Landuyt et al. 2014; Wohlleben, W., Kuhlbusch, T. A. J., Schnekenburger, J., Lehr 2015). Table 2 presents a list of relevant activities which may expose workers to NBMs at different life cycle stages of the MD and ATMP.

Table 2. Examples of occupational exposure routes and targets for tasks/activities identified within life cycle of NBMs used in MD and ATMP.

Life cycle stages	**Tasks/activities**	**Exposure route**	**Target**
Synthesis of NBMs	Weighing operations Mixing operations Purification Collection and sorting Packing / Re-packing	Inhalation Dermal	Workers in medical research labs
Product manufacturing	Dissolution preparation Sampling Packing / Re-packing *In vitro* & *In vivo* testing Cleaning and maintenance Waste management Collection and sorting Packing / Re-packing	Inhalation Dermal	Workers in medical research labs Workers in pharma industry Facility maintenance staff
Use	Flask filling and mixing operation Syringe filling (1 – 60 mL) Maintenance of drug preparation devices Waste management	Inhalation Dermal	Health care workers Home healthcare workers Waste management workers
End of Life	Handling patient excreta Spills treatments Waste management	Inhalation Dermal	Health care workers Waste management workers

There is a variety of approaches to assess the exposure to NBMs for the formulated exposure scenarios, which involve direct measurements or modelling. Site-specific measured data are typically preferred over model estimates and are needed to validate and improve the exposure models. Such data can be generated by portable or stationary monitoring and sampling instruments (e.g. Condensation Particle Counter (CPC), Scanning Mobility Particle Sizer (SMPS), Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) or electron microscopes (SEM/TEM)) following different measurement strategies (Eastlake et al. 2016;

Organisation for Economic Co-operation and Development (OECD) 2015; Ramachandran, Park, and Raynor 2011). To adequately quantify occupational exposure a multimetric approach (covering different parameters such as particle number, mass and surface-area concentrations, particle mean diameter) using a combination of instruments is recommended (Kuhlbusch, Wijnhoven, and Haase 2018). In the absence of measurements, exposure models for NBMs can be adapted from such models for chemicals and engineered nanomaterials (e.g. NanoSafer, iEAT, Dermal Advanced REACH Tool – dART, Stoffenmanager Nano) (McNally et al. 2019; Riedmann, Gasic, and Vernez 2015; Spinazzè et al. 2019).

Exposure assessment also requires the quantification of the bioavailable fraction that passes across the mucosal barriers (i.e., lung and intestinal epithelia). This can be estimated through experimental *in vivo* or *in vitro* testing. Moreover, using Physiologically Based Pharmacokinetic models (PBPK) the results of rodent studies or data obtained from *in vitro* transport studies can be used to extrapolate a human internal exposure by simulating the absorption, distribution, metabolism, excretion of NBMs. Typically PBPK models require physiological (tissue volumes, flow rates, metabolism of chemicals, etc.), biochemical, and material specific data (i.e., physicochemical properties) (Li et al. 2017; Miller et al. 2019).

In general, hazard assessment of NBMs is carried out by gathering/generating and evaluating relevant physicochemical and toxicological information from *in vitro* and *in vivo* tests to assess the intrinsic hazard of a substance and to establish a dose-response relationship (Drasler et al. 2017; Dusinska et al. 2015). Toxicological approaches to assess hazards of NBMs can either be based on methods adopted from classical toxicology or on alternative methods, including *in vitro* and *in vivo* testing and *in silico* modelling (e.g., QSAR models, grouping and read-across methods).

In fact, systems biology approaches and other advanced methods are gaining traction in the field of nanosafety research (Fadeel et al. 2018), though regulatory acceptance is needed in order to implement such approaches in hazard assessment of NBMs for MDs and ATMPs. ECHA identified relevant toxicological endpoints of concern for NMs (e.g., cytotoxicity, inflammation, oxidative stress, genotoxicity), and a list of related appropriate *in vitro* and *in vivo* tests (e.g., *in vitro* mammalian cell gene mutation test, bacterial reverse mutation test) (OECD 2018; Park, M., VDZ., Lankveld, D., PK., van Loveren, H., de Jong, W. 2009). Furthermore, the adverse outcome pathway (AOP) framework provides pragmatic insights to promote the development of alternative testing strategies (Halappanavar et al. 2020). Several

EU-funded projects, not least in FP7 (e.g., SUN, MARINA, NANOMILE, NANoREG projects (Lynch 2017)), have developed and evaluated different *in vivo* and *in vitro* protocols for investigating the hazard of nanomaterials, in principle applicable also to NBMs. These efforts have addressed the importance of assessing *in vitro* assays with respect to material interference (Guadagnini et al. 2013). Furthermore, the importance of using multiple cell types to properly evaluate the toxicity of NMs has been demonstrated in the MARINA and NANOMILE projects, and high-throughput screening approaches have been developed (Hansjosten et al. 2018). It is important to point out that while there is a strong (scientific and societal) incentive to move towards an animal free testing and the development of alternative test methods is highly recommended, these assays nevertheless need to be validated. The current situation however is that there are only a very limited number of validated *in vitro* and *in silico* test methods that can be used in regulatory toxicology. The exception might be the availability of *in vitro* (and *ex vivo*) approaches to evaluate acute effect on the skin.

The importance of data management also needs to be underlined and efforts are being made to harmonize procedures for data collection and data warehousing (Fadeel 2018). The nano-bio community is currently debating the need for minimum information requirements when reporting research results, with the goal to improve reproducibility, increase quantitative comparisons of NMs, and facilitate meta analyses and *in silico* modelling (Faria et al. 2018).

3.2.1.2 Environmental risk assessment

The environmental risk assessment (ERA) of NBMs contained in MDs and ATMPs is generally based on the REACH Chemical Safety Assessment, but there are prominent differences compared to chemicals, e.g., a focus on the aquatic environment with limited number of test systems (EMA 2018; SCENIHR 2015). It is well known that NMs behave differently from "regular" chemicals, in regard to environmental fate, subsequent exposure, and mode of toxic action. Further, the ultimate fate of NMs is generally considered to be solid environments, like soils and sediments, rather than aquatic systems (Sun et al. 2016). Hence, a comprehensive ERA strategy for NBMs should consider these compartments. Additional to these, there should also be a focus on sludge born materials, as sewage sludge is in many countries applied to soils (Irizar et al. 2018; Kraas et al. 2017). The ecotoxicological approaches of choice to assess the hazard of NBMs can be derived from classical ecotoxicology (EMA

2018) or on newer alternative approaches that provide more adequate information (Amorim, Roca, and Scott-Fordsmand 2016), including longer term testing (Bicho et al. 2016, 2018; Goncalves et al. 2017; Mendes, Amorim, and Scott-Fordsmand 2018) or mechanistic endpoints (Gomes et al. 2018; Maria et al. 2018). The development of alternative test methods has been highly recommended, also by regulatory agencies. For example, in the context of REACH Regulation, ECHA and EFSA have proposed the use of omics data for risk assessment purposes (Authority et al. 2018).

The identified relevant endpoints of concern for NMs (i.e., chronic, longer term and mechanistic) and a list of appropriate testing methods (Amorim et al. 2016), should in principle also be applicable to NBMs, as long as the required adaptations are included as suggested by Hund-Rinke et al. (2016) and Amorim et al. (2018). For the latest update including the recommended adaptations to OECD guidelines for testing the environmental hazard of NBMs please see Amorim et al. (2020). To increase the efficiency of testing, these methods should be implemented onto IATAs, where grouping and read-across approaches are combined with testing methods and non-testing *in silico* models to generate data for ERA according to a tiered approach (Scott-Fordsmand et al. 2014, 2017; Semenzin et al. 2015). A wide number of *in silico* tools for NMs have been developed in recent years (Oksel, Ma, and Wang 2015; Scott-Fordsmand, Amorim, and Sørensen 2018) and can be applied to derive data of relevance for risk assessment. Moreover, omics-techniques, including key initiating events and pathway analyses, hold great potential (Bicho et al. 2016). These techniques are highly relevant for NBMs used in MD and ATMP, as they have been developed with a specific biological purpose and therefore prior information on their mode of toxic action is available. Further, *in vitro* approaches in environmental organisms have also gained increasing interest, as they allow for a quick identification of relative toxicity, of possible mode of action and of what happens with a NBM when in contact with environmental media and after uptake by organisms, e.g. corona formation (Hayashi et al. 2015). The latter will not only inform on possible mode of action, but also be highly relevant for supporting read-across between species. Furthermore, since MDs and ATMPs will obviously be, in most cases, in contact with human tissues, excreted NBMs will have a biological corona formed that will influence their behaviour and toxicity, and therefore their environmental risks.

As for the *in situ* exposure, there are advanced exposure models being developed, but these require refinement and validation especially for NBMs released from MDs and ATMPs.

Therefore, the bioaccumulation/trophic transfer potential is not well understood for nanomaterials and hence also not for NBMs used in medicine. Although substantial research efforts have focused on bioaccumulation (Petersen et al. 2019), there are currently no good models available, partly because nanomaterials are difficult to detect in complex media, partly because nanomaterials are not assumed to follow equilibrium paradigms, which hampers the estimation of bioaccumulation factors.

Since NBMs are synthesised and the MDs and ATMPs are produced in controlled environments, where any released NBM is adequately managed and waste is properly dealt with, it can be excluded that NBMs will reach the natural environment during synthesis and formulation, hence the life cycle stages of concern are the use and end-of-life processing and disposal. In these life cycle stages, predicted environmental concentrations (PEC) need to be estimated for relevant exposure scenarios. The detection of NBMs at trace concentrations in natural samples is in most cases not yet possible as the available analytical tools are not capable of distinguishing the NMs from natural background nanoparticles at the low NBMs concentrations expected in complex environmental matrices (Montaño et al. 2014; Navratilova et al. 2015). Therefore, the exposure assessment of these materials relies on environmental exposure modelling by means of Material Flow Analysis (MFA) to predict releases from products, fate in technical systems and final release to the environment, and Environmental Fate Models (EFM) that describe the fate of NBMs in the environment and their distribution within environmental compartments (Nowack 2017).

Several MFAs have been conducted for ENMs such as Ti_2O, Ag, ZnO, SiO_2, Al_2O_3, quantum dots, iron oxides, carbon nanotubes, fullerenes, using static or dynamic models assessing accumulation of ENMs in environmental compartments over several years (Adam, Caballero-Guzman, and Nowack 2018; Gottschalk, Scholz, and Nowack 2010; Sun et al. 2014, 2017; Wang et al. 2016; Wang and Nowack 2018). So far, only two studies are available that modelled the flows of NBM to the environment. Mahapatra et al. (2015) investigated the flows of nano-gold from medical applications in the United Kingdom and the United States using a bottom-up approach for the calculation of the prospective maximal consumption. Using the same approach, Arvidsson et al. (2011) calculated the environmental release of nano-silver from wound dressings in Europe in a worst-case scenario.

Before existing models for ENMs can be used for NBMs, several adjustments need to be made. In general, these models are also applicable to NBM as most of the parameters are based on

the applications of the materials and are not particle-specific properties. A few parameters on fate in technical systems are specific to the type of particles and need to be adjusted to the type of NBM. As NBMs are mostly applied in a hospital setting, waste and wastewater from hospitals need to be included. Health care waste is treated differently to municipal waste due to their often-hazardous character. This means that the flows to alternative treatment or hazardous waste incinerators need to be included in the MFA model. NBMs can also be applied inside the body and stay there until the patient's death, e.g., from their use in implants. Thus, the inclusion of crematoria or burial in cemeteries in the model is necessary. A generic model for the flows of NBM through all life-cycle stages is shown in Figure 4 (Hauser and Nowack 2021). The main release point is the use of the NBM in a hospital setting, with releases to waste treatment and wastewater treatment specific for the use of the investigated NBM. Depending on the type of materials (organic/ inorganic), transformation can occur in several compartments such as wastewater treatment or waste incineration.

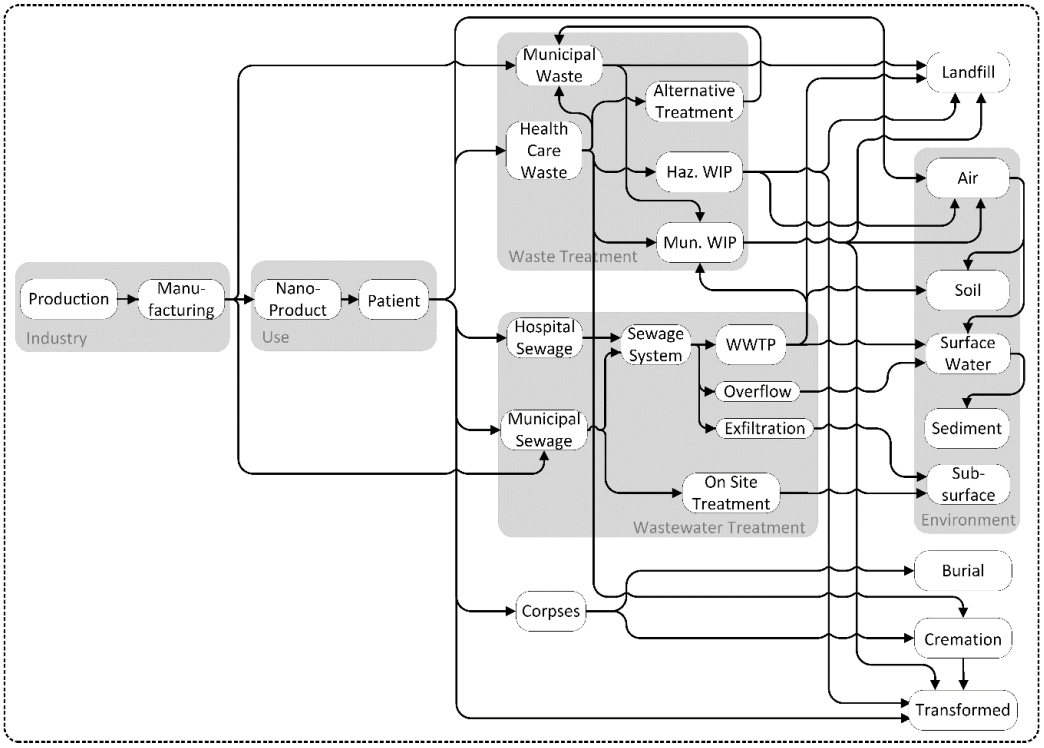

Figure 4. Material flow diagram for NBM from production to the intended use in patients, the wastewater and waste treatment specific to the use in hospitals and finally to the environment (adapted from Hauser and Nowack 2021).

Using the environmental flow data from the MFA modelling, worst-case PECs that exclude any fate processes in the environment can be directly calculated. These values have also been referred to as “release concentrations” as they do not include processes such as sedimentation or biodegradation that would decrease the environmental concentrations of the NBM. In order to consider these fate processes, EFM models developed for ENM need to be adjusted and parameterized for NBM. Such fate models are for example SimpleBox4Nano (Meesters et al. 2014) or MendNano (Liu and Cohen 2012). Whereas the processes that need to be considered in the fate models for NBM are the same as those for ENM, there is so far almost no data available to parameterize models for NBM. With an increased harmonization of test protocols and test media (Geitner et al. 2020) and the use of functional assays (Hendren et al. 2015), also data for NBM can be obtained so that fate models can be parameterized.

The PEC values derived from MFA or EFM models can then be compared to predicted no-effect concentrations (PNECs) derived from hazard assessments. Hauser et al. (2019) conducted a first environmental hazard assessment of organic and inorganic NBMs used for drug delivery based on a meta-analysis of previous ecotoxicity studies. Data are only available for a small subset of NBM, mainly for chitosan, polyacrylonitrile and hydroxyapatite. Mahapatra et al. (2015) conducted the first full environmental risk assessment of an NBM, focusing on the use of nano-gold in medicine. Based on PEC values derived from MFA and a probabilistic species sensitivity distribution, it could be shown that there is no overlap between predicted exposure of nano-Au and the concentrations where adverse effects on organisms can be observed.

3.2.2. Strategy for risk prevention and control

Once all risks along NBM life cycle have been assessed, adequate measures to avoid or limit/control these risks must be identified to ensure safer production, handling, and disposal of NBMs. The traditional risk management of chemical substances applies to the NBMs used in medical sector. It relies on the implementation of safe by design and risk reduction and control measures based on the hierarchy of controls (e.g., NIOSH 2013), following the so-called STOP principle: Substitution, Technical measures, Organisational measures and

Personal protection measures which can be applied throughout the life cycle of a specific material.

3.2.2.1. Safe by material and process design

The elimination or substitution of hazardous constituents by means of safe by material design (SbMD) strategies is the first line of defence (Brunelli et al. 2016; Costa 2016; Le et al. 2016; Wang et al. 2014; Wilkins et al. n.d.). The SbMD approach is based on the control of nano-bio reactivity since the early stages of R&D. In a recent review Hjorth et al. (2017) (Hjorth et al. 2017) clearly demonstrated how the SbMD approach is inspired by safety testing and assessment practices in drug discovery and development (DDD). The authors also outlined the limitations that still delay the creation of "design guidelines" for nanomaterials. The SbMD approach is based on the concept that safety is not an intrinsic property of material but can be built in through the manufacturing chain from raw materials to finished products, by adding SbMD criteria to Quality Assurance (QA) and Good Manufacturing Practice (GMP) specifications.

The context and logic of implementing the SbMD idea for nanomaterials have been described in the "Safe-by-Design (SbD) Implementation Concept" of the EU FP7 ProSafe project (Höhener, Höck, and Lehmann 2016). This idea builds on the SbD concept of the EU FP7 NANoREG project and the Safe Innovation Approach (SIA) of the EU H2020 NANoReg2 project. The main outputs expected by the implementation of a SbMD approach are decision criteria for selecting safer options in the early R&D steps. This requires adoption of screening-level approaches from predictive toxicology (*in vitro* and *in silico* tools) in order to speed-up the process and decrease the costs of generating material safety profiles, while taking quality and performance requirements into consideration. Indeed, as pointed out in a previous EASAC-JRC Report, 2011 (Joint EASAC-JRC Report 2011) on benefit *versus* risk of nanomaterials: "Successful innovation, if it is to encompass both regulatory and consumer approval, must incorporate safety by design."

Overall, the SbMD approach addresses safety issues at the early design stage of nano-enabled products. These issues should be formally assessed at the appropriate "Design Reviews", mandatory for EMA and Food and Drug Administration (FDA) compliance and "Best Practice" for new product developments. Nevertheless, safety is not an intrinsic property of materials,

and the goal of safer materials can only be achieved if predictive risk assessment tools are also available that are robust and easily implementable to guide material selection and product design. These concepts are not new in both a) drug discovery and developments and b) ATMP where early *in vitro* or *in silico* screening is used as part of an overall risk reduction or risk mitigation strategy. It is expected that they can be successfully introduced into NBMs manufacturing processes prior to addressing detailed toxicological testing and regulation. Within the hierarchy of controls, SbMD can be allocated at the substitution level where a more hazardous material is replaced by a less hazardous material.

The fundamentals for safety by process design (SbPD) lay in the evolution of engineering principles initially developed in the mid-1990s for the chemical industry for manufacturing nanotitanium dioxide (Besson, J. P., King, P. W. B., Wilkins, T. A., McIvor, M. C., Everall 1996) and integrated into pharmaceutical industry manufacturing (U.S. Food and Drug Administration (FDA) 2004). A holistic approach is adopted which starts with standard medical product GMP and QA procedures. Safety with respect to nanomaterials is built in right through the manufacturing chain from raw materials to finished products. SbPD seeks to maintain the much safer properties of the previously optimized raw materials by SbMD methods throughout the production process by optimization of all processing steps, with their respective production quantities.

The research and development processes for *in vivo* medical products deploying nano- and bio- technologies differ for products that are made by materials processing (e.g., drugs and *in vivo* imaging agents) and by discrete product fabrication (e.g., implanted joints or devices).

SbD of medical products made by materials processing is built on the principles of the US Food and Drugs Agency (FDA) regulatory guidelines of 2003 and adopted by EMA in 2003 as described by Brenderlberger 2003.

Since 2003, pharmaceutical companies have adopted the FDA scheme for Process Analytical Control (PAC) of active pharmaceutical ingredients (APIs). To control complex batch or continuous processes to manufacture API, a large number of inputs from low-functionality process sensors (e.g., pressure, temperature, flow, level and mass) is collected. These are linked to electrical controllers in nested hierarchical systems which may contain several distributed control systems coordinator control systems and in the case of very large manufacturing plants, super coordinator control systems.

In PAC strategies it is essential to have specific high-information content analysers on-line at critical stages in the process. These are coupled to closed-loop control systems. Such analysers might be Fourier transform infrared spectroscopy (FTIR), Raman and IR spectrometers, or Mass Spectrometers. They could also involve process tomography or automated sample and flow injection analysis through "lab-on-a chip" biosensor devices or particle sizers. During the pilot plant development stage, the process parameters (P, T, F, L & M) together with reagent quantities and additives, are varied to enable the process to be optimised simultaneously for product yield, quality, and process profitability. Importantly, *in silico* methods (e.g., nano-QSARs) are included in these calculations to minimise risks. Once optimised and tested by multivariate process modelling for the full-scale plant, all manufacturing specifications and control set points are then fixed. This guarantees that both "pilot" and "full scale" process plants will produce products of identical quality. These approaches collectively form the core part of safe by process design. It allows batches of products to be prepared for: i) safety ("*first in man*" trials); ii) clinical trials and iii) the EMA/FDA regulatory claims support file. This work can continue whilst the full-scale plant is being built and commissioned, thus shortening the time to market but guaranteeing the best possible products within a process intensification perspective (Babi, Cruz, and Gni 2016; Strube et al. 2018).

To achieve "safe-by-design" of products, additional characterisation and measurements tests and *in vitro* toxicology tests are added into the raw materials and pilot plant development stages (shown in green in Figure 5). These can be performed off-line or at-line using high-throughput parallel processing analysers including electron microscopy. On-line analysers are chosen for the pilot scale to enable full process analytical and product quality control. The results are analysed by nano-QSARs data analytics (Gajewicz et al. 2018; Oksel 2016; Tantra et al. 2015) and critical characterisation and measurement parameters together with those from the high information content on-line analysers, e.g., Raman, FTIR, mass spectrometers(Besson et al. 1997) to build multivariate statistical process control models. Together these devices are then used to maintain optimised SbD performance throughout the whole manufacturing chain from raw materials to full scale manufacturing.

Safe by Design for discrete object fabricated products begins with the same paradigm illustrated above for products that are made by materials processing. A significant range of materials must be tested at the raw materials stage to minimise product toxicology

downstream. The approach differs at the pilot plant stage where initially material "test coupons" are produced on prototype fabrication unit operations. The latter are small but mimic the automated device used in the full manufacturing plant. Critical in this early stage is to test the biocompatibility of the materials to be used for 'implantability' of such devices. Once suitable materials have been chosen, the implantable device geometry and performance characteristic are mathematically modelled, and the first manufacturing devices are engineered. Full scale manufacturing for fabricated products is achieved by "scale out" rather than "scale up". The imperative is to ensure the unit operations are fully optimised. These are then replicated to create a manufacturing line with banks of identical unit operations accurately producing the product in parallel to a full set of specifications, including NBM hazard reduction. Using nano-enabled SbD replacement hip joints, this generic SbD process development has been described for the first time in the BIORIMA project by Wilkins and colleagues (Wilkins et al. n.d.) and is illustrated in Figure 5 below.

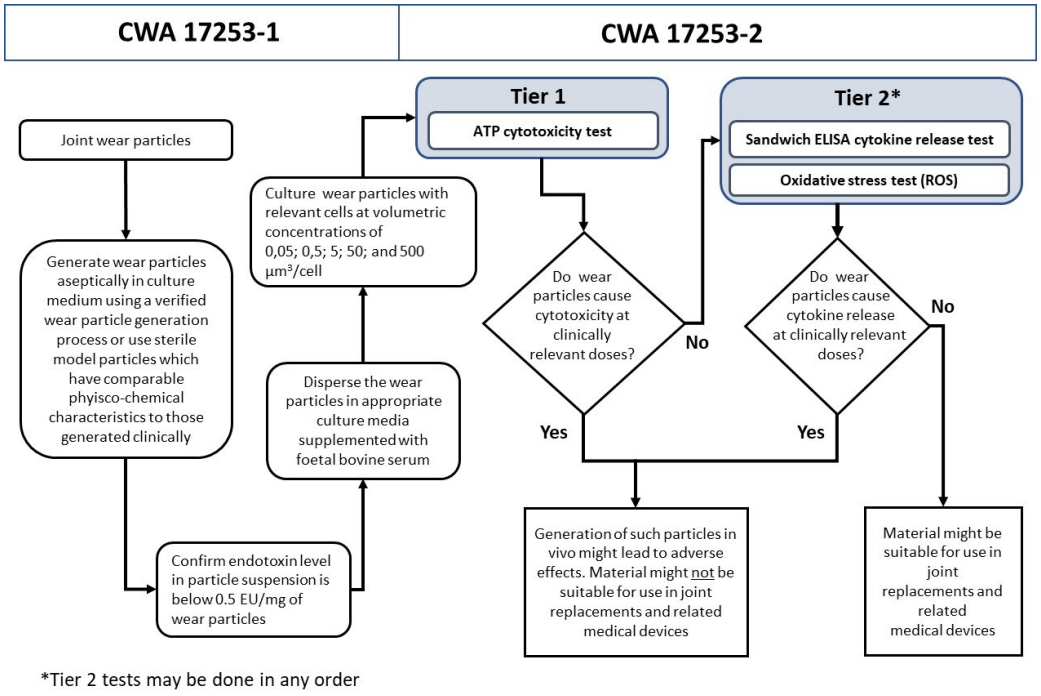

Figure 5. Tiered toolkit flow diagram for evaluating the biological impact of polyethylene wear particles from joint replacements and related medical devices.

As part of the manufacturing fabrication development, product performance is assessed by simulation and accelerated mechanical testing for *in vivo* use. In addition, these authors noted

that international standards (ISO, CEN, etc.) addressed only the risks of bulk materials of devices and not the risk of NBM used in the devices or created during lifetime wear. They have set a benchmark for standards (CWA: 17253-1 and CWA: 17253-2) respectively for NBM wear characterisation and toxicology testing, to be applied to all nano-enabled replacement joint devices.

The above same principles can also be applied to personalised medicine applications such as patient-specific 3D printed implants, containing nanohydroxyapatite to replace bone loss, following maxillary facial surgery to remove bone affected by cancer (Vazquez-Vazquez et al. 2019).

3.2.2.2 Risk reduction & control

In many cases it is not possible to substitute a specific substance and/or process and thus the further measures have to be applied during production and use. For risk reduction and control during the production phase, first of all targeted and well-defined technical measures (engineering controls applying closed processes, fume hoods, enclosed glove boxes, etc.) have to be applied (NIOSH 2014). These must be process and material specific and should be supported by on-site measures that may decline or support an initial suspicion of material release. If a measurement does not reveal any release during normal processing (e.g., higher concentration than the background and meeting the legal restrictions) no further acute action is needed. Nevertheless, the technical measures should be conceived as far as possible to also cover accidental scenarios. Organisational measures include administrative controls like operational procedures such as HEPA-filtered vacuum cleaning, regular wet wiping of surfaces and equipment but also periodical checks on the effectiveness of these procedures and training of the involved personnel. Further risk reduction and control is achieved by (additionally) using personal protective measures, that is equipment like eye protection, gloves, and respirators with different adjusted protection levels. Furthermore, discontinuous control measures as well as a continuous exposure monitoring on site (see the next paragraph) with appropriate measurement equipment for identification and quantification might be needed.

The above-described hierarchy of controls is also applicable during the use phase, at least for professionals working with the NBMs. It then has to be modified to cope for the material and

use specific properties in case of medical devices (e.g., scaffolds, implants) or the direct application of materials (e.g., food supplements, medicine, etc.): i) engineering controls can minimise abrasion, dissolution, and local exhaust during mechanical treatments; ii) administrative control can involve operational procedures for preparation and application of ATMP, specific cleaning procedures, waste handling, training of involved personnel; iii) personal protective equipment can involve the use of gloves and face masks by healthcare staff, while for patients use of tissue barriers, direct removal of debris, etc. In addition, health surveillance (continuous medical supervision) might be needed. Employees working with NBMs should be informed, trained, and supervised regularly.

3.2.3. Benefit-risk analysis for managing risks for patients

Innovative MD and ATMP containing NBMs could address current unmet therapeutic or diagnostic needs, however they may be developed, manufactured, and used in completely new ways compared with conventional medicinal technologies and this can challenge their market authorization. Therefore, to properly support the translation of NBMs used in MD and ATMP into clinical use, careful assessment of their benefit-risk balance is required (EMA, 2015) and this task is covered by the benefit-risk analysis component of the RMF (right-side pillar, cf. Figure 3).

When assessing the use of NBMs in ATMP and MD, it is important to underline that they can pose specific regulatory challenges related to their inherently complex nature and their relative novelty in the medical field. These materials involve complex nanostructures that can possibly trigger a wide range of biological responses. The broadly adopted “conventional” approaches for physicochemical and toxicological testing of medicinal products have been accepted also for nanoparticle medicinal products (Boisseau and Loubaton 2011). However, it has been acknowledged that these methods are not yet fully adapted to address the inherent complexity of these nano-bio systems, which raises concerns about how reliable is the current dataset for regulation (Boisseau and Loubaton 2011).

In simplified terms benefit-risk analysis is performed to answer the question - do the benefits of a NBM outweigh the risks to the target individual or population and are the uncertainties reasonably low (European Medicines Agency (EMA) 2010)? The answer to this question is important for both the industry developing these NBMs and the regulatory authorities.

Benefit-risk analysis is generally performed when applying for market approval for a medicine following clinical trials, however it has been recognised that the approach should be performed throughout the whole R&D phases of the medicinal product, including the non-clinical discovery phase, the clinical phases (phase I, II, III) and the post-marketing pharmacovigilance (Cavero 2009; Curtin and Schulz 2011).

In order to carry out a benefit-risk analysis according to regulatory requirements, a review of the scientific data should be performed, taking into account both benefits and risks of a given NBM for the target population (EMA 2011; Hughes et al. 2013). Figure 3 (right side) describes this task and depicts the main steps of a benefit-risk analysis.

First the results of the decision context, encompassing the analysis of the therapeutic context, the available comparators, the horizon and the stakeholder perspectives (Coplan et al. 2011) provide suitable information to properly identify the benefits and risks associated to the specific treatment. Benefits and risks are then weighted and compared in order to evaluate if the benefits outweigh risks. When considering benefit-risk analysis, it is important to have consistent definitions of the related terms. Here “benefit” relates to a favourable outcome (e.g., increased efficacy) of a given medical application, while “risk” is used to denote adverse effects defined by severity and probability of occurrence (EMA 2008b; Hughes et al. 2013). In contrast to occupational or environmental risks, which are calculated as absolute quotients that are strictly acceptable or non-acceptable, the risks from MD or ATMP are always relative to the expected therapeutic benefits and to the potential consequences the specific health problem can bring to the patients (e.g., death, impairment). Therefore, for medical applications such as MD and ATMP, the estimation of the dose-response relationship of possible adverse effects needs to be coupled with a benefit-risk analysis, which considers additional criteria such as the nature and severity of the disease to be treated, the possible benefits of the treatment to the patient, and the levels of risk acceptance on both the community (societal) and patient (personal) levels. For both benefit and risk, it is recommended that uncertainties such as variation, methodological flaws or deficiencies unsettled issues, limitations of the data set be considered during benefit-risk analysis (U.S. FDA 2018). IATA, as already discussed in Section 3.2.1, can support the analysis of existing information available along the product development phases, with the aim of guiding the selection of the most suitable and effective tests to provide the information needed to perform an effective benefit-risk analysis.

Benefit-risk analysis can be adapted considering the R&D phase of the MD or ATMP. Indeed, risks can be detected in non-clinical phase and continue throughout the development of the MD or ATMP in order to prevent and minimise risks when possible (EMA 2008a).

In the non-clinical discovery phase, also known as Go/No-Go decision, a MD or ATMP containing NBMs needs to pass through several steps, which include determination of drug availability, absorption, distribution, metabolism and elimination and preliminary studies to investigate safety aspects such as genotoxicity, mutagenicity, safety pharmacology and general toxicology (Andrade et al. 2016). Moreover, in this phase, the application of *in silico* and *in vitro* tests, complemented by *ex vivo* and *in vivo* assays (if necessary) can help to recognise safety/toxicity issues early in the process to correct those prior to the final selection of clinical candidates (Cavero 2009). In the pre-clinical phase, information of the disease obtained from animal models are compared with data from toxicological studies to determine whether (or not) a candidate type of NBM can be administered for the first time in humans (Curtin and Schulz 2011). In this phase, hypothetical benefits are assessed based on current understanding of the mode of action for the NBM identified in animal or *in vitro* tests, along with a nonclinical and *in vitro* safety evaluation (Sashegyi, Felli, and Noel 2014). During the clinical development, the registration process and the marketing period, benefit-risk analysis evolves identifying potential efficacy and safety endpoints and other surrogates as well as a more precise safety profile and the identification of adverse effects.

According to the ATMP guideline (EMA 2008a), after the development of a benefit-risk analysis, the applicant should provide a Risk Management Plan (RMP) for obtaining the market authorisation. In the RMP, safety specification, pharmacovigilance plan and risk minimisation activities need to be assessed. Safety specifications consists in the identification of risks to be minimised and/or characterised during the post-marketing phase considering risks derived from the product manufacturing, handling, application, and clinical follow-up (i.e., risks to patients due to interaction with other medicinal products or maladministration and risks to healthcare professionals). Pharmacovigilance activities consist in the identification, quantification, and characterization of safety hazard and the measurement of effectiveness of risk-management measures, while the risk minimisation plan includes risk minimisation measures such as supplement information about conditioning of the patient, product characteristics, adverse drug reactions, health care professional protection measures. Safety and Efficacy (S&E) follow-up data need to be provided to support the

marketing authorisation application considering the ATMP characteristics and its intended indication, while long-term S&E follow-up activities are only related to ATMP.

After the clinical studies of a MD, a risk management file must be developed for the post-market surveillance, including results from the benefit-risk analysis and risk minimisation measures. The risk management file should contain definitions of possible hazardous situation associated to the use of the investigated MD and all the possible applicable risk minimisation measures for patients as well as healthcare personnel. This includes the risk analysis, risk evaluation, the implementation and verification of the risk control measures and the final assessment of the acceptability of residual risks (Pane et al. 2019).

In 2009, EMA established a 3-year research program whose purpose was "to develop and test tools and processes for balancing multiple benefits and risks as an aid to informed regulatory decisions about medicinal products" (Sashegyi et al. 2014). According to the results of this study, the prominent form of benefit-risk analysis framework is multicriteria decision analysis (MCDA) (Mt-Isa et al. 2011), although these have been mostly academic to date. The EMA has participated in the development of MCDA frameworks such as PrOACT-URL (Problem formulation, Objectives, Alternatives Consequences, Trade-offs, Uncertainties, Risk tolerance) (EMA 2010). Additionally, the pharmaceutical industry has also developed benefit-risk assessment such as the PhRMA BRAT framework (Pharmaceutical Research and Manufacturers of America, the Benefit-Risk Action Team), a six step process that focuses on documenting rationale for decisions (Coplan et al. 2011). However, ideally a systematic, transparent, and structured regulatory decision-making process is required that is of use to all stakeholders. The development of the UMBRA (Universal Methodology for Benefit-Risk Assessment) framework makes steps towards this structured regulatory decision making process as it incorporates several frameworks (PrOACT-URL, PhRMA BRAT and FDA 5-step framework) (Walker and Mcauslane 2016). The UMBRA framework uses benefit-risk summary template and corresponding user manual to clearly communicate benefit-risk analysis to all stakeholders and upon review was found to be of value by several regulatory agencies (Leong, Walker, and Salek 2015; McAuslane et al. 2017).

As far as MD are concerned, the use of relevant harmonised standards are required to demonstrate conformity with the general safety and performance requirements and other legal requirements, such as those relating to quality and risk management (Geremia 2018; International Organization for Standardization (ISO) 2015, 2016). A benefit-risk ratio needs to

be estimated, which requires that all known and foreseeable risks shall be minimized and weighed against the evaluated benefits to the patient and/or user of the MD during normal conditions of use.

However, as pointed out by Halamoda-Kenzaoui et al. 2019, for some endpoints such as drug release/loading and the interaction of nanomedicines with the immune system no standards are available so far. This creates a potential Catch-22 situation inasmuch as the anticipation of standardization needs require a good understanding on the regulatory information for nanomedicines while, on the other hand, robust datasets allowing firm conclusions in regard to regulatory demands are not yet available (Halamoda-Kenzaoui et al. 2019). Efforts are currently being made across several EU-funded projects including BIORIMA to develop robust test methods for hazard assessment of NBMs to set the stage for standardization of NBMs in MD and ATMP while the REFINE project recently issued a report to highlight regulatory needs in nanomedicine (Halamoda-Kenzaoui et al. 2019).

To properly identify, assess and manage risks, ECHA along with the FDA, Health Canada, Australia Therapeutic Goods Administration, Japan Ministry of Health, Labour and Welfare (MHLW) endorsed ISO 14971:2007(ISO 2007). ISO 14971 applies only to manufacturers placing MD on the market in Europe, since it introduced three new annexes (ZA, ZB, and ZC) specifically developed to align with the EU MD directives. It is expected that revised versions of these annexes will be soon available to include the requirements of the new EU Medical Device Regulation (European Commission 2017).

3.3 Perspectives on the implementation of the Risk Management Framework

The BIORIMA RMF has been developed to be flexible and efficient (Bos et al. 2015). It is flexible enough to address different assessment goals depending on user needs. It is efficient in collecting information for risk assessment based on specific user goals (i.e., targeted testing) by means of optimal IATA, instead of fulfilling predefined data requirements. This is intended to ensure an optimal balance between compiling the data needed for a targeted

and accurate risk assessment and for selecting adequate risk control measures, and the efforts and cost required to collect these data.

In addition to the safety for patients, the occupational and ecological risks from NBMs used in medical applications need to be thoroughly assessed and managed. For example, the extensive use of nano-Ag in biomedical applications (e.g., wound dressing, catheters) is motivated by the increased antimicrobial activity if compared to the bulk form, but toxicity and inflammatory response in humans need to be controlled, and the possible contribution to silver resistance in bacteria in the long term raises concerns (Seltenrich 2013).

If the assessment of risks for different targets can be considered adequately addressed through distinct regulations, there is a need for a scientific framework and guidance on the best experimental and modelling approaches to do it. For instance, in absence of sufficient environmental monitoring data, the application of Material Flow Modelling could help investigating the expected concentrations of nano-Ag in different environmental compartment based on average production data(Hauser and Nowack 2021).

The RMF aims to help industry in the fulfilment of regulatory requirements and, at the same time, in the development of safer NBM applications while retaining their efficacy, performance and quality, so that they can successfully enter the market. It will provide guidance to identify the specific regulatory requirements in each step of the supply chains of these products and will suggest how to address them by means of appropriate safety testing and assessment strategies based on the state-of-the-art scientific knowledge.

It should be recognised that the benefit-risk analysis process required for the market authorization of a new MD or ATMP, and the process of assessing occupational and environmental risks (e.g., REACH, EHS regulations) are inherently different and require distinct strategies. This is not only because of the different regulatory regimes, but also because the very concept of “risk” and its perception and acceptability are different in these two areas. The risks for workers and the environment are determined by unintentional exposure to the materials, they are absolute in nature, assessed based on conservative assumptions and any risks above the exposure/hazard risk ratio are considered unacceptable. In contrast, the risks for patient posed by medical applications are always compared to their clinical benefits and can be accepted if the benefits significantly outweigh the safety concerns. These fundamental differences have determined different obligations in the respective regulations that require different testing strategies. However, some of the (standard) testing

methods, modelling tools and data can be used across these domains. The implementation of the RMF can facilitate this by identifying areas of cross-fertilisation to promote sharing of ideas, data and tools. In this sense, the RMF can promote and facilitate the communication and collaboration between scientists from different fields.

One important area of cross-fertilisation between the assessment of risks for workers, patients or the environment is physicochemical characterization. In this area the RMF will offer guidelines for testing of both intrinsic and extrinsic properties as the relevant standards are still in early stages of development (OECD 2019) while this is crucial baseline information on how to proceed with the evaluation of potential risks.

Moreover, guidelines are needed also to integrate the outcomes of physicochemical characterization with (eco)toxicological assessment. Therefore, the RMF provides such guidance in the form of a set of structured IATAs, presented as decision trees to guide the selection of *in vitro* and *in vivo* tests based on material identity. The outlook is to deliver IATA through a software-based BIORIMA Decision Support System for risk assessment and management of NBMs used in MDs and ATMPs, that is freely accessible on-line to end-users. Moreover, the RMF has been also implemented by the BIORIMA DSS through the development of a risk assessment and control described in chapter 4.

The adoption of a life cycle approach in the assessment of occupational and environmental risks is a key element as in the formulation and use stages significant exposure of workers and healthcare staff could occur if there are no adequate risk management measures in place.

For example, the use of hydroxyapatite-based dental composite in the dental sector requires the development of tailored occupational exposure scenario. This paste can be grinded, polished or shaped during the application by the dentist, who could be exposed to airborne nanoparticles if not adequately protected (Van Landuyt et al. 2014). This and similar exposure scenarios involving processing of artificial joints during replacement surgeries are of potentially high concern and their possible risks should be investigated, especially in the case the relevant exposure routes are different from those already assessed for intentional administration/use of the products by patients.

Healthcare professional can therefore benefit from the implementation of the RMF in terms of increased awareness about potential occupational risks and improved knowledge of adequate prevention and control measures. To develop an effective risk prevention and control strategy it is important to clearly define the target groups of concern for each specific

intended use of the NBM (e.g., doctors, dentists, surgeons, nurses) and train them in working with these substances and using the relevant safety measures, especially in view of personal protective equipment, which is only effective if properly used. The known level toxicity of the potentially released NBMs is a key starting point to determine the needed level of controls. Moreover, such a strategy should be based on reliable onsite measurements of emissions/release and exposure. Even though some processes might be evaluated by lab-scale simulations, the complexity and maybe also uniqueness of a specific workplace requires exposure measurements based on a mixture of personal and area sampling using appropriate equipment and including background measurements. These exposure measurements can then provide guidance for the evaluation of risk management measures and their effectiveness, which should consider specific characteristics of the health care sector, such as the transfer of NBMs from high security areas to uncontrolled areas or waste management practices where day-by-day exposure to NBMs is possible to occur. The effectiveness of protection measures already in place in hospitals (e.g., for handling of cancer treatment medicine) should be carefully evaluated and similar measures should also be adopted for NBM-based MDs and ATMPs. In other words, a read-across from the handling of other hazardous substances like anti-cancer drugs, biological agents and/or radioactive materials can be beneficial to set the frame for handling of potentially less toxicologically potent NBMs.
In the use phase, to ensure patient safety, benefit-risk analysis should be performed when progressing to *in vivo* pre-clinical and clinical trials and should be benchmarked against current treatment for the same disease. Such an analysis could also be performed in the pre-commercial steps, during the early stages of the innovation process when new NBM-enabled applications are developed and there is the need to understand the balance of their anticipated clinical benefits and possible safety implications.
In this context, the RMF could also represent a useful tool to improve the communication to patients about evidence on risks and benefits and how they are weighted and judged. There is indeed a growing awareness of the role that active patients and public participation can play in improving decision making on health technologies, that would eventually help improving adherence to treatments (Mühlbacher et al. 2016).

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Chapter 4

Occupational risk of nano-biomaterials: assessment of nano-enabled magnetite contrast agent using the BIORIMA Decision Support System

Contents included in:

Cazzagon V., Giubilato E.*, Pizzol L., Ravagli C., Doumett S., Baldi G., Blosi M., Brunelli A., Fito C., Huertas F., Marcomini A., Semenzin E., Zabeo A., Zanoni I., Hristozov D.* (2022). *Occupational risk of nano-biomaterials: assessment of nano-enabled magnetite contrast agent using the BIORIMA Decision Support System.* NanoImpact, 25, 100373. https://doi.org/10.1016/j.impact.2021.100373

Specific contribution of the PhD candidate:

The work performed within the PhD thesis included:

- the methodological design of the study.
- a literature review on occupational risk assessment of NMs and NBMs, with a focus on exposure assessment models and monitoring techniques.
- the design and administration of the questionnaire for healthcare personnel and data treatment.
- the organization and implementation of the monitoring campaign at the production site.
- the collection, critical review, and selection of all data needed for the risk assessment.
- the testing of the first version of the occupational risk assessment module of the BIORIMA DSS.

Information on physico-chemical characteristics of the investigated material was provided by project partners and the software implementation and refinement of the BIORIMA DSS was carried out by project partners.

4.1 Introduction

In the RMF presented in chapter 3 emerges the need to develop methodologies and approaches specific for the evaluation of risks of NBMs. The established regulatory risk assessment paradigm for chemicals can be applied to assess the risks of NMs (Dekkers et al., 2016; ECHA, 2016; ECHA 2014; Grieger et al., 2019; SCENIHR, 2009a; Hristozov et al., 2016, 2012 ;OECD, 2012) and, considering that the NBMs used in the medical sector are a special category of NMs, nano-specific approaches can be applied also to the risk assessment of these materials. However, specific considerations are needed for the assessment of risks, especially for workers exposed to NBMs along the entire life cycle of the MD or MP containing NBMs.

While some examples of occupational risk assessment of NMs used in industrial products can be found in literature (Hristozov et al. 2018; Pizzol et al. 2018; Silva, Arezes, and Swuste 2015), the occupational risks of NBMs used in medical applications have been far less investigated, especially for medical professionals, with few exceptions such as the assessment of the potential exposure to NPs of dentists (Van Landuyt et al. 2012, 2014). Therefore, it is necessary not only to perform more studies in this regard, but also to develop tools that can facilitate the occupational risk assessment for nano-enabled biomedical products (Leso et al., 2019; Murashov, 2009; Murashov and Howard, 2015).

To address this need, in the EU H2020 BIORIMA project, a Decision Support System (DSS) to support stakeholders from industry (especially SMEs), consultancy and regulation in occupational risk assessment and management of NBMs applied in medical applications, more specifically MDs and ATMP,s has been developed (https://biorimadss.greendecision.eu/). The use of this web-based system can facilitate the assessment of risks for product manufacturers, healthcare workers as well as end-of-life processing and waste disposal personnel through the application of up-to-date exposure and hazard assessment tools.

In this chapter, the RMF applicability is demonstrated through the development of an occupational risk assessment of NBMs using the BIORIMA DSS to a real case study: magnetite (Fe_3O_4) NPs coated with Poly (lactic-co-Glycolic Acid) (PLGA)-*block*- Polyethylene glycol (PEG)-carboxylic acid (PLGA-*b*-PEG-COOH) used as contrast agent for the diagnosis of solid tumours in Magnetic Resonance Imaging (MRI).

To obtain estimation of occupational risks of magnetite NPs used as contrast agent, specific considerations need to be done to fit the NBMs peculiarities, such as the identification of ad hoc exposure scenarios for healthcare personnel as well as the use of models for exposure and hazard estimations. Moreover, in the proposed probabilistic approach, quantitative estimates of hazard and exposure and their uncertainties are evaluated using the BIORIMA DSS, showing the DSS ability to clearly communicate sources of uncertainty.

4.2 Materials and methods

4.2.1 Case study material

The investigated case study is a dispersion of magnetite (Fe_3O_4) NPs coated with PLGA-*b*-PEG-COOH and its physico-chemical characteristics are reported in Table 3. Details on physico-chemical characterization performed by project partners are reported in Annex 1, where the work performed by Song et al., 2008 was used as a reference to obtain effective density value.

Table 3: Physicochemical characteristics of magnetite NPs coated with PLGA-*b*-PEG-COOH.

Parameter	**Technique**	**Results**
Particles size distribution (nm)	TEM	23 ± 6
Shape	TEM	Monodispersed and spherical particles
Hydrodynamic diameter (nm)	DLS	51 ± 1
Z potential (mV)	ELS	-53 ± 2
Effective density (g/cm^3)	Volumetric centrifugation	1.12

Due to magnetic properties, biocompatibility, and biodegradability, these superparamagnetic iron oxide nanoparticles (SPIONs) have been used in several types of application in oncological medicine (Ansari et al. 2018; Soetaert et al. 2020) and their size permits to enhance contrast in MRI, while the biocompatible coating of PEG and PLGA improves tumour targeting and increases the circulation time (Cole et al. 2011; Kim et al. 2019). Moreover, under an alternating magnetic field, studies revealed that magnetite NPs can be used for localised

hyperthermia at the tumour site by transforming magnetic field into heat (Chatterjee, Diagaradjane, and Krishnan 2011).

The investigated magnetite NPs coated with PLGA-*b*-PEG-COOH have been designed and produced by Colorobbia Consulting industry (Vinci, FI, Italy) and are currently under the pre-clinical investigation for the market authorisation process. Specifically, the application of magnetite NPs as contrast agent in MRI for the identification of solid tumour is considered. In the current study, no material transformations along the life cycle of the product are investigated as they are not likely and/or significant in the assessed exposure scenarios.

4.2.2 BIORIMA Decision Support System

The BIORIMA Decision Support System (DSS) is an adaptation of the SUNDS system (Subramanian et al. 2016) designed to estimate occupational and environmental risks of NBMs used in MD and ATMP along their life cycle. SUNDS was designed with the aim of supporting the assessment and management of environmental and human health risks of nanomaterials used in industrial applications and consumer products along their entire life cycle. The system can be used at two levels of complexity. At the first level, the NanoSCAN tool (developed within the LICARA project) can check supplier risks, competing products, market opportunities or perform benefit-risk analysis and is targeted at SMEs for regulatory safety assessments and product innovation decisions, reducing R&D&I costs. The second level (Risk Assessment and Risk Control) performs quantitative (deterministic or probabilistic) risk assessment of nanomaterials along the lifecycle of nano-enable products and, if needed, supports the selection of appropriate risk control measures; it is intended mainly for application by industry. As for human health risks, as detailed in the works by Pizzol et al., 2018 and Hristozov et al., 2018 on nano-pigments used in automotive plastics and nano-scale copper-based wood preservatives, respectively, SUNDS allows users to assess risks for workers, consumers, and the general population. The BIORIMA DSS has, instead, been specifically designed with the aim of supporting NBMs manufacturers, regulatory bodies, and standardization authorities in assessing environmental and occupational risks associated with the unintentional exposure to NBMs used in biomedical applications, necessary to complement the benefit-risk analysis of these products for the patients to whom the products are intentionally administered/applied. The BIORIMA DSS, therefore, focuses on the

quantitative assessment of risks for workers and the environment considering the peculiarities of biomedical applications in terms of release and exposure scenarios. In cases of risks that are not adequately controlled, the system proposes to the end-user suitable risk management measures (e.g., engineering controls, Personal Protective Equipment), including information about their efficacy.

Specifically, the system is divided into two modules: Risk Assessment, which is subdivided in Occupational and Ecological Risk Assessment, and Risk Control. In the Occupational Risk Assessment section, which is demonstrated in this chapter, the user can input deterministic or probabilistic exposure values obtained, for example, from a monitoring campaign, or calculate them by applying occupational exposure models (i.e., a 2-box model for inhalation, iEAT for ingestion exposure). For the hazard assessment, deterministic or probabilistic Derived No-Effect Level (DNEL) values can be directly inserted as input or derived from raw toxicity data by applying dose-response and intra/inter-species extrapolation models (i.e., PROAST, APROBA).

The resulting estimation of human health risk is always quantitative through the identification of the Risk Characterisation Ratio (RCR) which is the ratio between the measured/estimated exposure value and the DNEL. The RCR can be either deterministic (risk acceptable when RCR <1, not acceptable ≥1) or probabilistic using the following classes: 1) acceptable (when the threshold of one is higher than the 95th percentile of the RCR distribution), 2) needs further consideration (threshold of one between the 90th and 95th percentile) and 3) not acceptable (threshold of one below the 90th percentile). If the resulting risks are unacceptable, Risk Management Measures (RMMs) and their corresponding efficacy values specific for each route of exposure can be selected from the Exposure Control Efficacy Library (ECEL) database, which is connected with the BIORIMA DSS.

4.2.3. Occupational risk assessment

The approach for occupational risk assessment of NBMs adopted in the DSS is based on the REACH Chemical Safety Assessment (ECHA 2016a), which was already applied to NMs in a number of studies such as Hristozov et al., 2018 and Pizzol et al., 2018. The Chemical Safety

Assessment approach implemented in the DSS includes three steps: (1) hazard assessment, (2) exposure assessment, and (3) risk characterization, including uncertainty analysis.

4.2.3.1. Hazard assessment

This step consists of hazard identification and dose-response assessment. The hazard identification involves the gathering and evaluation of the available information on the adverse health effects of the substance. The main issue to address is whether the existing evidence suggests a potential risk for the human health. To identify the relevant hazard information for magnetite NPs, a literature review focused on the following human health endpoints required in the REACH Chemical Safety Assessment guidance was performed: acute toxicity, irritation and corrosivity, sensitisation, repeated dose toxicity, mutagenicity, carcinogenicity, and reproductive toxicity (ECHA, 2017).

The dose-response assessment characterises the relationship between the dose of the substance administered during animal studies and the observed *in vivo* effects by means of statistical modelling. The final goal is to estimate an acceptable human exposure level such as the Derived No-effect Level (DNEL), which is defined by REACH (Annex I, 1.0.1) as the level of exposure above which humans should not be exposed (ECHA, 2012a). The DNEL can then be compared to measured or estimated exposure levels to calculate risks in specific exposure scenarios. The starting point for estimating DNEL is the Point of Departure (PoD), or in other words the highest safe dose based on which adverse effects are not likely to occur in the test animals. The PoD can be a No Observed Adverse Effect Level (NOAEL) or the lower confidence limit of the Benchmark Dose (BMD).

Two tools are included in the BIORIMA DSS to support dose-response assessment and extrapolation of the PoD to DNEL: PROAST and APROBA. When toxicological information from *in vivo* testing is available, a BMD can be estimated by using PROAST (c), a software package developed by the Netherland's National Institute for Public Health and the Environment (RIVM) for the statistical analysis of dose-response data. This model has been adopted in toxicological studies as it provides probabilistic distributions of BMD (Gosens et al. 2014, 2015, 2016). The BMD is estimated from the complete dose-response dataset by fitting dose-response models. Statistical uncertainties in the data are taken into account in the confidence interval around the BMD, whose lower limit (denoted as BMDL) is the PoD that is used as a

starting point for deriving the DNEL by applying inter- and intra-species extrapolation factors (EFs). This extrapolation is performed by using APROBA, which was developed by the World Health Organization International Programme on Chemical Safety Workgroup (WHO/IPCS). APROBA performs probabilistic (as well as deterministic) analysis of human dose extrapolation starting from animal dose-response results (e.g., NOAEL, BMDL) considering EFs distributions and based on the European Chemicals Agency (ECHA) guidelines (ECHA, 2012b).

4.2.3.2 Exposure assessment

The occupational exposure assessment is the process of characterizing, estimating, measuring, and modelling the magnitude, frequency, and duration of contact with a substance (including NM) as well as the number and characteristics of workers exposed considering the different route of exposure (Vallero 2014). Inhalation, dermal contact and ingestion are the main routes of exposure to be addressed for nanoforms under REACH regulation (EC, 2018). Inhalation is considered the primary route by which NPs in the form of free, unbound, airborne particles will enter the bodies of workers and, once inhaled, NPs will deposit in different regions of the respiratory tract, depending upon their particle size (ISO/TR 12885:2008). As there is insufficient information on the penetration of NPs through the skin, (EFSA, 2017; WHO, 2006) and local effects that NPs could create on the skin, dermal exposure also needs to be assessed by deposition from the air, by the direct contact with the substance or with contaminated surfaces (i.e., lab objects, clothing) (ECHA, 2016a) in each life cycle stages. Ingestion exposure typically occurs when substances are accidentally transferred from contaminated hands to the peri-oral region (ECHA, 2016).

4.2.3.2.1. Identification of exposure scenarios

For each stage of the life cycle of the NM under assessment, specific exposure scenarios (ESs) should be identified and described considering i) information on the NM, ii) the process and activities performed by workers, iii) the presence of any RMMs and iv) the estimates of exposure that can be quantified under the described conditions (Read et al. 2014). For each ES, a number of Contributing Exposure Scenarios (CES) can be identified as described in ECHA guidance documents (ECHA, 2016a, 2014b), which refer to specific activities where release of NMs may take place.

Estimation of exposure for each CES can be performed through direct monitoring as well as using exposure models. Site-specific measurements of known quality are often preferred over model estimates and are also needed to validate and improve models (Pizzol et al. 2018). However, for most exposure scenarios, such measurements are hardly available (ECHA, 2012), which requires the use of models to estimate exposure values.

In this work, the development of CESs along the life cycle of the investigated nano-enabled contrast agent was based on the recommendations of Read et al., 2014. Information on work cycle, substances, workplace conditions, targets and risk control measures were collected from the literature as well as from a developed questionnaire for healthcare personnel coupled with interviews to the contacted workers.

Due to confidentiality restrictions on industrial production, detailed information about the synthesis of magnetite NPs cannot be disclosed and the synthesis stage has been excluded from the assessment. For this reason, the occupational risk assessment was conducted for the following life cycle stages: product manufacturing, use and end-of-life. CESs of the product manufacturing were identified through several interviews with the nano-enabled product manufacturers. For the use stage, information was collected based on a questionnaire (Annex 2) following the recommendations described in Read et al., 2014 and listed in the table in Annex 6. This questionnaire was filled in by three medical radiologists, three radiology technicians and three nurses of the University Hospital of Padova (Italy) in order to define activities performed by healthcare staff during the administration of contrast agent as well as the use of specific risk management measures. CESs for the end-of-life were identified considering all the possible types of disposal of medical devices and from information obtained from semi-structured interviews to workers at the Department of prevention and public hygiene at the University Hospital of Padova, to healthcare waste disposal workers, and workers of the incinerators of Verona and Padova (Italy).

4.2.3.2.2. Monitoring campaign

A monitoring campaign was performed at Colorobbia Consulting industry with the aim of quantifying the release of magnetite NPs during the activities performed by workers in the product manufacturing stage. The tiered approach suggested by NIOSH and updated by OECD, 2015 for the evaluation of nanoparticles at workplaces was considered.

At Tier 1, information related to the workplace, specific characteristics of magnetite NPs and workplace activities were collected during a scoping visit at Colorobbia Consulting industry in 2019. At this stage, the identification of possible emission sources during the product manufacturing activities as well as the use of specific risk management measures were addressed. In general, activities such as weighing and mixing of suspension of magnetite NPs are performed by workers which can cause airborne NMs release (Ding et al. 2017). For this reason, a basic exposure assessment was performed using for online measurements an optical particle sizer (OPS) (TSI, Model 3330) and the Aerasense NanoTracer (Oxility, Eindhoven, The Netherlands) connected with a Tygon tube (length 1m) settled at 30 cm from the mouth of the worker to collect data near the breathing zone of the worker. Two high flow peristaltic pumps (Casella, model APEX) containing a polycarbonate HEPA filter were fixed on the lab coat of the worker settled at 30 cm from the mouth to collect particles in air. Filters were then observed by scanning electronic microscopy analysis using a Field Emission Scanning Electron Microscope, FESEM (Carl Zeiss Sigma NTS, Germany) for off-line measurements. Elemental analysis was performed by image analysis using FESEM coupled to an energy dispersive X-ray micro-analyser (EDS, mod. INCA). More information can be found in Annex 3.

As during the monitoring campaign the investigated NPs may have a similar size range derived from other industrial processes (Demou, Peter, and Hellweg 2008), in this study measurements of background were performed by monitoring workers' activities performed under the typical working conditions but without using the magnetite NPs. Moreover, as it is not clear whether a concentration of the investigated NPs can be considered 'significantly high' during an activity compared to the corresponding concentration without nano-activity, the practical approach proposed by Brouwer et al., 2013 was followed. Accordingly, three main parameters were defined for analysing time series measurements: i) the ratio of the average concentrations as determined by on-line instruments between nano-activity and non-nano-activity higher than 2, ii) the presence of nanoparticles on the SEM grids by EDX analysis, iii) the absence of other activities generating the investigated NPs. The evaluation criteria between nano-activity (A) and non-nano activity (B) can be defined as:

ratio A/B≥2: likely.

ratio A/B>1.05 and <2: possibly/not excluded.

ratio A/B<1.05: not likely.

Information related to other activities performed at Colorobbia Consulting during the monitoring campaign was collected to exclude possible release of iron in other compartments of the industry.

Transformation of the obtained values from particle concentration to mass concentration were performed by following the equation described in Annex 4.

4.2.3.2.3. Exposure models

When exposure measurements are not available, predictive models can be used to perform occupational exposure assessment for NMs. Indeed, as workplace measurements of NMs are relatively complex in healthcare sector and in waste disposal, exposure models may be required to provide estimates of exposure especially in those ESs when a direct monitoring is difficult to perform.

For this reason, in the context of BIORIMA project, a 2-box model was implemented for the quantification of the inhalation exposure for workers unintentionally exposed to NBMs along the life cycle of a MD or an ATMP containing NBMs, based on Ganser and Hewett, 2017. The inhalation model has been coded in Python and it is included with a graphical interface within the BIORIMA DSS. This model can simulate the particle behaviour in a well-mixed room predicting the near-field (NF, close to the emission source) and far-field (FF, inside the room but distant from the emission source) concentrations. This model considers some inputs parameters regarding the physico-chemical characteristics of the NM (i.e., particle size, density, and fraction of pristine NM), characteristics of the activity performed by workers (i.e., mass of material used, task duration, generation rate, number of repetitions, and the type of activity) and room conditions (i.e., room volume and number of air changes per hour). The first step is to calculate the total emission of NM during the activity. The activity release rate allows to calculate the total emission rate to the air (in mg/min and for the worst case) for different activities that could lead to a NM release in any of the life cycle stages (e.g., synthesis of a NM, handling or transferring, use or end-of-life). Then, different equations are applied to calculate the steady state concentration (in mg/m^3) assuming a constant emission rate, and the transient concentration that leads to a generation curve followed by a decay curve. The inhalation model provides as final exposure output the NF and FF concentration over time, which are then reported in the BIORIMA DSS as the average concentration during the work shift (mg/m^3). The most conservative value between NF and FF concentrations is then used

to calculate the final risk by dividing it by the DNEL for inhalation exposure, using a precautionary approach.

Considering a possible exposure of healthcare workers to NBMs from hand to mouth contact (Murashov and Howard 2015), the predictive model iEAT developed by Gorman et al., 2012 has been selected to estimate inadvertent ingestion exposure in the workplace, following the approach proposed by Pizzol et al., 2018 and Hristozov et al., 2018 (assuming that a person touches a surface contaminated with the investigated NMs and then touches inadvertently the area around the mouth with subsequent ingestion by licking). The iEAT model is included in the BIORIMA DSS. This model identified four compartments (i.e., the source, air, surface contaminant layer, oral cavity), nine processes of mass transport between the compartments (i.e., emission, deposition, resuspension or evaporation, transfer, removal, redistribution, decontamination, penetration and/or permeation, swallowing) and uses a database with more than 500 empirically measured transfer efficiencies in order to calculate the Lower Confidence Limit (LCL) and Upper Confidence Limit (UCL) of the ingestion dose in mg/kg bw/d (these values serve then in the DSS to calculate a normal distribution of the ingestion dose, used in the probabilistic risk assessment).

Due to the lack of dermal exposure models in literature for NMs, REACH equations for dermal exposure were used, based on the work performed in Goede et al., 2018; McNally et al., 2019 where the mechanistic Dermal Advanced REACH Tool (dART) is presented. This model has been developed to quantify dermal exposure for low-volatile liquid and in this work, its application in a dispersion of NPs is demonstrated. dART tool is based on three main equations where dermal exposure is calculated by a sum of i) the deposition of the investigated substance from the air to the hands, ii) the direct emissions and/or direct contact with the liquid, and iii) the transfer from contaminated surfaces. After the application of each equation, the final output is a deterministic estimation of hand exposure in mg/cm^2/d using the standardized value of hand surface found in ECHA, 2017b.

4.2.3.3 Risk characterisation and uncertainty analysis

Risks can be assessed in either deterministic or probabilistic terms and are considered acceptable when: i) exposure is below prescribed no-effect threshold (e.g., occupational

exposure limit - OEL), or ii) ESs have a negligible exposure, or iii) risk characterization ratio (RCR) is lower than 1.

If exposure cannot be excluded, the RCR value is calculated based on Equation 1:

$$RCR = \frac{EV}{HD} \quad (1)$$

where EV is the exposure value or the probabilistic distribution of exposure determined for a specific ES, and HD is the hazard dose represented by the DNEL that can be both a probabilistic distribution or a deterministic value.

The units of the exposure and the units used for deriving the DNEL must be the same (ECHA, 2014c). For systemic effects, the units of DNELs are mg/m^3 for inhalation, and mg/kg bw or mg/kg bw/day for oral and dermal exposure. For local effects, the unit of DNELs is mg/m^3 for inhalation, while for dermal exposure it is mg/cm^2 skin, mg/person/day (e.g., calculated based on the deposited amount per cm^2 times the actually exposed body area), or a measure of concentration (% or ppm) (ECHA, 2012).

Once the risk is estimated, its acceptability can be classified according to an approach based on confidence intervals. Specifically, in case the risk is presented deterministically, two classes are identified: acceptable (RCR<1) and not acceptable (RCR>1). As probabilistic risk distributions typically follow a right-skewed lognormal distribution, the risk is acceptable if the 90th percentile of the exposed target is safe, but conservative values can also be selected (i.e., the 95th percentile or the 99th percentile) (Pizzol et al. 2018).

To support risk communication of the obtained results, uncertainties need to be clearly assessed. In BIORIMA DSS, uncertainty contribution to RCR by each involved factor is estimated by means of the Monte Carlo approach with 10000 trials where the RCR was calculated as the ratio between exposure (assuming a normal distribution) and hazard (assuming a log normal distribution). At each trial, the RCR is numerically estimated by randomly sampling elements from the BMD/NOAEL distribution, exposure values, and from EF distributions used to derive the DNEL. The contribution to uncertainty of each factor is quantified by assessing the level of correlation between the factor and the resulting RCR by means of squared Spearman's rank correlation coefficient (Helton and Davis 2003).

Uncertainties related to the dose-response data has been performed by means of parametric bootstrapping. The contribution of each EF is selected as the arithmetic mean of each resulting curve and appropriate figures are developed for communication purposes.

4.2.4 Risk management measures

If the resulting risks are unacceptable, the adoption of RMMs can be selected based on route of exposure as well as its efficacy of protection of NMs.

Considerations on specific requirements during the preparation of drug containing NMs and its administration can be found in European Agency for Safety and Health at Work, 2013. For example, for inhalation exposure the use of HEPA filters, respiratory cartridges and masks with fibrous filtering materials are considered effective against airborne NMs (e.g., half- or full-face masks with P3/FFP3 or P2/FFP2 filters), while for dermal exposure, the adoption of two pair of gloves is considered effective for the protection from NMs and the use of protective clothing made with cotton fabrics should be avoided.

In the BIORIMA DSS, specific RMMs can be selected from an inventory of Technological Alternatives and Risk Management Measures (TARMM) from the ECEL database (Fransman et al. 2008), which permits to select the best RMM considering not only its efficacy value, but also its cost of implementation as well as its average life duration.

4.3 Results

4.3.1 Hazard assessment

Relevant information on the toxicity of the magnetite for the inhalation, ingestion and dermal contact exposure routes was found in the European Union Observatory for Nanomaterials (EUON) website (https://echa.europa.eu/registration-dossier/-/registered-dossier/15989/7/6/1) and extracted from the respective REACH registration dossier. The magnetite NPs considered in the dossier can be used as a reference for hazard assessment of the dispersion of magnetite NPs coated with PLGA-*b*-PEG-COOH as these polymers have been approved by the Food and Drug Administration (FDA) as biocompatible because they can be

degraded into non-toxic lactic acid and, accordingly, these polymers should not be considered in the risk assessment (Liang et al. 2019).

For inhalation exposure, a sub-chronic study performed by Pauluhn, 2012 was selected. In this study, rats were exposed to powder of magnetite (Fe_3O_4) for 6 hours per day, 5 days per week, for 13 weeks at target concentrations of 0 (dry air), 10, 15 and 50 mg/m^3 (20 rats per sex per group) which correspond to a near field exposure determined by gravimetric analysis of 0, 4.7 ± 0.6, 16.6 ± 3.0 and 52.1 ± 6.4 mg/m^3 respectively. The NOAEL was determined after the identification of significative pulmonary effects through five different endpoints: histopathology, changes in bronchoalveolar lavage (BAL) protein, increase in total cell counts in BAL, and increase of absolute and relative neutrophilic granulocytes in BAL. Based on these endpoints, the NOAEL value of 4.7 mg/m^3 was proposed by the author for sub-chronic inhalation. This value was firstly corrected to derive a Point of Departure (PoD) which considers the effective exposure of the target (ECHA, 2012b). Indeed, the exposure duration in animal testing is 6 h/d and need to be modified to reflect the 8 h/d of workers exposure. To achieve this, ECHA suggests to apply a correction factor of 0.75 to obtain the corrected NOAEL for 8 h/d (ECHA, 2012b) obtaining the corrected NOAEL of 3.5 mg/m^3. Then, this value has been used as PoD to calculate the corresponding DNEL using APROBA in the BIORIMA DSS.

In order to estimate the oral and dermal toxicity, the study by Remya et al., 2016 was selected. In this study, the authors performed prolonged and repeated administrations for oral exposure (low dose of 500 mg/kg bw; medium dose – 1000 mg/kg bw, and high dose – 2000 mg/kg bw) in rats of a mean weight of 0.2 g following the OECD 453 guideline for 90 days (OECD, 2018). Results showed an increase in glutathione reductase activity in high dose treated animals, which suggests that the system is combating some oxidative stress but in a controlled manner. Indeed, there is no significative difference in the antioxidant parameters of the treated animals compared to the control. The value of 2000 mg/kg bw was used to estimate the corresponding DNEL value.

As for dermal toxicity, Remya et al., 2016 performed sub-acute studies by exposing three rats to different concentrations of NPs (Low-25 mg/kg, Medium-50 mg/kg, and High–100 mg/kg) 6 h daily for 28 days. Results revealed no observable signs of tissue damage in kidney, liver or spleen, and no noticeable change in the haematological and biochemical parameters of

treated animals. The authors affirmed that no skin sensitization or irritation can be observed. For this reason, the corresponding DNEL value was calculated using a NOAEL value of 100 mg/kg as point of departure, firstly multiplying the NOAEL for 0.75 obtaining the corrected NOAEL value of 75 mg/kg for 8 h/d (ECHA, 2012b).

The NOAEL values extracted from the above studies were used to derive DNELs for each exposure route, by applying APROBA, using the interspecies and intraspecies scaling and extrapolation factors reported in Table 4 and performed over 10000 Monte Carlo simulations to derive lognormal distributions of $DNEL_{long\text{-}term}$ for local and systemic effects due to inhalation, ingestion and dermal exposure to magnetite.

DNEL values in mg/kg/d were obtained (LCL: 0.08, UCL: 31.6) using APROBA tool. However, as hand dermal exposure is measured in mg/cm^2/d, DNEL values were modified following ECHA, 2012b document by using the standardised body weight for workers (70 kg) and total body surface (16600 cm^2) defined in ECHA, 2017b, obtaining the final hand dermal DNEL values of LCL: 0.003, UCL: 1.33 mg/cm^2/d.

Table 4. Inputs and outputs of the APROBA tool for each route of exposure.

Inputs		**Route of exposure**		
		Inhalation	**Dermal**	**Ingestion**
Type of PoD		Continuous	Continuous	Continuous
Magnitude of Effect		0.05	0.05	0.05
PoD		NOAEL	NOAEL	NOAEL
Value of PoD		3.525	75	2000
Study type		Subchronic	Subacute	Chronic
Test species		Rats	Rats	Rats
Species weight (kg)		0.35	0.2	0.2
Human weights (kg)		70	70	70
Population Incidence Goal		0.05	0.05	0.05
Probabilistic Extrapolation Factors	Uncertainty in NOAEL as surrogate of BMD	LCL: 0.07 UCL: 1.57	LCL: 0.07 UCL: 1.57	LCL: 0.07 UCL: 1.57
	Inter-species scaling	LCL: 1 UCL: 1	LCL: 4.59 UCL: 7.33	LCL: 4.59 UCL: 7.33

	Remaining inter-species toxicokinetic/toxicodynamic differences	LCL: 0.33 UCL:3	LCL: 0.33 UCL: 3	LCL: 0.33 UCL: 3
	Uncertainty in exposure duration	LCL: 0.5 UCL: 8	LCL: 0.63 UCL: 40	LCL: 1 UCL: 1
	Uncertainty for intraspecies variability	LCL: 1.77 UCL: 14	LCL: 1.77 UCL: 14	LCL: 1.77 UCL: 14
Output		**Inhalation (mg/m^3)**	**Dermal (mg/kg/d)**	**Ingestion (mg/kg bw/d)**
DNEL		LCL: 0.08 UCL: 13.80	LCL: 0.08 UCL: 31.60	LCL: 23.6 UCL: 1830

PoD: Point of Departure, LCL: Lower Confidence Level, UCL: Upper Confidence Level

4.3.2 Exposure assessment

The description of all the CESs, the assessed exposure routes and related exposure estimations identified along the life cycle of the case-study NBM is reported in table 5 and in details in Annex 5.

Table 5. Life cycle stages and Contributing Exposure Scenarios (CES) assessed for the magnetite NPs used as contrast agent, the source of information for each route of exposure and the final exposure estimation in mg/m3 for inhalation, mg/cm2/d for dermal and in mg/kg bw/d for ingestion exposure.

Life cycle stages	**Contributing Exposure scenario**	**Exposure route**	**Estimation method**	**Exposure estimation**
Product manufacturing	CES1: Weighting, solution preparation and mixing	Inhalation	Monitoring campaign	NE
		Dermal	dART equations	1.47E-03
		Ingestion	iEAT model	LCL: 1.87E-04 UCL: 2.92E-03
	CES2: Formation of coated NPs in a mixing chamber	Inhalation	Monitoring campaign	NE
		Dermal	dART equations	NE
		Ingestion	iEAT model	NE
	CES3: Dialysis and concentration	Inhalation	Monitoring campaign	NE
		Dermal	dART equations	NE

		Ingestion	iEAT model	NE
	CES4: Filtration and packaging in glass bottles	Inhalation	Inhalation model	NF: 6.15E-06 FF: 2.46E-06
		Dermal	dART equations	3.19E-03
		Ingestion	iEAT model	LCL: 2.00E-04 UCL: 3E-03
	CES5: Cleaning and maintenance	Inhalation	Monitoring campaign	NE
		Dermal	dART equations	2.46E-3
		Ingestion	iEAT model	LCL: 2.00E-04 UCL: 3.08E-03
Use	CES6: Injection administration	Inhalation	Questionnaire	NE
		Dermal	dART equations	0.80E-06
		Ingestion	iEAT model	LCL: 2.00E-04 UCL: 3.08E-03
	CES7: Cleaning and waste disposal	Inhalation	Questionnaire	NE
		Dermal	dARTequations	7.97E-03
		Ingestion	iEAT model	LCL: 2.00E-04 UCL: 3.08E-03
End-of-life	CES8: Incineration	Inhalation	Interviews	NE
		Dermal	dART equations	0
		Ingestion	iEAT model	LCL: 6.71E-03 UCL: 9.34E-02

NE: negligible exposure, LCL: Lower Confidence Limit, UCL: Upper Confidence Limit, NF: Near Field, FF: Far Field

Specifically, as reported in Table 5, for inhalation exposure, results obtained from the questionnaire for healthcare personnel showed a negligible exposure during CES6 and CES7 (additional specifications in Annex 6). Exposure during product manufacturing for CES1, CES2, CES3 and CES5 was also evaluated as negligible based on the results of the monitoring campaign. Indeed, although the ratio of the average concentrations determined between nano-activity and non-nano-activity (as measured by the NanoTracer) was a value >2 in CES1 and CES5, morphological analysis and chemical analysis of the filter during nano and non-nano activities revealed the absence of iron and the only presence of ceramics in both the

filters (Annex 7). For this reason, it was possible to exclude a release of magnetite NPs during activities performed in CES1, CES2, CES3, CES5.

For CES4, the inhalation model was applied (see input data in Annex 8), and the concentration of magnetite NPs estimated in the Near Field and Far Field is reported in Table 5.

Considering the end-of-life stage (CES8), once the investigated material is injected to the patient using a syringe, it is classified as highly hazardous health-care waste containing sharps contaminated with blood and need to be treated in incinerator (WHO, 2014). The understanding of NMs behaviour during solid waste incineration is still at an early stage (OECD, 2016). Combustion temperature and melting/boiling points, chemical composition, size, and oxidation state of the nanomaterial are significant determinants of the fate of the NM during incineration. Inorganic NPs that escape destruction during the incineration process will mostly end up in the bottom ash (UNEP, 2018).

As for the exposure of workers employed at incineration facilities, in general occupational exposure levels to airborne dust can be considered negligible during routine operations of an incineration plant, while significant exposure to airborne dust could occur during cleaning and maintenance operations where workers are handling air pollution residues (fly ash) or bottom ash created during the combustion process (IOM, 2012). This also applies to nanomaterials: because of their large surface-to-volume ratios, NPs tend to adhere to surfaces in the furnace chamber, boilers, heat exchanger tubes and the wet scrubber (Walser et al. 2012), and then removed with compressed air which can disperse residual NPs. Therefore, as concluded by Walser et al., 2012, attention should be paid during maintenance operations, when exposure to NPs trapped in the system may increase. However, incinerator maintenance activities are not routine operations and the quantity of magnetite NPs currently used as contrast agent is very limited. Thus, in the absence of additional literature data or predictive models and given the fact that an ad-hoc monitoring was not feasible, in this work we assumed that a negligible inhalation exposure in CES 8 was a reasonable conclusion. These assumptions have been also confirmed by the two directors of incinerators consulted for this specific case-study, as they stated that the use of appropriate emission control technologies at the incinerator prevent a release of contaminated air in the workplace.

Dermal exposure for each CES was calculated using the equations defined in dART tool and reported in Annex 9. Results showed the lowest value of exposure during the administration of the contrast agent (CES6) due to the semi-automatic process of injection, while the highest

value is obtained in CES7, when healthcare personnel clean contaminated surfaces (Table 5). A negligible exposure was assessed for CES2 and CES3 because these activities are performed in a closed reactor.

Oral intake through the hand-to-mouth exposure was quantified using the iEAT model (see input data reported in Annex 10) while exposure estimates are reported in Table 5. Results revealed no differences in the oral exposure during activities performed by workers along the entire life cycle, except for workers at the incinerator who may be more exposed to contaminated objects containing NPs compared to all the other CESs. Indeed, all the activities during product manufacturing and in the use stage are performed in a 'clean workplace where surfaces are regularly decontaminated' (as defined by the iEAT). Therefore, the lowest value of surface contamination (represented by the hand loading parameter in Annex 10) was assigned to these CESs, while, since the incinerator can be considered as a 'clean industrial environment' as well as a 'dirty industrial environment with visible contamination', higher values of surface contamination were selected as input in CES8.

4.3.3 Risk characterisation and uncertainty analysis

The results of the hazard and exposure assessment are integrated during the risk characterization to obtain RCR probability distributions. For all the CESs where an exposure cannot be excluded, the RCR probability distributions as a result from over 10000 Monte Carlo simulations are reported in Figure 6, 7 and 8 for inhalation, dermal and ingestion respectively, where the RCR was calculated as the ratio between exposure (assuming a normal distribution) and hazard (assuming a log normal distribution). In Table 6 the mean values of RCR for each CES are summarized.

As can be seen from Table 5, a negligible inhalation exposure is obtained for all CESs except for CES4, where an acceptable risk is obtained as the RCR is less than 1 for >95% of the sensitive population (Figure 6a). The uncertainty associated to the risk estimations can be assessed considering the probabilistic distributions used for the derivation of the long-term human dose for inhalation. Indeed, as the deterministic value of the near field has been used to derive exposure estimation, no uncertainties are obtained for exposure assessment. Uncertainties related to the derivation of the DNEL can be ascribed to the choice of using a NOAEL instead of the BMD as Point of Departure for a 36%, the duration extrapolation from

sub chronic to chronic for a 29%, while 18% and 26% are associated to inter- and intraspecies extrapolation factors, respectively (Figure 6b).

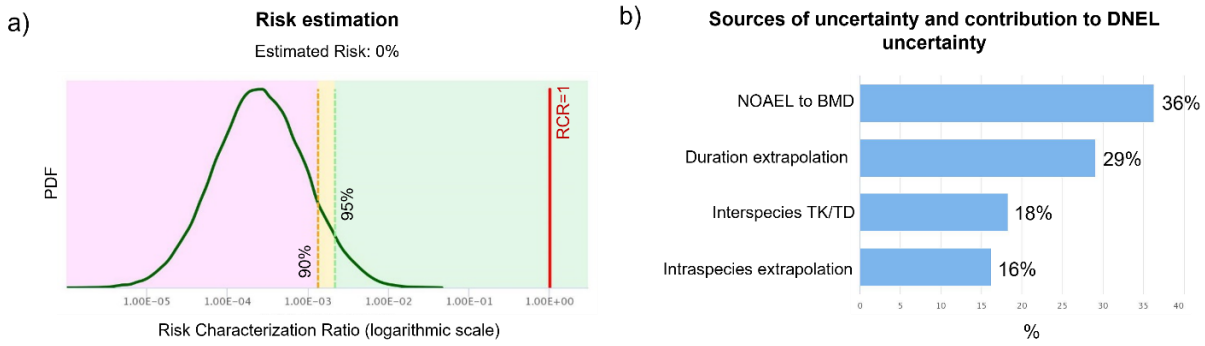

Figure 6. a) Risk Characterisation Ratio distribution of CES4 for inhalation exposure and b) contributions of the different sources of uncertainties to the total uncertainty related to the derivation of the DNEL for inhalation. PDF: probability distribution function, TK: toxicokinetic, TD: toxicodynamic.

As can been seen from figure 7, all the reported CESs have an acceptable risk for dermal exposure as the RCR is below1 for > 95% of the sensitive population (Figure 7a-d), except for dermal exposure in CES7 (Fig. 7e) where the RCR is 0.6 ± 3.0. For this reason, the application of proper RMMs to control the risk is needed and a possible choice is to consider the use of Personal Protective Equipment. In this regard, a pair of nitrile gloves was selected from the ECEL library with an efficacy of 97% for NMs. After recalculating the RCR with the new scenario with PPE, an acceptable risk value of 0.02 ± 0.1 is obtained (Figure 7f).

As for inhalation exposure, risk uncertainties are related only to the derivation of the DNEL for dermal exposure (Figure 7g) which can be ascribed to the extrapolation factors used to derive DNEL from a subacute NOAEL for a 48%, the choice of using a NOAEL instead of the BMD as Point of Departure for a 26%, while 13% and 12% are associated to inter- and intraspecies extrapolation factors, respectively.

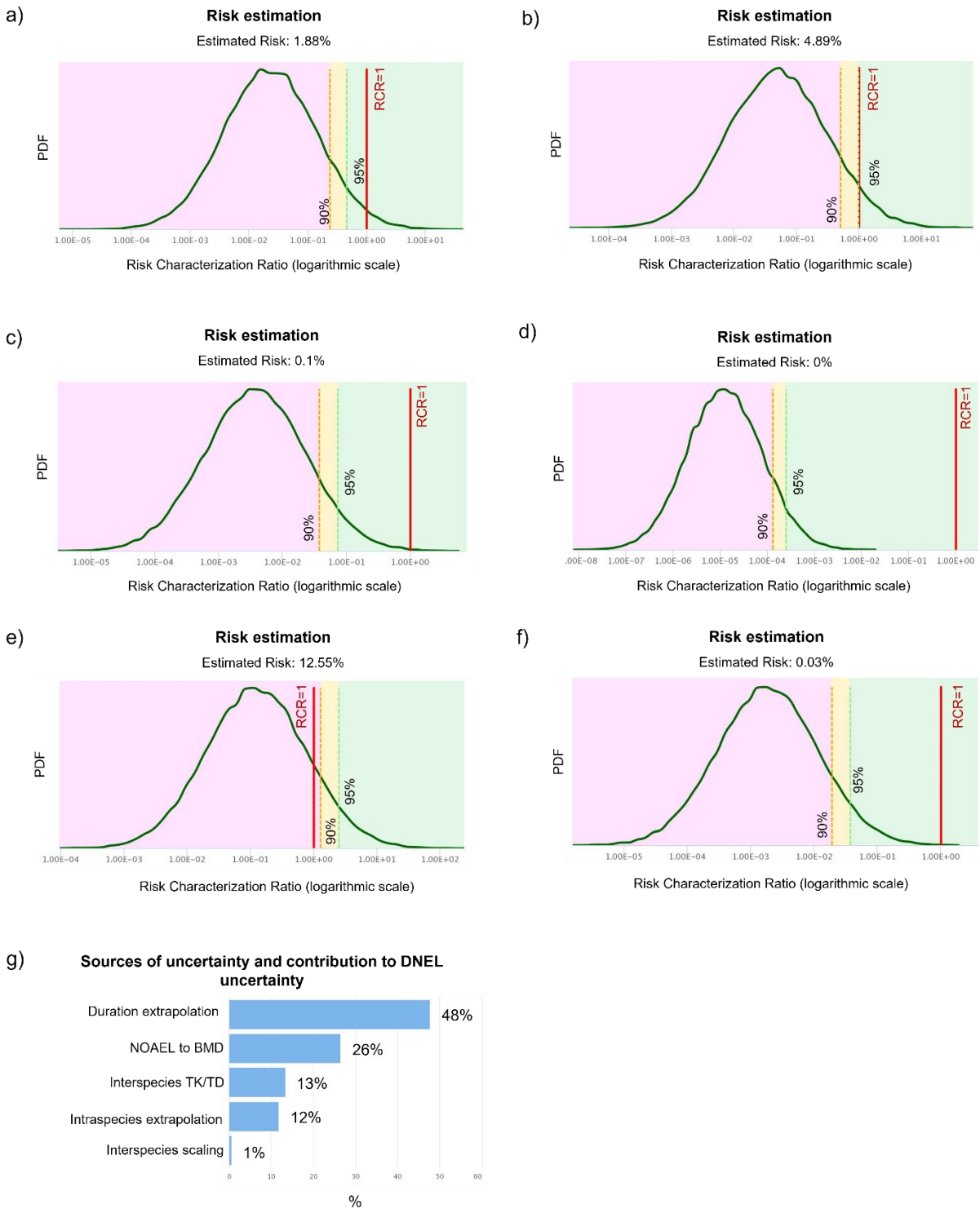

Figure 7. RCR distribution for dermal exposure in a) CES 1, b) CES4, c) CES5, d) CES6, e) CES 7, f) CES 7 adding proper RMMs and g) contributions of the different sources of uncertainties to the total uncertainty related to the derivation of the DNEL for dermal exposure.

As for oral exposure, an acceptable risk is obtained for all the CESs since the RCR is always below 1, where Figure 8a represents risk distribution for CESs 1-7 and Figure 8b for CES8.

Once the iEAT model is used, the BIORIMA DSS permits to identify the uncertainties related to the use of this model in the risk evaluation. Indeed, uncertainty associated to the use of iEAT model in the risk evaluation can be defined as a 25% in CES 1-7 (Figure 8c) and for a 19% in CES 8 (Figure 8d).

Uncertainties related to the derivation of the DNEL can be ascribed to the choice of using a NOAEL instead of the BMD as Point of Departure for a 51%, while 25% and 23% are attributed to inter- and intraspecies extrapolation factors, respectively (Figure 8e).

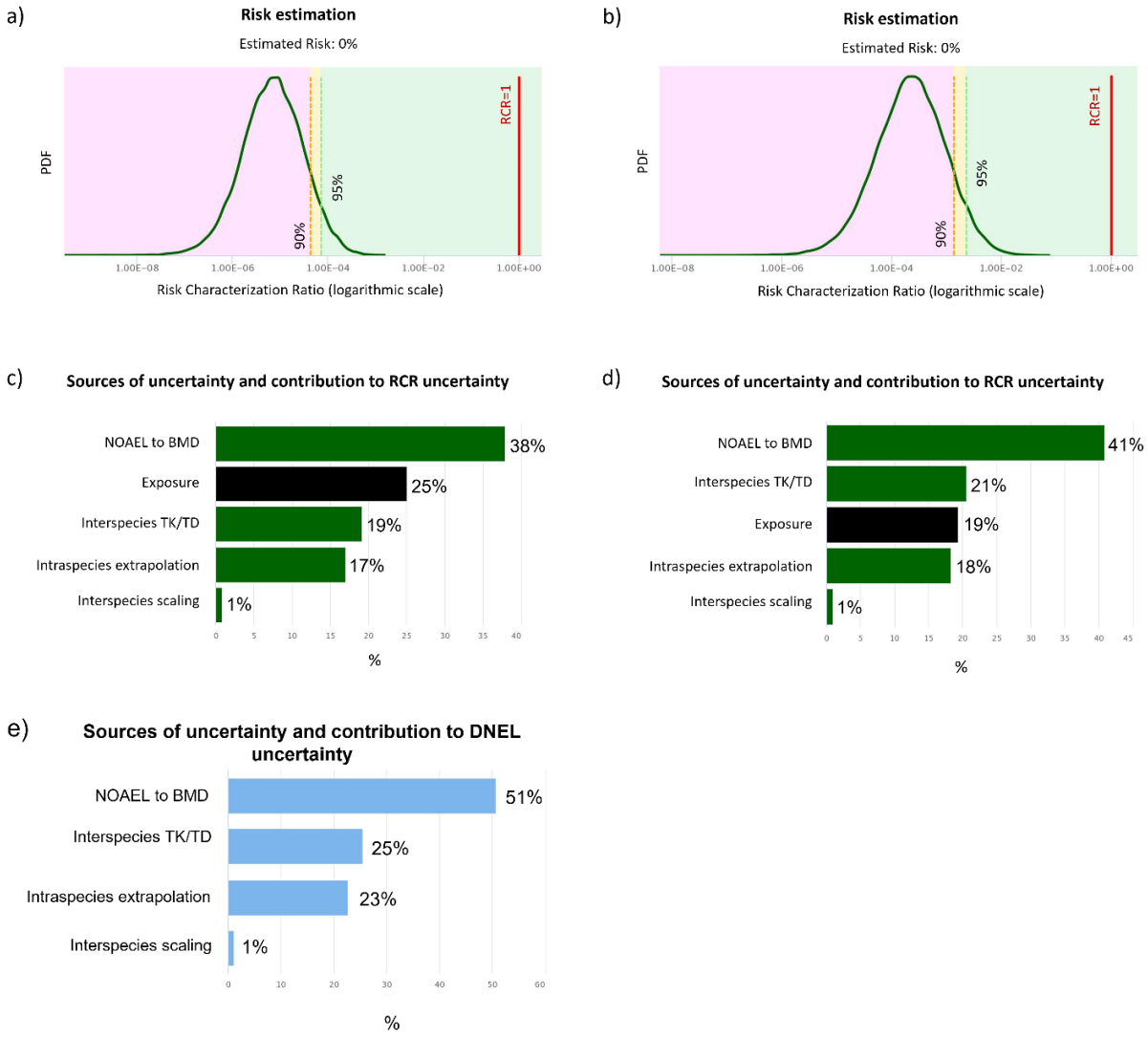

Figure 8. RCR distribution for ingestion exposure in a) CES 1-CES7 and b) CES8, c) uncertainty related to the derivation of RCR in CES1-CES7 and d) in CES8, e) contributions of the different sources of uncertainties to the total uncertainty related to the derivation of the DNEL for ingestion exposure.

Table 6. Risk values for Contributing Exposure Scenario for each route of exposure.

Life cycle stages	Contributing Exposure scenario	Exposure route	Risk value (Mean ± SD)
Product manufacturing	CES1: Weighting, solution preparation and mixing	Inhalation	0
		Dermal	3.4E-01 ± 8.0E-04
		Ingestion	1.85E-05 ± 4.47E-05
	CES2: Formation of coated NPs in a mixing chamber	Inhalation	0
		Dermal	0
		Ingestion	0
	CES3: Dialysis and concentration	Inhalation	0
		Dermal	0
		Ingestion	0
	CES4: Filtration and packaging in glass bottles	Inhalation	0.10E-01 ± 0.20E-01
		Dermal	7.30E-01 ± 0.20E-02
		Ingestion	1.95E-05 ± 4.43E-05
	CES5: Cleaning and maintenance	Inhalation	0
		Dermal	5.0E-02 ± 1.0E-04
		Ingestion	1.95E-05 ± 4.43E-05
Use	CES6: Injection administration	Inhalation	0
		Dermal	4.90E-05 ± 2.17E-04
		Ingestion	1.95E-05 ± 4.43E-05
	CES7: Cleaning and waste disposal	Inhalation	0
		Dermal	**6.3E-01 ± 3.0E+0**
		Dermal after RMMs	2.0E-02 ± 1.0E-01
		Ingestion	1.95E-05 ± 4.43E-05
End-Of-Life	CES8: Incineration	Inhalation	0
		Dermal	0
		Ingestion	5.94E-04 ± 1.48E-03

SD: Standard Deviation

4.4. Discussion and conclusion

In this study, a probabilistic occupational risk assessment approach for NBMs has been applied to a nano-enabled biomedical product: i.e., a dispersion of magnetite NPs coated with PLGA-*b*-PEG-COOH used as contrast agent in MRI applications.

The strength of the proposed probabilistic approach (in comparison to the more conventional deterministic ones) is its ability to clearly communicate sources of uncertainty in the quantitative estimates of hazard and exposure.

In case qualitative information is obtained and used in the assessment (for example, when exposure is characterized through questionnaires or interviews, for data-scarce scenarios), the BIORIMA DSS cannot incorporate and evaluate the associated contribution to the overall uncertainty. This current limitation of the DSS could be tackled in future development of the tool, for example implementing Value-of-Information approaches (Zabeo et al. 2019) that could be used to quantify how targeted collection/generation of additional information may achieve optimal (cost-efficient) reduction of uncertainty in the risk assessment results.

In this work, important sources of uncertainty can be ascribed to hazard assessment, namely i) the choice of the PoD to derive a DNEL (e.g., NOAEL, BMD), ii) the type of toxicological tests performed (e.g., acute, sub-acute, sub-chronic or chronic tests) and iii) the inter- and intra-species variability.

In details, important considerations are needed for the choice of the PoD. Indeed, as the NOAEL value is dependent on experimental study design (e.g., selection of dose levels, the range between doses), while the BMD is derived from the complete dataset of dose-response data, ECHA guidance suggested that BMD is preferred over NOAEL for the derivation of the DNEL (ECHA, 2012a). Indeed, advantages of using a BMD instead a NOAEL are that i) a BMD is derived using all experimental data and reflects the dose-response pattern to a greater degree, ii) the BMD is independent of predefined dose levels and spacing of dose levels, iii) the BMD makes more reasonable use of sample size, with better designs resulting in higher Benchmark doses (ECHA, 2012a).

As no NOAEL values for ingestion and for dermal exposure of magnetite NPs are currently available in the literature, concentrations which represent the highest tested concentrations that do not cause long term effects are considered as NOAEL using a conservative approach. From the obtained results, the choice of using a NOAEL value as an estimate of the BMD is

the largest source of uncertainty in the derivation of the $DNEL_{ingestion}$ and $DNEL_{inhalation}$, while it is the second largest source of uncertainty in $DNEL_{dermal}$ derivation.

When a PoD from a chronic study is available, then using this value to derive a DNEL should be preferred as this would require no use of assessment factors to extrapolate for the duration of the study (e.g., from sub-acute or sub-chronic to chronic) (ECHA, 2012a). In our assessment, due to the lack of relevant chronic data, we derived a chronic PoD starting from a sub-acute study using a probabilistic EF with a confidence interval equal to 0.62 and 40. This extrapolation is a major source of uncertainty for the $DNEL_{dermal}$ that we used in our risk assessment. To derive $DNEL_{inhalation}$, a sub-chronic PoD was used (EF: LCL: 0.5, UCL:8), while no extrapolation factors were needed for the ingestion route of exposure where a suitable chronic study was available and therefore was used to derive the $DNEL_{ingestion}$. In conclusion, to increase the confidence in the evaluation of toxicological effects of magnetite NPs for dermal exposure, it is important to repeat the assessment once sub-chronic or chronic data become available, identifying not only local but also systemic effects. However, as the dispersion of magnetite NPs coated with PLGA-*b*-PEG-COOH can be classified as non-soluble in water, its formulation is expected not to lead to a significative dissolution of the NPs once it reaches the workers' skin and if the skin of workers is expected not to be seriously damaged, dermal adsorption of magnetite NPs coated with PLGA-*b*-PEG-COOH can be considered very low, taking into account the classification provided in the ECHA guideline for dermal adsorption of NPs (ECHA, 2020).

The sources of uncertainty related to intra- and inter-species variability (i.e., the differences between animals and humans and between humans) were also considered in the study but using the default EF values proposed for chemical substances, which may not be fully adequate for nanomaterials. To reduce the uncertainty related to intra- and inter-species extrapolations, it is worth investing future efforts into deriving nano-specific EFs by applying *in silico* tools to the large body of toxicity data already available in the literature.

Uncertainties of this assessment can also be attributed to the use of toxicological data of a substance similar to the investigated material instead of the substance itself. Indeed, this work uses toxicity data not derived from ad-hoc studies on magnetite NPs coated with PLGA-*b*-PEG-COOH, but data for iron oxide (not in nano form) for inhalation toxicity and iron oxide NPs coated with dextran for dermal and oral toxicity respectively, which may cause an increase of the uncertainty on the final risk evaluation. However, as ECETOC (ECETOC 2013)

concluded that local toxicity of the poorly soluble particles of low toxicity (PSPs) is independent of the particle size (i.e., micro or nano-sized material) and is threshold related, and since magnetite NPs can be classified as PSPs (Pauluhn, 2012), local toxicity at different concentrations investigated by Pauluhn, 2012 was considered as starting point to define a $DNEL_{inhalation}$ even if it is not in the nano-range (the average diameter of the tested particles was 981nm). In order to estimate the oral and dermal toxicity, the study by Remya et al., 2016 was selected as the investigated material (dextran stabilized iron oxide NPs) may be considered similar to the magnetite NPs investigated in this paper, as both are in nano-form and coated with a polymer used for medical applications.

Considering exposure assessment, the quantification of dermal exposure was determined based on dART equations for low volatile liquids (instead of applying a nano-specific exposure modelling approach) and this choice could lead to an approximative estimation of hand exposure to magnetite NPs, which probably causes an overestimation of dermal exposure in CES7. However, the use of specific RMMs resulted effective in the reduction of the final risk value. Indeed, after the application of a pair of nitrile gloves, the resulting risk is considered acceptable. Therefore, the applications of a dermal exposure model specific for NPs is advisable and will help risk assessors in obtaining a more realistic dermal exposure estimation.

The use of the iEAT model demonstrates its applicability to estimate ingestion exposure of magnetite NPs from hand-to-mouth contact. However, the iEAT model uses a reduced set of parameters to characterize ESs, and this does not allow to differentiate CESs with different characteristics. For this reason, no significant differences were obtained for the selected CESs.

Results obtained from the monitoring campaign in product manufacturing stage demonstrates the importance of following the OECD tiered approach when planning occupational exposure monitoring. As shown in the current study, the use of particle counters without the characterization of the particles sampled could lead to an incorrect interpretation of results, causing an overestimation of the effective workers exposure of the investigated NP. Indeed, results revealed that the combination of online and offline measurements permits to distinguish between background particles and a (no) release of magnetite NPs. When monitoring campaigns were not possible to perform, the 2-box model was used to quantify inhalation exposure, revealing its ability to quantify near field and far field exposure of NBMs.

The final risk evaluation permits to conclude that risks for workers who use or manage magnetite NPs coated with PLGA-*b*-PEG-COOH used as contrast agent in MRI along its entire life cycle are not significant.

However, as specific tasks performed by healthcare personnel are currently not represented by models showed in this work, further improvements need to be done in order to quantify exposure of healthcare personnel in the use stage. The same observation applies to workers involved in waste management during end-of-life processes. This would require performing extensive research of all the activities performed by healthcare personnel who are managing NBMs, or workers involved in the disposal of waste incorporating NBMs, as well as to perform occupational monitoring campaigns to obtain experimental data. Moreover, given the diversity and the high number of tasks performed by the different categories of healthcare personnel and the continuous changing of type of applications of nano-enabled biomedical products, the development of specific ESs or CESs would require their continuous evaluation, improvement, and verification. It is worth highlighting that due to the increasing interest in NBMs and their medical applications, the development of occupational risk assessment of NBMs will be an essential task for their market authorization, investigating not only the safety of patients but also workers who may be potentially exposed to these nano-enabled biomedical products.

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Chapter 5

Identification of the Safe(r) By Design alternatives of Ag NPs-enabled wound dressings

Contents included in:

Cazzagon V., Giubilato E., Blosi M., Zanoni I., Bonetto A., Vineis C., Varesano A., Marcomini A., Hristozov D., Semenzin E., Badetti E. (in preparation). *Identification of the Safe(r) By Design alternatives of nanoSilver- enabled wound dressings*.

Specific contribution of the PhD candidate:

The work performed within the PhD thesis included:

- a literature review on SbD approaches for NMs and NBMs.
- the design of the SbD procedure for nano-enabled wound dressing.
- a literature review on wound dressings containing Ag NPs similar to the investigated case studies, with a focus on products already on the market.
- the collection and selection of the information related to the investigated materials needed for the SbD procedure.
- the assessment of leaching tests of Ag from the investigated case studies and colloidal characterization of NPs.

Information on physico-chemical characteristics of the investigated materials was provided by project partners involved in this work together with the development of antimicrobial tests and morphological characterization of the wound dressings.

5.1 Introduction

The use of silver nanoparticles (Ag NPs) in medical devices (e.g., coatings on implants, catheters and medical bandages) (SCENIHR, 2015) is constantly increasing due to the well-known antimicrobial properties of silver (António et al., 2015). In this context, the

antimicrobial action of wound dressings (WDs) containing Ag NPs is mainly related to the release of Ag in the ionic form (Nešporová et al., 2020), which can interact with components of the bacteria cells, reducing respiration and provoking their subsequent inactivation and lysis (Musino et al., 2021; Yetisen et al., 2016). Because of Ag mode of action, the choice of specific type of Ag and its content in WDs need to be properly evaluated adopting a life cycle perspective, by investigating not only the safety aspects during the use (i.e., the application of the wound dressing on the injured skin), but also in the end-of-life, through the assessment of the Ag released in environmental compartments and its effects.

Indeed, Ag NPs used in consumer products reach the wastewater treatment plants where Ag can be retained in sewage sludge which is then used as fertilizer for agricultural soils. Through runoff water, Ag NPs can reach aquatic environments (McGillicuddy et al., 2017; Zhu et al., 2019) with possible hazardous effects for organisms.

Effects of silver on aquatic and terrestrial organisms were exhaustively investigated in the past. At the concentration between 1 and 5 µg/L (expressed as free Ag^+), Ag NPs have been found to be lethal for representative species of aquatic plants, invertebrates and fishes (SCENIHR, 2014), causing bioaccumulation processes on liver, gills, intestine of *C. carpio* at 90 µg/L after long term ingestion exposure (Kakakhel et al., 2021). Indeed, according to the proposal of the Harmonized Classification and Labelling, silver can be classified as substance with acute and long-term aquatic hazards (ECHA, 2020). In the soil organism *E. crypticus*, upon Ag NPs exposure, body Ag concentrations keep increasing for longer time leading to a higher risk of longer-term exposure of Ag NPs compared to Ag^+ (Santos et al., 2021).

Fate and behaviour of Ag NPs, which are key points in the assessment of risks of Ag used in WDs, are strongly influenced by many physical, chemical and biological processes such as particle dispersion, aggregation and agglomeration, adsorption, sedimentation, dissolution, precipitation, speciation and bio-transformation (Shevlin et al., 2018). Moreover, as Ag NPs are added in WDs in large excess to exert a long-term and constant antimicrobial effect, an excessive instantaneous release of Ag^+ or the persistence of unused Ag NPs can be observed during the use of WDs (Musino et al., 2021). During the end-of-life, the excess of Ag can interact with sulphur that naturally exists in anaerobic environments to form Ag_2S in soil, or with Cl^- forming AgCl in aquatic environments (Zhang et al., 2018). In this regard, leaching tests of NPs from nano-enabled products provides a valuable support to the investigation of release of Ag into different environmental compartments (i.e., surface water, soil, air, ground

water). As explained in the review of Brunelli et al., 2021, leaching tests can be performed by a partial or a total immersion of the nano-enabled product and by quantifying NPs and/or its ionic form at different time of immersion (for a maximum of 4 weeks).

In order to reduce the potential risks posed by these NPs to both human health and the environment, Safe-by-Design (SbD) approaches can be considered. These approaches have been indeed identified as very promising within the Risk Prevention, Control and Monitoring strategy of the Risk Management Framework (see chapter 3.2.2), because they allow NBM developers to identify and obtain safer alternatives already at the early stage of the innovation process (Soeteman-Hernández et al., 2020).

Such strategies aim at reducing potential human health and environmental risks while optimizing efficacy and costs of the product (Schmutz et al., 2020). In the last years, the SbD concept has been applied in many sector of nanotechnology, such as nano-enabled products used in the conservation of works of art (Semenzin et al., 2019), paints, biosensors or automotive applications (Sánchez Jiménez et al., 2020), or smart nanomaterials used in agriculture, food, food packaging and cosmetics (Gottardo et al., 2021).

In the context of nanomedicine, it is worth citing the SbD approach recently proposed by Schmutz et al., 2020 within the GoNanoBioMat project ("GoNanoBioMat SbD approach" hereafter). It consists of an iterative approach for developing nanomedicines (with a focus on polymeric NBMs for drug delivery, but applicable to NBMs in general) and it is built on three main pillars: 1) "Safe Nanobiomaterials" including Material Design, Characterization, Human Health and Environmental Risks Assessment, 2) "Safe Production", focused on Manufacturing and Control, 3) "Safe Storage and Transport". For each pillar, specific methods and tools or endpoints to be considered are proposed, with the aim of evaluating and handling the safety of nanomedicines along the product development process. Within the first pillar, if the investigated material does not meet the health and environmental safety criteria, SbD actions are identified as means to maximize safety while optimizing efficacy and costs (by going back to the Material Design step). The process ideally leads to the selection of the Safer-By-Design alternative among a set of material design options. Eventually, solutions for the safe production, storage, and transport of the SbD alternative are investigated in the second and third pillars.

In the current work, the GoNanoBioMat approach, and specifically its Pillar I "Safe NBM", inspired the development of a SbD procedure aimed at guiding the identification of the safest

alternatives among five WDs containing silver NPs. As wound dressings containing Ag NPs (indicated as Ag-WDs hereafter) should have a sufficient antimicrobial effect but simultaneously could release significative amount of Ag into the environment, a trade-off between the factors affecting the performance and the safety of WDs should be pursued. The main aim of this work was to define a framework to support the identification of Safe(r) By Design alternatives based on multiple criteria. For the framework, three main steps have been identified: material design, material characterization and SbD evaluation. In the first step, considerations and information about the selected wound dressings are investigated; moreover, three SbD objectives have been identified: i) maximisation of the antimicrobial activity of the Ag-WD, ii) reduction of possible Ag released into the environment, iii) optimization of the cost-effectiveness of the Ag-WD.

For the second and third steps, ad-hoc experimental tests have been selected and some of them have been used as criteria associated to one of the three objectives. For example, leaching of Ag from the total immersion of Ag-WDs in environmental media has been used as criterion to verify to which extent the objective "reduction of possible Ag released into the environment" was satisfied.

The SbD procedure was then used to identify the safer alternatives between five topical Ag-WDs, which differ in i) the polymer used for the matrix, ii) the type and iii) the quantity of Ag NPs incorporated in the Ag-WD. Moreover, this SbD procedure was also applied to two commercial Ag-WDs, ActicoatFlex 3 and ActicoatFlex 7.

5.2 Methods

5.2.1 SbD procedure for the comparison of Ag NPs-enabled wound dressings

A procedure has been developed to support the comparison of different Ag-WDs and guide the selection of the best option(s) based on SbD principles (Figure 9). The procedure takes inspiration from the GoNanoBioMat SbD approach because it promotes the consideration of material properties and efficacy, health, and environmental safety, as well as costs for nanomedicines' production. However, the original approach has been adapted to allow for the comparison in parallel of several alternatives and to take into account the peculiarities of

the target product, considering that Ag-WDs are classified as medical devices (ISO 10993-22:2017), and the specific issues of the NBM of interest (Ag NPs). The presented procedure can therefore be considered as a preliminary analysis to guide the selection of the safest Ag-WDs alternatives. Once the best alternative(s) are identified, further investigations on possible human health and environmental risks should be considered before proceeding with the safe production and the safe storage and transport of Ag-WDs, but these aspects are beyond the objectives of the current work and will not be discussed.

Material Design

The first step of the SbD procedure corresponds to the "Material Design", which is guided by considerations and information about the investigated/desired biomedical product, such as its classification (i.e., medical device), the type and the duration of application (i.e., topical application up to 7 days), the administration route (i.e., dermal) and its mode of action (i.e., a controlled release of Ag^+ up to 7 days). Specific information on the material design in this work can be found in Paragraph 5.2.2.

During the material design, it is important to define the SbD objectives and for our case they are: i) maximisation of the antimicrobial activity of the Ag-WD, ii) reduction of possible Ag released into the environment, iii) optimization of the cost-effectiveness of the Ag-WD. These three main objectives are interconnected as the SbD approach requires to optimize the balance between safety, efficacy, and costs.

Material characterization

Once the SbD objectives are fixed, the "Material characterization" step aims at understanding if the material has the desired physico-chemical characteristics considering specific tests.

In this study, three main characteristics have been selected: i) mechanical strength, assessed by performing morphological analysis through SEM analysis of the Ag-WD before and after the total immersion of the Ag-WD in synthetic sweat (see Paragraph 5.2.3.1), ii) total Ag content in Ag-WDs after their total immersion in acidic conditions, investigated by using Inductively Coupled- Plasma Mass Spectrometry (ICP-MS) (see paragraph 5.2.3.2), iii) leaching of Ag from Ag-WDs immersed in synthetic sweat at different time of immersion, investigated

by ICP-MS(see paragraph 5.2.3.3). If the investigated characteristics do not correspond to the desired ones, the material is modified (i.e., by returning to the Material Design step of the framework) or discarded (i.e., not considered in the third step).

SbD evaluation

Once the required physico-chemical characteristics are verified, the SbD criteria are assessed in the "SbD evaluation" step to investigate the trade-off between benefits for human health (efficacy), reduction of environmental exposure and cost-effectiveness.

In this work, efficacy is considered as the capacity of the Ag-WD to exert an antimicrobial effect. For this reason, antimicrobial tests in *E. coli* were performed (Paragraph 5.2.4). If the material does not exert a sufficient antimicrobial effect, it is modified (i.e., by returning to the Material Design step of the framework) or discarded (i.e., not considered in further analysis).

The second SbD criterion is related to the effectiveness in terms of cost of the Ag-WD, represented by the ratio between the total Ag content of the Ag-WD and the Ag released from the Ag-WD during the period of application (see Paragraph 5.2.5).

Considering possible exposure on the different environmental compartments, leaching tests of Ag from the complete immersion of Ag-WDs were also performed using three environmental media (i.e., freshwater, marine water, and a soil-water extract) in order to quantify Ag released in a worst-case scenario (i.e., assuming an incorrect disposal of Ag-WDs through their littering on soil and water). As a support, colloidal characterization was also performed in the same environmental media used in the leaching tests to study the behaviour of Ag once dispersed in the environment (see Paragraph 5.2.6).

For the identification of the safer alternative, as no threshold can be defined to determine a range of safety values for each of the selected SbD criterion of the SbD evaluation step, the Ag-WDs were ranked according to each criterion evaluating 1) their antimicrobial efficacy, 2) their cost-effectiveness value and 3) leaching of Ag from Ag-WDs during the total immersion of Ag-WDs in environmental media. For both the antimicrobial effect of Ag-WDs in *E. coli* and their cost-effectiveness, SbD alternatives were ordered from the highest to the lowest antimicrobial effect and CE % values, respectively, while for leaching of Ag from Ag-WDs

immersed in environmental media, the best alternative was considered the Ag-WD with the lowest value of release of Ag immersed in the different environmental medium.

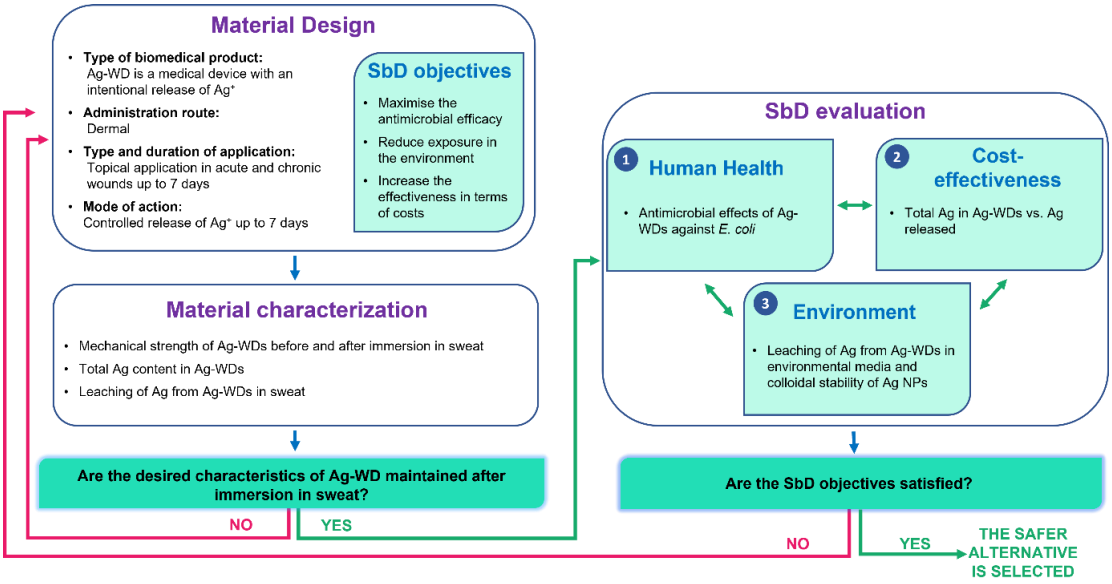

Figure 9. SbD procedure for topical wound dressing containing Ag NPs.

5.2.2 Material Design and test samples

The investigated Ag-WDs were developed using the electrospinning process, a technique capable of producing fibres from polymers with diameters in the nano- to micrometer range containing NPs (Rujitanaroj et al., 2008). Such process ensures the development of uniform and stable fibrous scaffolds (An et al. 2013, Alberti et al. 2020) and fibers that act as an effective barrier for damaged skin to prevent pathogens (Augustine et al., 2018).

Because of their biocompatibility, biodegradability and low cost (Augustine et al., 2018; Gökmeşe et al., 2013; Thamarai Selvi et al., 2018), polyvinyl alcohol (PVA) and poly-L-lactide (PLLA) has been used as matrix for the Ag-WDs (Ambekar & Kandasubramanian, 2019), while the antimicrobial efficacy was exerted by two types of Ag NPs: uncoated Ag and Ag coated with hydroxyethyl cellulose (HEC).

According to ISO 10993-22:2017 on the “Biological evaluation of medical devices. Part 22: Guidance on nanomaterials”, the five Ag-WDs alternatives can be classified as medical devices

with an intentional release of NPs. These Ag-WDs were designed to release Ag up to 7 days in acute and chronic wounds. Since the concentration range of Ag NPs needed to reach an antimicrobial efficacy is not reported in the literature (White & Cutting, 2006), the optimal amount of Ag NPs that has to be contained within the Ag-WDs cannot be established before preforming the antimicrobial tests. Therefore, the five SbD alternatives differ not only in the type of Ag NPs but also in the total Ag content.

The first type of Ag NPs considered in this study are commercial NPs from Sigma Aldrich (referred to as Ag Sigma NPs). These NPs are uncoated and with a diameter by TEM of around 19 nm.

The second type of NPs are Ag NPs coated with hydroxyethylcellulose (HEC) synthetized following a patented procedure (Costa & Blosi, 2016). The coupling of Ag NPs with a positively charged polymer provided a key synergistic effect in antimicrobial activity with enhanced performances against pathogenic strains compared to commercial Ag NPs. Furthermore, the HEC coating enhances the Ag interaction with polymeric/organic formulations and contributes to improve the Ag biocompatibility. The synthesis of AgHEC NPs is an eco-friendly process, entirely carried out at room temperature, by using safe reagents and water as solvent. The synthesis enables the achievement of high concentrated and stable suspensions (0.1-0.5% wt) with sizes ranging from 10 to 20 nm.

Considering the polymer matrix, commercial PVA with an average molecular weight of 130000 g/mol was purchased from Sigma-Aldrich (Italy). PVA powder was dissolved as received in water at 90°C, under mild stirring, until the solution appeared transparent (about 2 h). PVA solution was slightly cooled down at ambient temperature (about 20 °C) under stirring. Then, 5 mg of Ag Sigma NPs powder were added to 10 mL of PVA solution. PVA-Ag solution were electrospun using electrospinning equipment consisting of a high-voltage generator (Spellman SL300P, USA) electrically connected to a 27G metal tip (Butterfly infusion set by Hospira, UK), a metering pump (KDS 200 from KD Scientific) feeding the solution to the metal tip (0.4 mm internal diameter) and a flat metal collector (50 x 50 cm) electrically grounded (for Ag Sigma) and A 23 gsm polypropilene spunbonded non-woven with an average fiber size of 16 ± 4 µm (supplied by Soft NW, Italy) were cut in squares with the same size of the collector and stuck on it as a substrate suitable for handling the nanofiber layers. The PVA-Ag solutions were processed at voltages of +30 kV at the tip with a working distance from the tip to the collector of 20 cm and a flow rate of 0.02 mL/min. The ambient conditions were 21 ± 2

°C temperature and 35 ± 2 % relative humidity. Electrospun nanofibers were collected on the non-woven and the PVA-Ag WD was obtained.

Colloidal AgHEC NPs dispersion was added to the PVA solution at a volume ratio 1:1. The final hybrid PVA-AgHEC solutions were kept under stirring for at least 2 h in order to ensure complete mixing before electrospinning. The PVA-AgHEC solutions were processed at voltages of +30 kV at the tip and −5 kV at the collector with a working distance from the tip to the collector of 20 cm and a flow rate of 0.02 mL/min. The ambient conditions were 22 ± 2 °C temperature and 35 ± 5 % relative humidity. Electrospun nanofibers were collected on the non-woven. Each deposition lasted 1 h and 2 h, developing wound dressing called PVA-AgHEC.1h and PVA-AgHEC.2h respectively.

Ag Sigma and AgHEC NPs were then incorporated into electrospun PLLA fibers with an AgHEC content of 4 %wt obtaining PLLA-AgHEC and Ag Sigma in a concentration of 5 % wt, leading to PLLA-Ag WDs.

Two commercial wound dressings containing AgNPs, namely Acticoat Flex3 and Acticoat Flex7, were selected, analysed, and compared with the different SbD alternatives due to their ability in constantly release silver during their application period (i.e., 3 and 7 days respectively).

In Table 7, the investigated Ag-WDs and their main characteristics are reported.

Table 7. Ag-WDs and their main characteristics.

Ag-WDs	**Type of Ag coating**	**Type of matrix**	**WD type**
PLLA-Ag	Uncoated	PLLA	SbD alternative
PLLA-AgHEC	HEC	PLLA	SbD alternative
PVA-Ag	Uncoated	PVA	SbD alternative
PVA-AgHEC.1h	HEC	PVA	SbD alternative
PVA-AgHEC.2h	HEC	PVA	SbD alternative
Acticoat Flex 3	Unknown	Polyester	Commercial WD
Acticoat Flex 7	Unknown	Polyester	Commercial WD

5.2.3 Material characterization

5.2.3.1 Morphological characterization of Ag-WDs

SEM images and Energy Dispersive X-ray (EDX) spectra of each Ag-WDs before and after immersion in synthetic sweat were obtained. Specifically, one piece of Ag-WD (2.5x2.5 cm) of each sample was totally immersed for 24 hours in 10 mL of synthetic sweat, and then dried at ambient air for 2 days.

Morphological investigation was performed by means of a JSM-6010PLUS/LA SEM with an EDX spectrometer (Oxford INCA-350). The specimens were cut and by a double-stick carbon tape attached to an aluminium stub; the specimens were also coated with a thin film of carbon (10 nm thick), using a Carbon Coater-Balzers CED-010. The resulting SEM images were assessed using ImageJ software to obtain fibres diameter reported as mean ± standard deviation (in µm) of 100 measurements of each sample.

5.2.3.2 Quantification of Ag content in Ag-WDs

The total silver content in Ag-WDs was determined by ICP-MS (NexION 350D, Perkin Elmer). Before ICP-MS analysis, one piece of each Ag-WD (5x5 cm) was weighted and immersed in 5 mL of HNO_3 and after 6 hours total dissolved Ag content (both particulate and ions Ag) was measured using ICP-MS equipped with a seaFAST autosampler. A calibration curve with 7 points in the range of 0.5-50 ppb was used by adding the stock standard solution of Ag 1000 ppm in a solution of pure HNO_3 (69 %). Duplicates were performed for each Ag-WD and presented as mean ± standard deviation of Ag content for each piece of Ag-WD (indicated as µg/WD hereafter). Analysis was conducted in KED (kinetic energy discrimination) mode by using He as collision gas. Samples were automatically diluted 10 times and Y at 10 ppb was used as internal standard. The Limit of Detection (LoD) and the Limit of Quantification (LoQ) were automatically calculated by the software of the ICP-MS technique as the average of blanks + 3 standard deviation (SD) and as the average of blanks + 10 SD, respectively, obtaining an LoD of 0.095 ppb and an LoQ of 0.26. As no reference certified materials are available on the market containing AgHEC NPs, accuracy has been assessed adding Ag and

AgHEC NPs at the concentration of 100 mg/L in a solution of pure HNO_3 (69 %) and analysing samples after 6 hours, obtaining a mean concentration of 95 ±4 %.

5.2.3.3 Leaching tests of Ag from Ag-WDs during immersion in synthetic sweat

As a controlled release of Ag needs to be guaranteed along the entire duration of application, leaching tests of Ag after 1, 3 and 7 days of total immersion of the Ag-WD in synthetic sweat (simulating the worst-case scenario) were performed.

As demonstrated by Midander et al., 2016, the use of a comprehensive artificial sweat containing amino acids, vitamins, organic acids and carbohydrates for the evaluation of metal release do no significantly differ from the EN artificial sweat protocol, and for this reason, synthetic sweat was prepared according to the EN 1811:2011 protocol by mixing urea (0.1 wt%), sodium chloride (0.5 wt%) and lactic acid (0.1 wt%) in deionized water. A Scaltec SBA41 balance (readability: 0.001 g) was used for the weighing of the chemicals. The pH of the solution was adjusted with 1 M NaOH to reach the pH of 6.5 ± 0.05 (Hanna Instruments HI-5522-02 multiparameter meter).

One piece of each wound dressing (5x5 cm) was weighted and immersed in 50 mL of synthetic sweat, and it was stored without agitation. Duplicates were performed for each Ag-WD and presented as mean ± standard deviation of Ag content for each piece of Ag-WD (µg/WD). When the selected time of immersion was reached, 0.25 mL from each sample were mixed with 2.25 mL of nitric acid (HNO_3) 2% and analysed.

For the analyses, an ICP-MS equipped with a seaFAST autosampler was employed and a 6 points calibration curves in the range of 0.5-50 ppb was used to quantify the total dissolved Ag content (both particulate and ions). Calibration curve was determined by adding 0.5-1-5-10-25-50 ppb of Ag from a stock standard solution of Ag at 1000 ppm at a solution containing 200 ppm of NaCl, 300 ppm of $CaCl_2*2H_2O$ and 100 $MgCl_2*6H_2O$, for simulating the salts contained in the synthetic sweat. Detection Limit automatically calculated by ICP-MS technique (as explained in paragraph 5.2.3.2) was 0.08 ppb and a LoQ of 0.22 for Ag element. Analyses were conducted in KED (kinetic energy discrimination) mode by using He as collision gas. Samples were automatically diluted 10 times and Y at 10 ppb was used as internal standard.

5.2.4 Human health SbD criterion

Antibacterial tests of Ag-WDs against E.coli

The antibacterial activity was evaluated according to ASTM E 2149-01 "Standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions". This method is designed to evaluate the resistance of antimicrobial treated specimens to the growth of microbes under dynamic contact conditions. The bacterium was *Escherichia coli* ATCC 11229. The incubated test culture in a nutrient broth was diluted to give a concentration of 1.5–3.0×10^5 CFU/mL (working dilution). Each sample (i.e., 5x5 cm piece of each Ag-WD) was contacted to the working dilution at the ratio 1 g of material in 50 mL of solution. To evaluate the bacterial action of the dispersions, the equivalent amount of Ag contained in the electrospun nanofibers was calculated. All flasks were shaken for 1 h at 190 rpm. After a series of dilutions, 1mL of the solution was plated in nutrient agar. The inoculated plates were incubated at 37 °C for 24 h and surviving cells were counted. The antibacterial activity was expressed in % reduction of the organisms after contact with the test specimen compared to the number of bacterial cells surviving after contact with the control, according to the Equation 2:

$$\text{Reduction } (\%) = \frac{B-A}{B} \times 100 \quad (2)$$

where *A* is CFU/mL after contact (end test) and *B* is CFU/mL at zero contact time.

5.2.5 Cost-effectiveness SbD criterion

To estimate the effectiveness of the Ag-WDs as a function of costs, the ratio (%) between the total Ag content in a 5x5 cm piece of Ag-WD and the Ag released from the same piece was calculated by using equation 3:

$$CE_{t_i} = \frac{[\text{Ag tot}]}{[\text{Ag released}_{t_i}]} * 100 \quad (3)$$

Where CE_{t_i} is the % ratio cost-effectiveness calculated at different time of immersion of the Ag-WDs, *[Ag tot]* is the total Ag concentration in wound dressings (µg/WD) obtained in chapter 5.2.3.1 and $[Ag\ released_{t_i}]$ is the concentration of Ag released from the Ag-WDs (µg/WD) at *i* time of immersion in synthetic sweat (e.g., after 1, 3 and 7 days) as described in chapter 5.2.3.2.

5.2.6 Environmental SbD criterion

Leaching tests of Ag from Ag-WDs during total immersion in environmental media

To estimate the amount of silver released during the end-of-life stage considering a worst-case scenario, leaching tests of Ag from the total immersion of Ag-WDs in Artificial Fresh Water (AFW), Artificial Marine Water (AMW) and soil:water extract were conducted. AFW was synthetized following OECD 203:1992, while AMW was prepared following ASTM D1141-98:2021. Soil: water extract was obtained by mixing LUFA 2.2 soil (LUFA Speyer, Germany) and ultrapure water in a proportion of 1:5 (w/v) with an orbital shaker for 5 min, at 250 rpm. After that, the mixture was centrifuged for 20 min. at 2000 rpm. The supernatant was collected and filtered through a 0.7 µm filter to avoid larger surface material. The pH of this medium resulted 5.4 ± 0.2 according to the analysis performed (Irizar et al., 2018).
ICP-MS analysis were performed following the same procedure described in chapter 5.2.3.3. Samples (i.e., 5x5 cm pieces of each Ag-WD) were analysed at days 1-3-7-14-21 and 28, where 28 days corresponds to the duration time of the sub-acute ecotoxicological tests, and additional samplings from day 1 and day 28 was performed to measure Ag released during time. Duplicates were performed for each Ag-WD at each time of immersion are presented as mean and standard deviations of Ag released from each piece of Ag-WD (in µg/WD). LoD values automatically calculated by the software (as explained in paragraph 5.2.3.2) were 0.09 ppb,0.18 ppb and 0.49 ppb for AFW, AMW and soil:water extract, respectively, while LoQ were 0.24 ppb for AFW, 0.5 ppb for AMW and 1.3 for soil:water extract.

Colloidal characterization of NPs in environmental media

The concentrations of Ag and AgHEC NPs used for the analyses (i.e., 1-10-100 mg/L of Ag NPs) were selected in order to reach the lowest concentrations of NPs detectable from DLS, ELS

and CSA techniques and to be as close as possible to the concentrations of Ag released from the pieces of Ag-WDs immersed in the three environmental media.

Ag and AgHEC were weighted using a Cubis Sartorius balance and dispersed in AFW, AMW, soil:water extract, and in ultrapure water (to investigate the behaviour of Ag NPs in the absence of salts). Ultrahigh-pure water (UPW, minimum resistivity: 18.2 MΩ·cm) was produced by a MilliQ water purifier system (Millipore, Bedford, MA, USA).

As the use of sonication in NPs dispersions (required by dispersion protocol such as NanoGenotox) can increase particle dissolution and change surface properties of metal NPs (Pradhan et al., 2016), Ag and AgHEC NPs were added in the medium and then manually shaked.

DLS measurements were performed by means of the multi-angle Nicomp ZLS Z3000 (Particle Sizing System, Port Richey, FL, USA) to determine the particle size distribution of the NPs. Hydrodynamic diameter (d_H) was measured with an optical fiber set at 90° scattering angle (W=25 mW and λ=639 nm) at room temperature. Refraction index of 1.333 and viscosity value of 0.993 cP were used as formulations are dispersed in water-based media.

ELS measurements were obtained using a Nicomp ZLS Z3000 (Particle Sizing System, Port Richey, FL, USA). A 5V electric field was applied and a zeta potential (ζ-pot) was determined from the mean phase shift with respect to time. The Smoluchowski equation was applied to convert the electrophoretic mobility to the zeta potential.

Both d_H and ζ-pot values were obtained according to three independent measurements, with each measurement consisting of three individual readings and presented as mean ± standard deviation.

CSA was employed to assess dispersion stability of NPs in terms of sedimentation velocity (V-sed) by using the Multiwavelength Dispersion Analyzer LUMiSizer® 651. This technique allows to compare different colloidal dispersions and to establish a stability ranking under specific experimental conditions. The temperature was set at 25°C throughout the time span of analysis. Sedimentation velocity values was achieved at 4000 Rotation Per Minute (RPM), which corresponds to a Relative Centrifugal Force (RCF) of 2146 at 120 mm far from the rotor of the centrifuge. Sedimentation velocity data can be calculated from the transmittance values obtained setting the wavelength of the transmitted light at 470 nm and collecting the transmittance (%) over time at three different positions (115, 120 and 125 mm far from the rotor) over the length of the cuvette. The runtime of each analysis was chosen according to

the lowest time needed to reach the plateau, i.e., the maximum transmittance values, indicating the complete sedimentation of NPs. V-sed values are presented as mean ± standard deviation of three independent measurements.

5.3 Results

5.3.1 Material characterization

5.3.1.1 Morphological characterization of Ag-WDs

SEM images were obtained for each wound dressing before and after the total immersion of the material in synthetic sweat for 24h. PLLA-Ag (Figure 10a) and PVA-Ag (Figure 121a) are composed of uniformly distributed electrospun fibers, while PLLA-AgHEC (Figure 10b), PVA-AgHEC.1h (Figure 12a) and PVA-AgHEC.2h (Figure 12b) showed an excess of polymer in small regions of the Ag-WD.

Fibres in PLLA-Ag sample are quite homogeneous in terms of size with a diameter of 2.5 ± 0.4 µm, and the observed morphology is preserved even after 24 h of immersion in synthetic sweat obtaining a value of 2.7 ± 0.3 µm (Figure 10c). In PLLA-Ag HEC (Figure 10b), PLLA fibres have a lower size than PLLA-Ag (i.e., diameter size of 70 ± 23 nm), and once they are immersed in synthetic sweat, shape of fibers is not preserved, probably due to a low mechanical strength of the fibers and HEC polymer (Figure 10d), which could not permit to calculate the diameter of fibers.

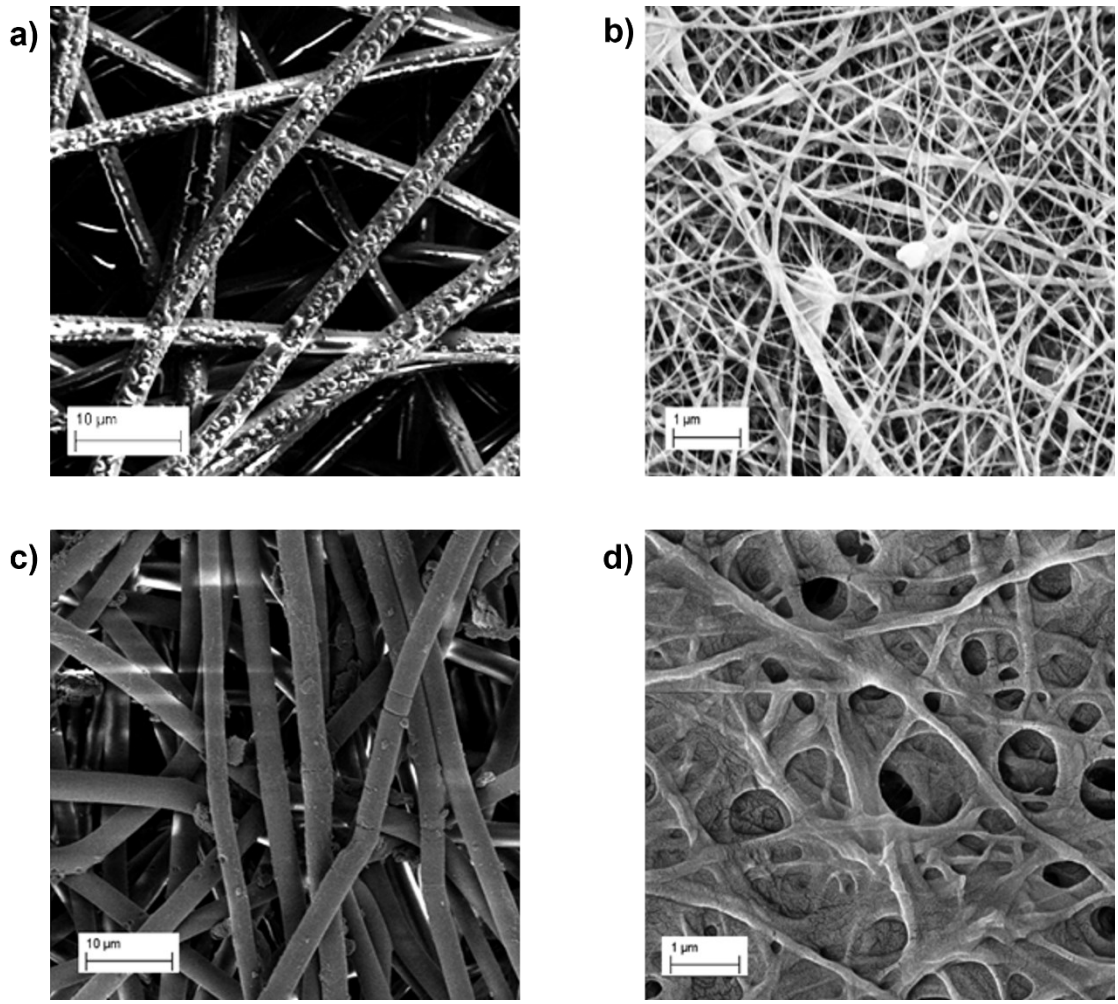

Figure 10. SEM images before immersion of a) PLLA-Ag, b) PLLA-AgHEC, and after 24h of immersion in synthetic sweat of c) PLLA-Ag and d) PLLA-AgHEC.

Considering PVA-Ag SbD alternative, shape and dimension of the fibers are maintained even after immersion in synthetic sweat (Figure 11b). Indeed, a diameter size of 15.4 ± 1.3 µm of this sample was obtained before immersion (Figure 11a) which is close to the value of 16.4 ± 2.1 µm after immersion (Figure 11b). From EDX analysis, both Ag and Cl were detected before (Figure 11c) and after immersion (Figure 11d) in synthetic sweat, suggesting the presence of AgCl on the surface of the fibers, probably as residues of Ag NPs synthesis.

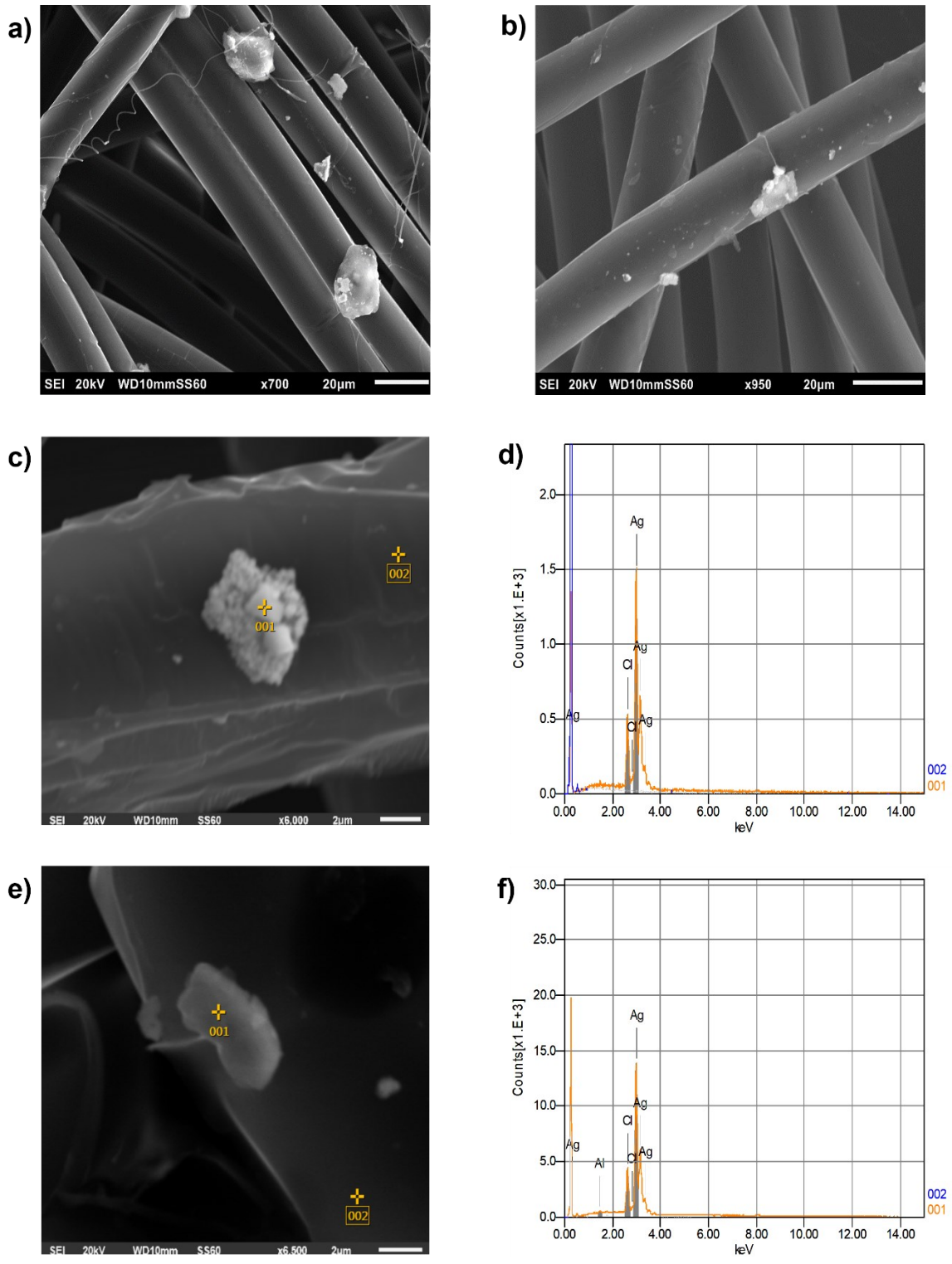

Figure 11. SEM images of PVA-Ag a) before and b) after 24h of immersion in synthetic sweat, and EDX spectrum of PVA-Ag c) before and d) after 24h of immersion in synthetic sweat.

Also in PVA-AgHEC.1h sample (Figure 12a), shape and dimension of the fibres are preserved after immersion (Figure 12c), as indicated by the obtained fibres diameter values (i.e., 16.4 ±

1.8 µm and 15.5 ± 1.8 µm before and after immersion, respectively). No significative differences are observed in PVA-AgHEC.2h sample before (Figure 12b) and after immersion (Figure 12d), as confirmed by the obtained fibres diameter values (i.e., 16.2 ± 1.5 µm and 16.3 ± 1.5 µm before and after immersion, respectively).

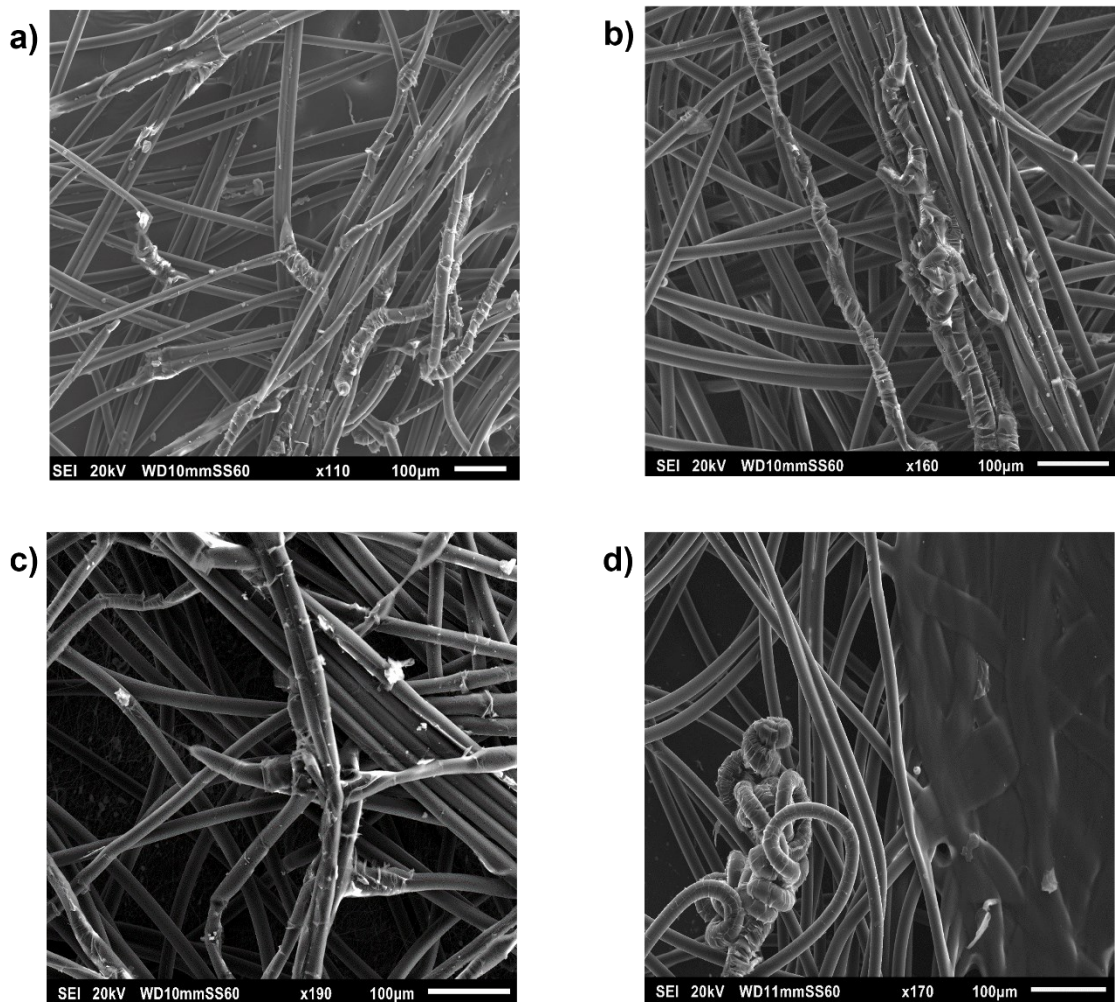

Figure 12. SEM images before immersion of a) PVA-AgHEC.1h, b) PVA-AgHEC.2h, and after 24h of immersion in synthetic sweat of c) PVA-AgHEC.1h and d) PVA-AgHEC.2h.

Considering commercial Ag-WDs, fibres of both Acticoat Flex 3 and Acticoat Flex 7 are homogeneous in size and shape (Figure 13a and 13b). However, after immersion in synthetic sweat (Figure 13c and 13d) a degradation of the fibers' surface is observed. This degradation is probably related to a detachment of the protective layer present on the fibers, because of the interaction with the synthetic the sweat. Comparing the values of fibres diameter before

and after immersion in the simulated medium, a slightly increase in fiber size for both Acticoat Flex 3 and Acticoat Flex 7 after immersion was observed (e.g., for Acticoat Flex 3, an increase from 6.8 ± 1.9 µm to 7.7 ± 0.8 µm was observed, considering for samples after immersion only the part of the fibers without the protective coating).

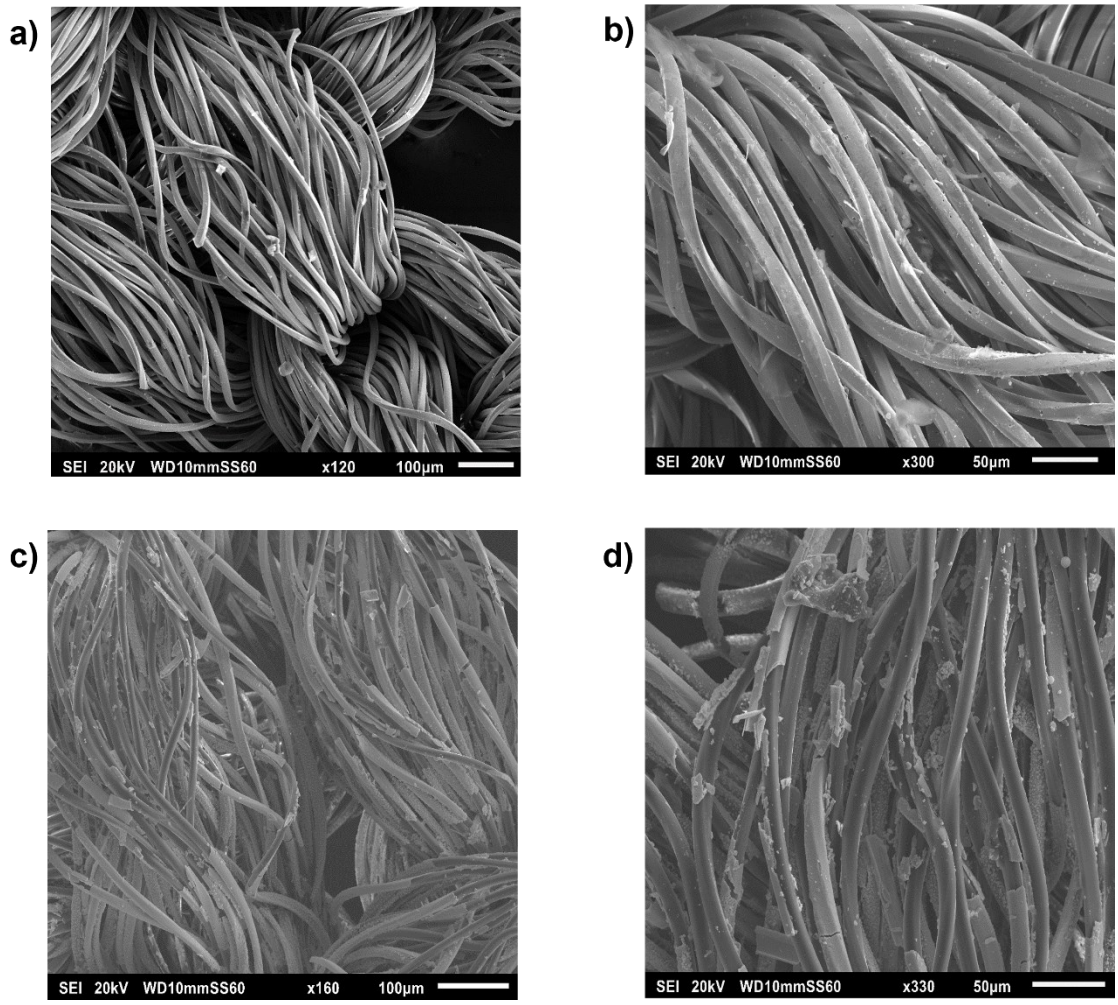

Figure 13. SEM images before immersion of a) ActicoatFlex 3, b) ActicoatFlex 7, and after 24h of immersion in synthetic sweat of c) ActicoatFlex 3 and d) ActicoatFlex7.

To conclude, because of the low mechanical strength of fibers after immersion in synthetic sweat, PLLA-AgHEC is not further investigated as SbD alternative.

5.3.1.2 Quantification of Ag content in Ag-WDs

The amount of Ag contained in each piece of Ag-WDs (5x5 cm) was determined by ICP-MS analysis, and the results obtained are reported in Table 8 as µg/WD. As can be observed from the table, the total amount of silver contained in the commercial Ag-WDs is higher than those determined in the SbD alternatives selected (30 mg/WD of Ag for the commercial Ag-WDs vs. amounts ranging from 40 µg/WD to 2 mg/WD for the SbD alternatives). Moreover, as expected, Acticoat Flex 7 showed a higher amount of Ag than Acticoat Flex 3, since its controlled release of Ag up to 7 days. Considering SbD alternatives, PLLA-Ag showed the highest Ag content (around 2 mg/WD) compared to the other Ag-WDs (between 40 and 125 µg/WD), followed by PLLA-Ag > PVA-Ag > PVA-AgHEC.2h > PVA-AgHEC.1h.

Table 8. Total Ag content in one piece of Ag-WDs (5x5 cm).

Ag-WDs	**Ag content in Ag-WD (µg/WD) Mean ± SD**
PVA-Ag	124 ± 25
PLLA-Ag	1912 ± 148
PVA-AgHEC.1h	41 ± 7
PVA-AgHEC.2h	89 ± 14
Acticoat Flex 3	27872 ± 1029
Acticoat Flex 7	31943 ± 1124

SD: Standard Deviation

5.3.1.3 Leaching tests of Ag from Ag-WDs during immersion in synthetic sweat

The amount of Ag released from the selected Ag-WDs in the synthetic sweat was determined by ICP-MS at different immersion time. The overall results are reported in Table 9 and figure 13.

As it can be observed from Table 9, the concentrations of Ag released from the six samples analyzed at day 1 and 3 in the synthetic sweat, ranged from 1 to 80 µg/WD. The highest content of Ag was released from Acticoat Flex 3 and 7 (between 60 and 80 µg/WD). These results were expected since these materials present a higher initial amount of Ag than the

other SbD alternatives (see chapter 5.3.1.2). On the other side, the lowest release of Ag was detected for PLLA-Ag WDs (ranging from 1-10 μg/WD), while the amount of Ag released from PVA-Ag WDs ranged from 8 to 30 μg/WD.

Measurements obtained after 1, 3 and 7 days of immersion were always higher than those observed for the PLLA-Ag WDs, despite the initial Ag amount of PLLA-Ag which is one order of magnitude higher than PVA-Ag (see chapter 5.3.1.2). Considering AgHEC WDs, the amount of Ag released from PVA-AgHEC.1h WDs (between 10 and 18 μg/WD) was twice less than the Ag released form PVA-AgHEC.2h WDs (range between 25 and 50 μg/WD). These results can be ascribed to the different time used in the electrospinning process (1 vs 2 hours), which is also related to the initial Ag contained in the Ag-WDs.

Table 9. Ag content (μg) released from a piece of Ag-WDs (5x5 cm) at different time of immersion in synthetic sweat reported as Mean ± Standard Deviation (SD).

Ag-WDs	**Ag released (μg/WD) Mean ± SD**		
	1 d	**3 d**	**7 d**
PVA-Ag	8.5 ± 0.7	16 ± 3	30 ± 6
PLLA-Ag	1.6 ± 0.1	3.5 ± 0.3	8 ± 1
PVA-AgHEC.1h	10 ± 1	12.3 ± 0.7	18 ± 2
PVA-AgHEC.2h	28 ± 4	32 ± 4	46 ± 6
ActicoatFlex 3	68.8 ± 0.5	69 ± 2	76 ± 2
ActicoatFlex 7	64.5 ± 2.0	64.6 ± 0.3	77 ± 1

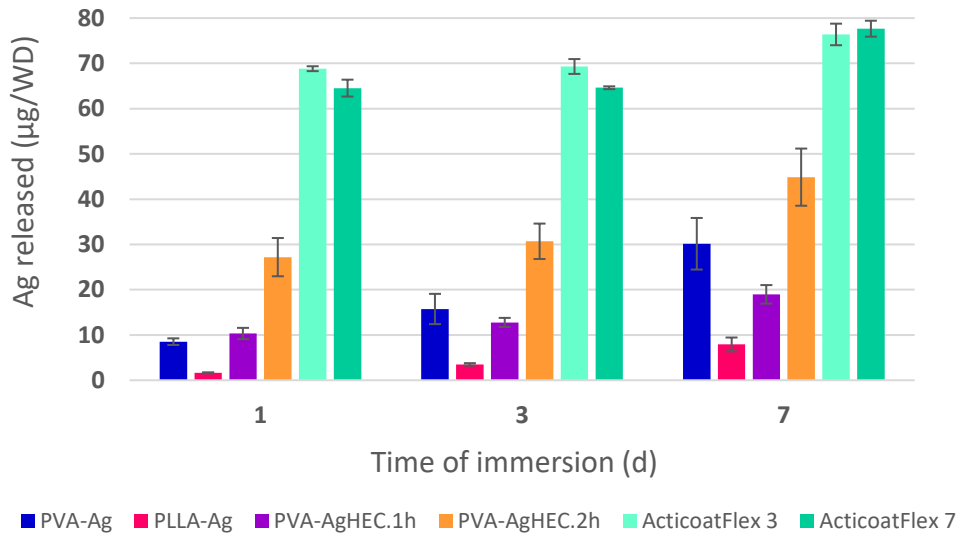

Figure 13. Concentration of Ag released (µg/WD) from PVA-Ag, PLLA-Ag, PVA-AgHEC.1h, PVA-AgHEC.2h, Acticoat Flex 3 and Acticoat Flex 7 Ag-WDs immersed in synthetic sweat at day 1, 3 and 7.

As the obtained results showed that Ag is released at all the tested time of immersion (i.e., 1-3-7 days) by each Ag-WDs, all the alternatives are further investigated in the SbD evaluation.

5.3.2 Human health SbD criterion

Antibacterial tests of Ag-WDs against E.coli

The antibacterial results showed that AgHEC suspension has an excellent biocidal action even after dilution (i.e., 100% antibacterial efficacy after 1:10 dilution) which remains unaltered even after the electrospinning process (Table 10). Indeed, the best antibacterial results against Gram-negative bacteria *Escherichia coli* reduction are PVA-AgHEC fibers (i.e., 100% antibacterial efficacy) followed by PVA-AgHEC>PLLA-Ag>PVA-Ag. The antimicrobial efficacy of PVA-AgHEC is induced by AgHEC, as PVA polymer did not show any antibacterial activities (i.e., 27%). Moreover, the antibacterial action of HEC polymer decreased with 1:10 dilution (i.e., from 94% to 34%).

Table 10. Bacterial reduction (%) in E. coli of Ag NPs and Ag-WDs.

Ag NPs and blank	**Bacterial reduction % (*Escherichia coli*)**	
	Amount equivalent to 1 g of nanofibres	Amount equivalent to 0.1 g of nanofibres (ratio 1:10)
Ag Sigma	99	64
AgHEC	100	100
Blank 1	94	34
Blank 2	27	-
Blank 3	69	-
Ag-WDs	**Bacterial reduction % (*Escherichia coli*)**	
PLLA-Ag	97	
PVA-Ag	89	
PVA-AgHEC	100	

Blank 1: HEC without Ag, Blank 2: PVA without Ag, Blank 3: PLLA without Ag

5.3.3 Cost-effectiveness SbD criterion

Values obtained from equation 3 are reported in Table 15, where % of Cost Effectiveness index (CE) is calculated for each Ag-WDs at day 1, 3 and 7. Considering SbD alternatives, PLLA-Ag showed the lowest %CE value (i.e., 0.4%), indicating that the high quantity of Ag NPs present on the PLLA fibers is almost not released. On the contrary, % CE of both PVA-AgHEC.1h and PVA.AgHEC.2h WDs were close to 26% at day 1, at 30% at day 3 and 45% at day 7, suggesting the high effectiveness of these Ag-WDs. For commercial Ag-WDs, the %CE was always very low (from 0.1 to 0.4 %), these results suggested that despite the high quantity of Ag added to the polymer, the Ag is released only in very small quantity.

According to the obtained results, SbD alternatives can be ranked as followed: PVA-AgHEC.1h>PVA-AgHEC.2h>PVA-Ag>PLLA-Ag.

Table 16. Cost-Effectiveness ratio (CE%) for each Ag-WDs.

Ag-WDs	**CE (%) at day 1**	**CE (%) at day 3**	**CE (%) at day 7**
PVA-Ag	6.9	12.7	24.2
PLLA-Ag	0.1	0.2	0.4
PVA-AgHEC.1h	26.4	33.8	49.0
PVA-AgHEC.2h	26.9	31.1	45.0
Acticoat Flex 3	0.2	0.2	0.3
Acticoat Flex 7	0.2	0.2	0.2

5.3.4 Environmental SbD criterion

Leaching tests of Ag from Ag-WDs during total immersion in environmental media

Leaching tests of Ag from Ag-WDs at different time of immersion were performed in AFW (table 11), in AMW (table 12), and in soil:water extract (table 13). In general, release of Ag from each SbD Ag-WD was not constant with time, due to possible Ag NPs transport and transformation processes of NPs (e.g., sedimentation, hetero/homo-aggregation, agglomeration) in the investigated environmental media, which could explain the total Ag variation over time.

Considering Ag released from SbD Ag-WDs alternatives immersed in AFW (figure 14), the lowest values of Ag were observed for PLLA-Ag WDs (range between 1 and 3 µg/WD), while the amount of Ag released from PVA-Ag WDs reached the highest value at day 28 (around 25 µg/WD). As far as the SbD WDs containing AgHEC NPs are concerned, the amount of Ag determined from both PVA-AgHEC.1h and PVA-AgHEC.2h was almost constant, showing negligible differences among the concentrations of Ag released at the different immersion time selected. While the release of Ag from the SbD WDs resulted in the range between 1 and 25 µg/WD, the release of Ag from the commercial Ag-WDs resulted much higher, with concentrations ranging from 230 to 400 µg (Figure 15). In addition, for both commercial WDs, the release of Ag slightly increased from day 1 and day 28 and the concentrations of Ag released from Acticoat Flex 7 were always higher than those measured for the Acticoat Flex 3 at each time of immersion.

According to the obtained leaching tests of Ag from Ag-WDs immersed in AFW, the SbD alternatives can be ranked as followed: PLLA-Ag>PVA-AgHEC.1h>PVA-AgHEC.2h>PVA-Ag.

Table 11. Leaching of Ag from each piece of Ag-WD immersed in AFW and reported as mean ± SD in µg/WD.

Ag-WDs	**Ag released (µg/WD) Mean±SD**					
	1 d	**3 d**	**7 d**	**14 d**	**21 d**	**28 d**
PVA-Ag	9.1 ± 0.7	10 ± 1	6.3 ± 0.5	13 ± 1	11.7 ± 0.3	23.5 ± 3.1
PLLA-Ag	0.79 ± 0.04	0.9 ± 0.1	0.02 ± 0.03	1.4 ± 0.1	1.0 ± 0.3	2.8 ±0.1
PVA-AgHEC.1h	7 ± 2	8 ± 1	7.8 ± 0.8	7.1 ± 0.5	6 ± 1	7.0 ± 0.9
PVA-AgHEC.2h	10.9 ± 0.8	12.8 ± 0.1	15.01 ± 0.3	14.3 ± 0.1	13.1 ± 0.2	13.8 ± 0.9
ActicoatFlex 3	231 ± 3	228.9 ± 0.2	276 ± 7	254 ± 10	298 ± 9	330 ± 3
ActicoatFlex 7	253 ± 27	265 ± 6	319 ± 25	297 ± 28	355 ± 28	382 ± 20

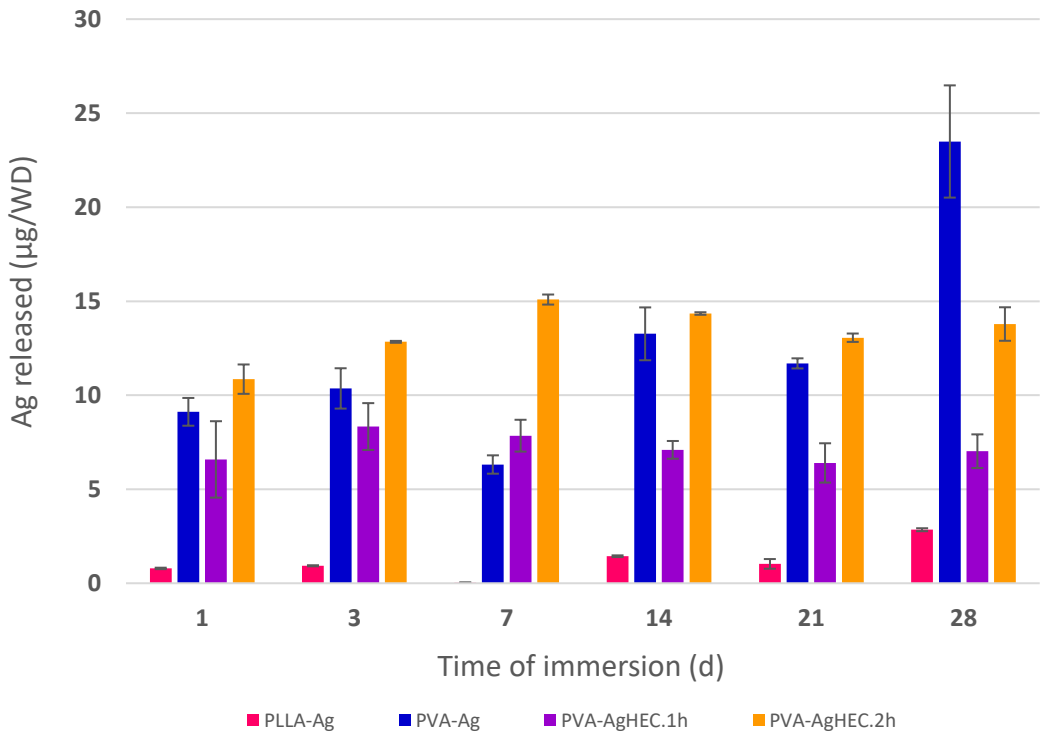

Figure 14. Leaching tests of Ag released (µg/WD) from PVA-Ag, PLLA-Ag, PVA-AgHEC.1h, PVA-AgHEC.2h Ag-WDs immersed in AFW at day 1, 3, 7, 14, 21 and 28.

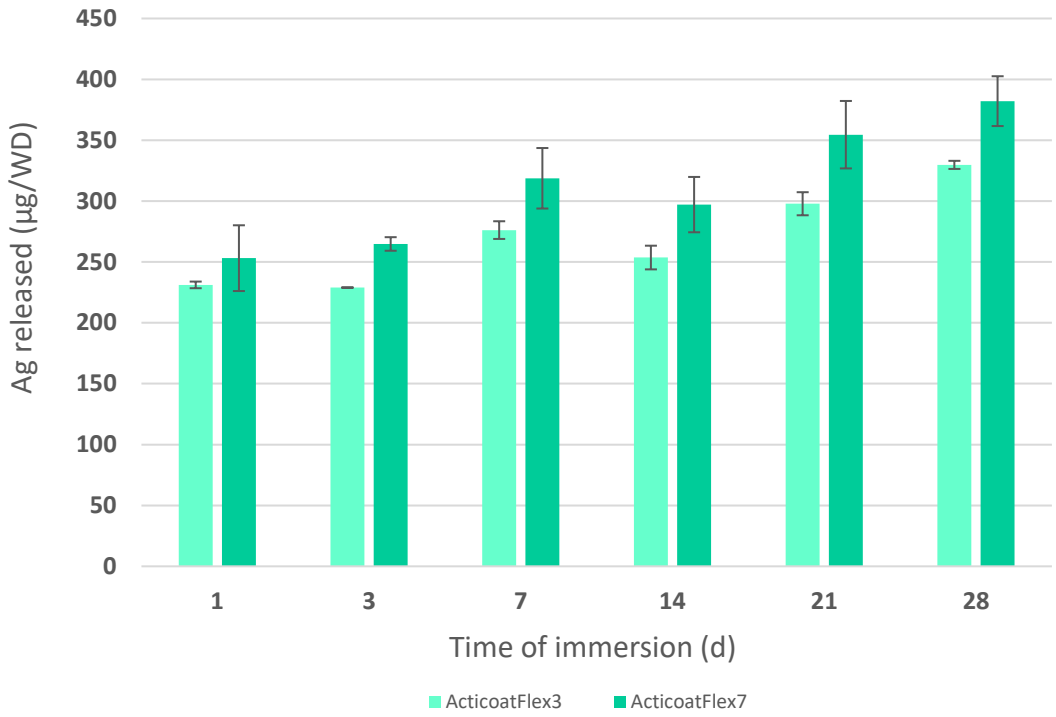

Figure 15. Leaching tests of Ag released (µg/WD) from ActicoatFlex 3 and Acticoat flex 7 immersed in artificial fresh water at day 1, 3, 7, 14,21 and 28.

Considering leaching tests of Ag from Ag-WDs in AMW (Figure 16), PVA-AgHEC.1h WDs showed the lowest Ag release values in almost all the time of immersion (between 7 and 26 µg/WD), while Ag released from PVA-AgHEC.2h WDs reached the highest values at each time of immersion (range between 25 and 82 µg/WD). Comparing WDs containing uncoated Ag NPs, the leaching of Ag from PLLA-Ag WDs was lower at day 1 and 3 than that observed for PVA-Ag WDs, while from day 7 up to day 28 an opposite behaviour was observed. These results can be ascribed to the different Ag-WDs fiber structure, which is related to the two different polymers used in the Ag-WDs. As already observed for AFW, the Ag released from the commercial Ag-WDs immersed in AMW (180-250 µg/WD) resulted always higher than those released from the SbD alternatives (4-58 µg/WD), and the concentrations determined from the immersion of Acticoat Flex 7 in AMW were always higher than those obtained from Acticoat Flex 3 at each time of immersion (Figure 17).

According to the obtained leaching tests of Ag from Ag-WDs immersed in AMW, the SbD alternatives can be ranked as followed: PVA-AgHEC.1h>PVA-Ag>PLLA-Ag>PVA-AgHEC.2h.

Table 12. Leaching of Ag from each piece of Ag-WD immersed in AMW and reported as mean ± SD in µg/WD.

Ag-WDs	Ag content (µg/WD) Mean±SD					
	1 d	**3 d**	**7 d**	**14 d**	**21 d**	**28 d**
PVA-Ag	9 ± 1	21 ± 3	15 ± 4	32 ± 6	11.5 ± 2	40 ± 5
PLLA-Ag	4.3 ± 0.4	14 ± 1	28.8 ± 2.5	49 ± 2	48.9 ± 0.4	57.6± 0.1
PVA-AgHEC.1h	10 ± 3	13 ± 4	16 ± 4	20 ± 5	17 ± 5	21 ± 5
PVA-AgHEC.2h	34 ± 9	42 ± 9	55 ± 11	66 ± 13	64 ± 10	67 ± 15
ActicoatFlex 3	188 ± 7	195 ± 6	221 ± 6	225 ± 5	216 ± 1	228.1 ± 0.5
ActicoatFlex 7	194 ± 1	206 ± 1	229 ± 7	226 ± 2	225 ± 6	239 ± 9

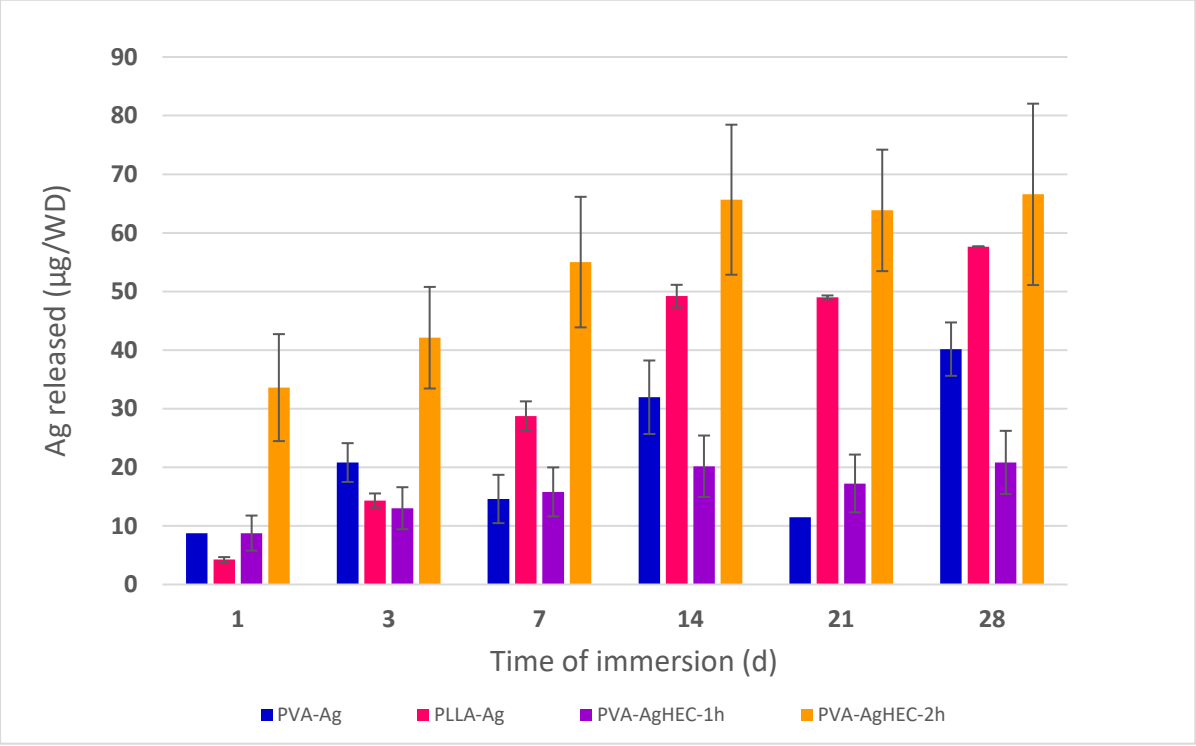

Figure 16. Leaching tests of Ag released (µg/WD) from PVA-Ag, PLLA-Ag, PVA-AgHEC.1h, PVA-AgHEC.2h Ag-WDs immersed in AMW at day 1, 3, 7, 14,21 and 28.

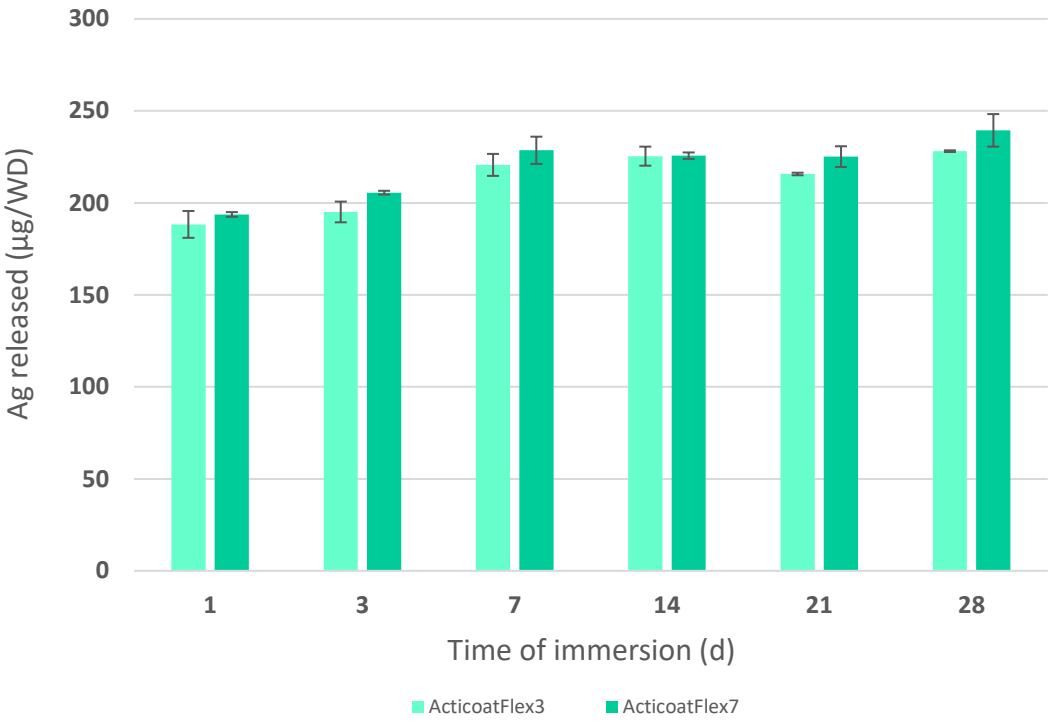

Figure 17. Leaching tests of Ag released (µg/WD) from ActicoatFlex 3 and Acticoat flex 7 immersed in artificial marine water at day 1, 3, 7, 14,21 and 28.

Leaching tests of Ag from SbD WDs immersed in soil:water extract revealed that the lowest concentration of Ag was released from PLLA-Ag at each time of immersion (range from 3 and 5 µg/WD), while the highest amount of Ag was released from PVA-AgHEC.2h WDs (range 25-34 µg/WD) (Figure 18). Similar results were observed for PVA-Ag WDs and PVA-AgHEC.1h WDs, showing releases ranging from 12 to 18 µg/WD and from 8 to 12 µg/WD respectively.

Similarly to what observed in AFW and AMW, the leaching of Ag from the commercial Ag-WDs immersed in soil:water extract were much higher than those obtained from the SbD alternatives. Acticoat Flex 7, for example, released 10 mg/WD of Ag after 28 days of immersion in the soil:water extract (Figure 19). As reported from the literature, the pH of the medium, as well as the presence of dissolved organic matter, can influence the stability of Ag NPs (Reidy et al., 2013), leading to higher dissolution of Ag NPs at lower value of pH (Liu et al., 2012; Liu & Hurt, 2010). Accordingly, the high release values of Ag from the two commercial Ag-WDs (3-10 mg/WD) can be related to the acidic pH of the soil:water extract.

From the obtained results, the SbD alternatives can be ranked as followed: PLLA-Ag>PVA-AgHEC.1h>PVA-Ag>PVA-AgHEC.2h.

Table 13. Leaching of Ag from each piece of Ag-WD immersed in soil:water extract and reported as mean ± SD in µg/WD.

Ag-WDs	**Ag content (µg/WD) Mean±SD**					
	1 d	**3 d**	**7 d**	**14 d**	**21 d**	**28 d**
PVA-Ag	28 ± 6	15±4	17 ± 2	18 ± 4	12 ± 3	14 ± 4
PLLA-Ag	4.9 ± 0.6	2.7 ± 0.1	5.2 ± 2.5	3.6 ± 0.8	3 ± 1	3 ± 1
PVA-AgHEC.1h	11 ± 4	8 ± 4	10 ± 4	11 ± 5	11 ± 7	12 ± 7
PVA-AgHEC.2h	26 ± 1	29 ± 1	30 ± 1	34.2 ± 0.6	26.5 ± 0.1	23.4 ± 0.5
ActicoatFlex 3	4889 ± 9	6182 ± 12	7108 ± 11	8126 ± 17	9236 ± 9	9776 ± 6
ActicoatFlex 7	3769 ± 2	6046 ± 3	7382 ± 6	8456 ± 2	9450 ± 3	10073 ± 4

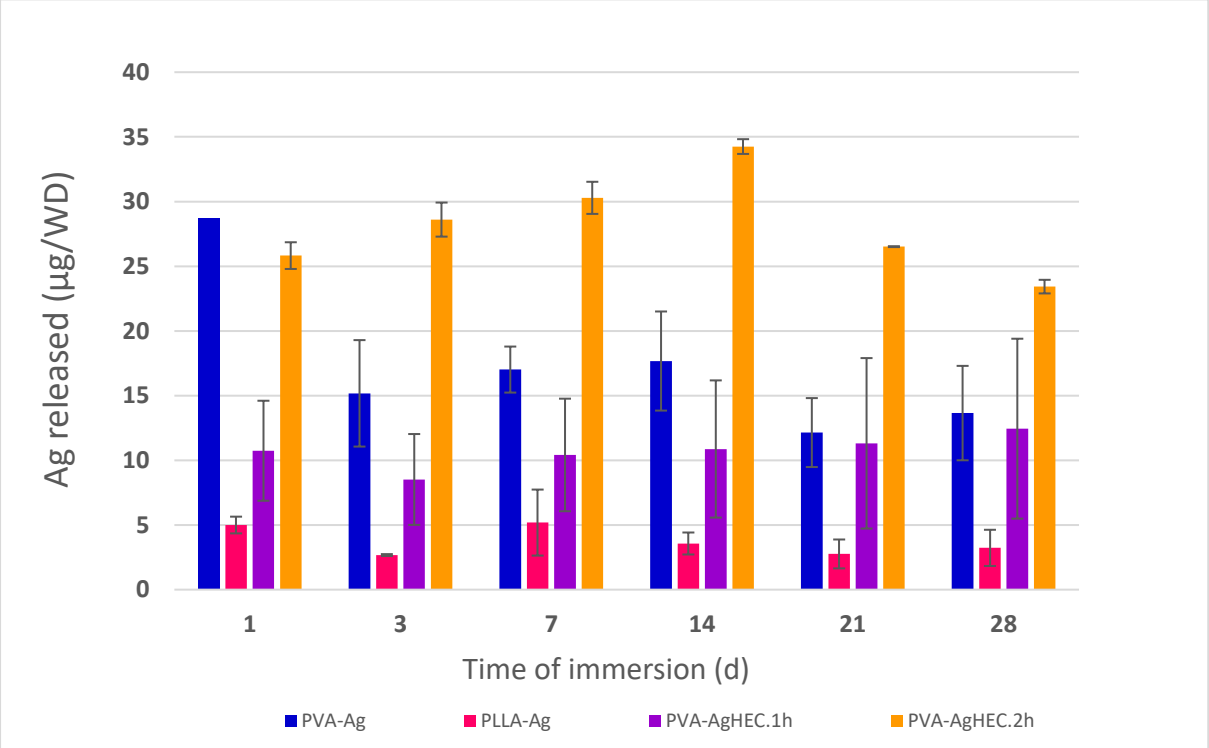

Figure 18. Leaching tests of Ag released (µg/WD) from PVA-Ag, PLLA-Ag, PVA-AgHEC.1h, PVA-AgHEC.2h WDs immersed in soil:water extract at day 1, 3, 7, 14,21 and 28.

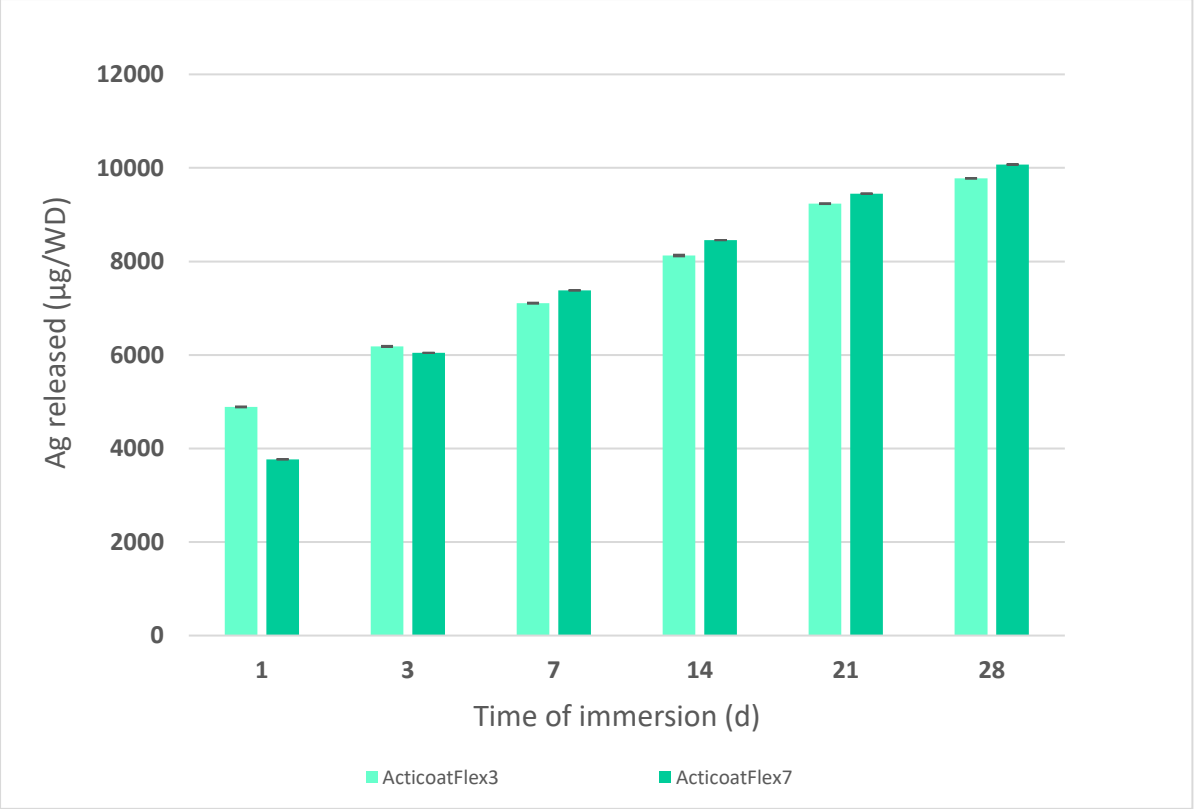

Figure 19. Leaching tests of Ag released (µg/WD) from ActicoatFlex 3 and Acticoat flex 7 Ag-WDs immersed in soil:water extract at day 1, 3, 7, 14,21 and 28.

Colloidal characterization of NPs in environmental media

The colloidal behaviour of Ag and AgHEC NPs in AFW, AMW, soil:water extract and in ultrapure water was investigated by means of DLS, ELS and CSA techniques. The overall data of sedimentation velocity, V-sed, hydrodynamic diameter, d_H, and zeta potential, ζ-pot, are reported in Table 14.

Table 14. Sedimentation velocity (V-sed), hydrodynamic diameter (d_H), zeta potential (ζ-pot) of Ag and AgHEC NPs in ultrapure water, AFW, AMW, and soil:water extract.

Sample name	NPs concentration (mg/L)	Medium	CSA		DLS		ELS	
			V-sed. (μm/s)	SD	d_H (nm)	SD	ζ-pot. (mV)	SD
Ag Sigma	100	AFW	Under LoD		421	305	-12	2
	10	AFW	Under LoD		Under LoD		Under LoD	
	1	AFW	Under LoD		Under LoD		Under LoD	
	100	AMW	Under LoD		510	87	-12	1
	10	AMW	Under LoD		Under LoD		Under LoD	
	1	AMW	Under LoD		Under LoD		Under LoD	
	100	Soil: water	0.66	0.2	1147	147	-10	1
	10	Soil: water	Under LoD		1493	208	-18.0	0.5
	1	Soil: water	Under LoD		1305	179	-4.0	0.6
	100	milliQ	Under LoD		796	134	-12	2
	10	milliQ	Under LoD		Under LoD		Under LoD	
	1	milliQ	Under LoD		Under LoD		Under LoD	
AgHEC	100	AFW	Under LoD		490	71	12.0	0.6
	10	AFW	Under LoD		Under LoD		Under LoD	
	1	AFW	Under LoD		Under LoD		Under LoD	
	100	AMW	Under LoD		730	120	7	2
	10	AMW	Under LoD		Under LoD		Under LoD	
	1	AMW	Under LoD		Under LoD		Under LoD	
	100	Soil: water	0.13	0.007	2209	329	12.1	0.6
	10	Soil: water	0.17	0.006	3024	464	-11.0	1
	1	Soil: water	0.14	0.02	1407	203	-10	1
	100	milliQ	Under LoD		970	152	6	1
	10	milliQ	Under LoD		Under LoD		Under LoD	
	1	milliQ	Under LoD		Under LoD		Under LoD	
Soil:water	Soil:water	Soil: water	0.26	0.02	1333	191	-11	1

AFW: Artificial Fresh Water, AMW: Artificial Marine Water, LoD: Limit of Detection, CSA: Centrifugal Separation Analysis, DLS: Dynamic Light Scattering, ELS: Electrophoretic Light Scattering, SD: Standard Deviation

AFW

Ag NPs dispersed in AFW were detected by DLS and ELS techniques only at the highest concentration tested (100 mg/L) with a d_H of around 420 nm and a negative ζ-pot value (-11.6 mV), the other tested concentrations were too low to be detected by these techniques. AgHEC could also be detected only by DLS and ELS at the highest concentration tested, showing a d_H similar to Ag NPs (of around 490 nm) but with a positive ζ-pot value (+12 mV), related to the presence of HEC polymer.

Comparing the behaviour of NPs in AFW and ultrapure water, d_H values of Ag Sigma NPs in AFW were observed smaller than in ultrapure water (around 796 nm) but with similar ζ-pot values (about -12 mV). Also for AgHEC NPs, smaller d_H values wer detected in AFW compared to d_H values in ultrapure water (around 970 nm), while the ζ-pot value increased from +6 to +12 mV moving from ultrapure to AFW.

As far as CSA results, transmission profiles of Ag NPs in AFW indicate that settling is occurring (Figure 20b). However, V-sed could not be measured for this sample since the initial transmission value was too high (at around 80%) – values would not be accurate. Moreover, transmission profiles of Ag Sigma NPs in AFW were similar to those detected for Ag Sigma in ultrapure water (Figure 20a). In the case of AgHEC NPs in AFW (Figure 20d), the transmission profiles showed by the CSA were similar to those of AgHEC in ultrapure water (Figure 20c) and do not permit to calculate a V-sed also for AgHEC even at 100 mg/L.

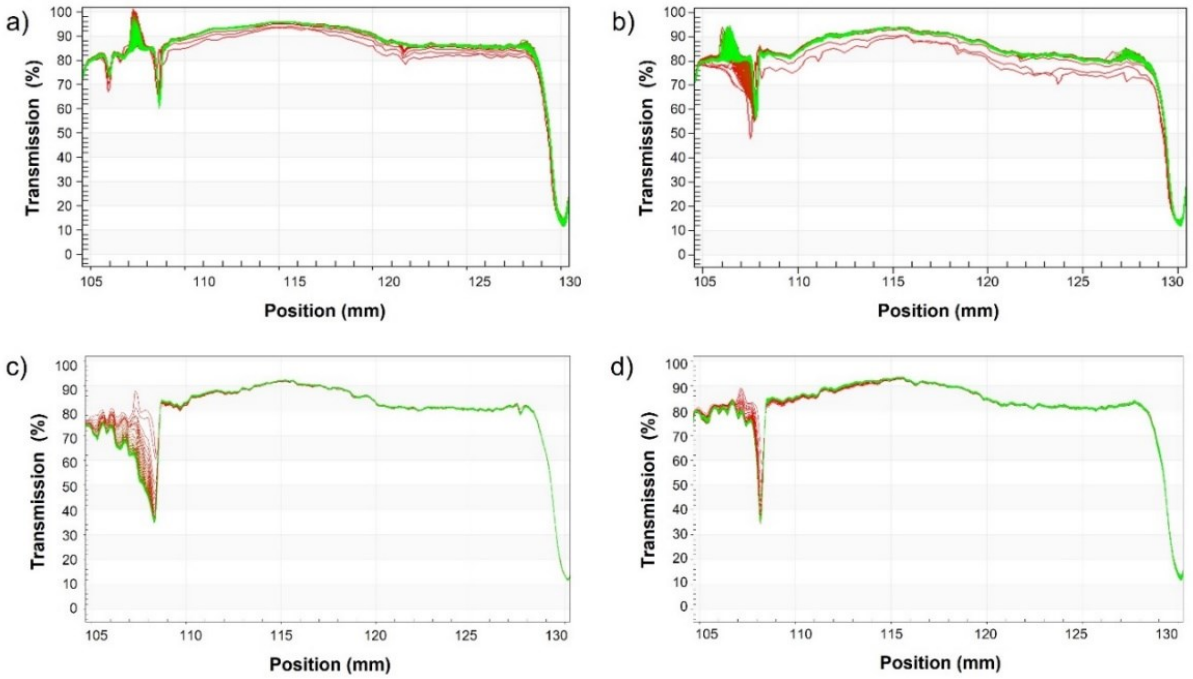

Figure 20. Transmission profiles at 100mg/L of a) Ag NPs in ultrapure water, b) Ag NPs in AFW, c) AgHEC NPs in ultrapure water, d) AgHEC NPs in AFW.

AMW

Because of the limit of detection of DLS and ELS techniques, d_H and ζ-pot values of Ag NPs in AMW were measured only at the highest concentration tested (100 mg/L) showing a d_H of around 510 nm and a negative ζ-pot value (-11.8 mV). AgHEC could also be detected only at the highest concentration tested, obtaining a d_H of around 730 nm and a positive ζ-pot value (+7.2 mV) because of the presence of positive charged HEC polymer. d_H values were slightly smaller than those obtaining at the same concentration in ultrapure water (i.e., 790 and 970 nm for Ag and AgHEC respectively), while ζ-pot values of both the dispersions at 100 mg/L in AMW were similar to those measured in ultrapure water (i.e., -12.6 for Ag and 6.3 for AgHEC). As far as CSA results, transmission profiles of Ag and AgHEC NPs in AMW at 100 mg/L (Figure 21a and 21b respectively) were similar to transmission profiles of ultrapure water (Figure 21a and 21c) and V-sed values cannot be measured for both samples. For Ag and AgHEC NPs dispersions at 1 and 10 mg/L, no measurements were obtained as the concentrations were too low to be detected by DLS, ELS and CSA techniques.

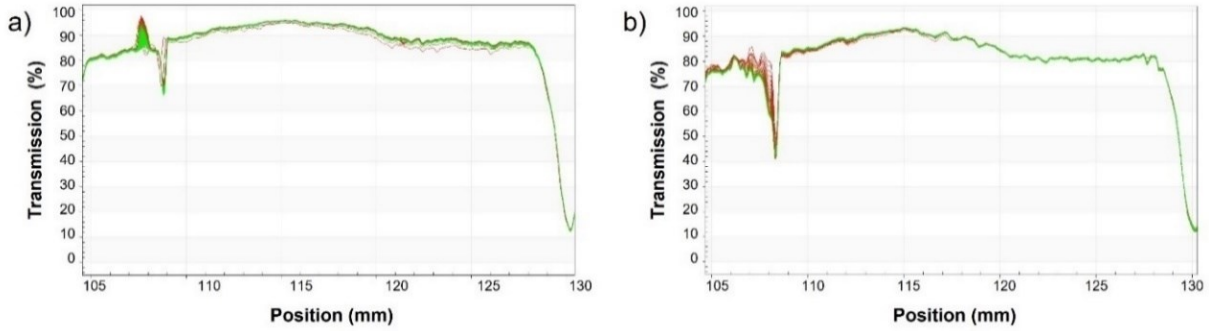

Figure 21. Transmission profiles at 100 mg/L of a) Ag NPs and b) AgHEC NPs in AMW.

Soil:water extract

Soil:water extract medium showed the presence of dispersed particles (according to the preparation protocol it can contain particles up to 50 μm). Therefore, the medium was characterized by DLS, ELS and CSA techniques without adding NPs. According to these techniques, particles with a hydrodynamic size of around 1300 nm, a negative ζ-pot value of around -7.0 mV and a V-sed value of 0.26 μm/s were observed (Figure 22a). These suspended particles can interact with NPs (e.g., heteroaggregation processes) influencing the measurements, and, depending on NPs concentration, soil particles can completely overlap the signals of the investigated NPs.

As it can be observed from Table 14, d_H values measured for both Ag NPs at 10 and 1 mg/L correspond to those of soil:water extract medium alone. A V-sed value of 0.66 μm/s was obtained only at the highest concentration tested (Figure 22b).

Once AgHEC NPs are dispersed in soil:water extract, the HEC polymer probably interacts with particles in the soil medium. Indeed, transmission profiles obtained at 100 mg/L(Figure 22c), at 10 mg/L (Figure 22d), at 1 mg/L (Figure 22e) showed a decrease of the sedimentation process of the sample, obtaining sedimentation velocity values of 0.13 μm/s at 100 mg/L, 0.17 μm/s at 10 mg/L and 0.14 μm/s at 1 mg/L. At these concentrations, hydrodynamic diameter values were higher than AgHEC in ultrapure water which can be detected only for 100 mg/L. Considering zeta potential values, the positive value of 12 ± 0.6 mV at 100 mg/L, the negative values of -11mV at 10 mg/L and -9 mV at 1 mg/L suggest that HEC was revealed at the highest tested concentration as HEC is a positively charged polymer. Indeed, a positive zeta potential value was obtained also in AFW (12 ± 0.6 mV) and in ultrapure water (6.3 ± 1 mV) at the same AgHEC concentration of 100 mg/L.

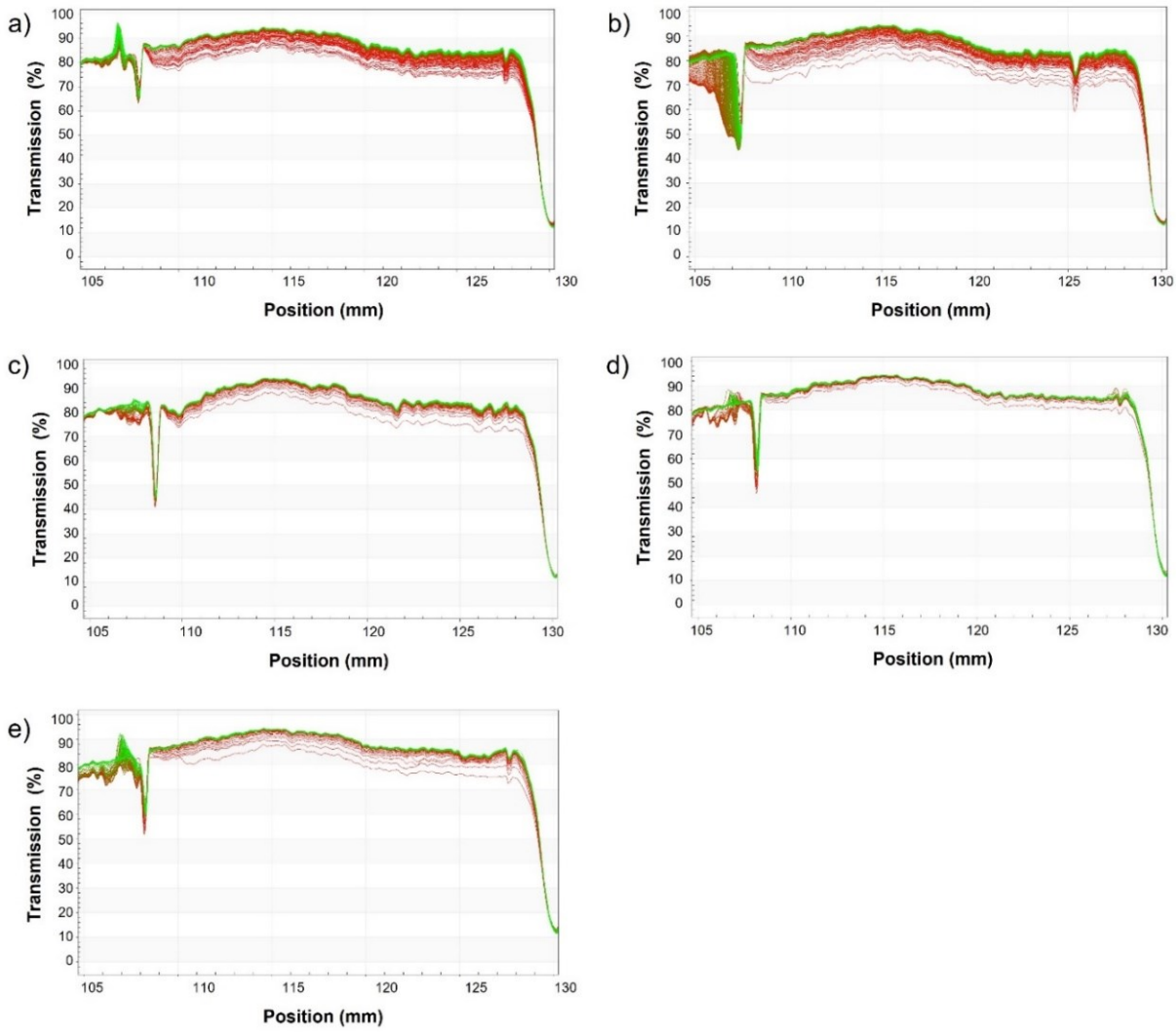

Figure 22. Transmission profiles of a) soil:water extract without NPs, b) Ag NPs at 100 mg/L in soil:water, c) AgHEC NPs at 100 mg/L in soil:water extract, d) AgHEC NPs at 10 mg/L in soil:water extract, e) AgHEC NPs at 1 mg/L in soil:water extract.

To conclude, because of the LoD of DLS, ELS and CSA techniques, colloidal characterization of NPs at the lowest investigated concentrations (1 and 10 mg/L) did not provide any results, confirming that colloidal characterization of Ag at the concentrations released from Ag-WDs cannot be performed using these techniques.

5.3.5 SbD evaluation: final results

For the identification of the safer solution, the ranking of Ag-WDs alternatives for each SbD criterion is reported in Table 16, where SbD alternatives are coloured from the dark green to

the light green. As can be seen from the table, the safer solution among the five SbD alternatives is PVA-AgHEC.1h. Indeed, this Ag-WD is the best alternative in antimicrobial efficacy, cost- effectiveness and leaching of Ag in AFW criteria and ranks very well also in leaching of Ag in AMW and in soil: water extract criteria.

Table 16. Ranking of the Ag-WD alternatives according to the investigated SbD evaluation criteria.

Wound Dressing	**SbD criteria**				
	Antimicrobial activity	**CE**	**Leaching tests in AFW**	**Leaching tests in AMW**	**Leaching tests in S:W**
PVA-Ag					
PLLA-Ag					
PVA-AgHEC.1h					
PVA-AgHEC.2h					

CE: Cost-effectiveness, S:W: Soil-water extract

5.4 Discussion and conclusion

In the current work, a SbD procedure for the identification of the safer alternative among five Ag-WDs was presented. Moreover, the procedure was also applied to two commercial wound dressings in order to compare SbD alternatives with Ag-WDs already on the market.

The procedure takes inspiration from the GoNanoBioMat SbD approach (Schmutz et al., 2020) developed for nanomedicines and it has been adapted to allow for the comparison in parallel of several Ag-WDs alternatives which are classified as medical devices. It is built on three main steps (i.e., material design, material characterization and SbD evaluation) where SbD objectives were identified and ad-hoc tests were selected according to them, namely i) maximisation of the antimicrobial activity of the Ag-WD, ii) reduction of possible Ag released into the environment, iii) optimization of the cost-effectiveness of the Ag-WD.

The application of the SbD procedure supported the selection of the best SbD alternative according to the following criteria: mechanical strength of the Ag-WDs, their antibacterial effect, the cost-effectiveness of each Ag-WDs, and the release of Ag from Ag-WDs immersed in environmental media. For each of them, Ag-WDs were ranked allowing to identify PVA-AgHEC.1h as the safer alternative.

Mechanical strength of Ag-WDs has been investigated through SEM analysis of Ag-WDs before and after 24 hours of immersion in synthetic sweat. Results revealed the importance of performing such screening tests at early stage of product development: in the current study, indeed, the low mechanical strength of PLLA fibers used in PLLA-AgHEC WDs allowed to discard it as a promising alternative.

Then, antibacterial tests of Ag-WDs in *E. coli* allowed to rank the Ag-WDs based on their antimicrobial efficacy and to demonstrate the antibacterial action of HEC polymer used as coating in Ag NPs.

The cost-effectiveness (CE) of Ag-WDs has been evaluated with the ratio between the concentration of total Ag content in Ag-WDs and the Ag released during the period of application. This criterion permits to characterise a key feature for the selection of the best alternative: the identification of the Ag-WD that presents the best balance between total Ag content and released Ag.

As WDs containing Ag NPs are produced for a controlled release of silver during their application on the ulcered skin, remaining Ag NPs can be released in the different environmental compartments during the end-of-life of the product (if not properly managed). In this regard, leaching tests of Ag from Ag-WDs immersed in AFW, AMW and soil:water extract were performed, revealing the heterogeneity of material behaviour in environmental systems. Indeed, release of Ag from each SbD Ag-WD is not constant in time, due to possible Ag NPs transport and transformation processes (e.g., sedimentation, hetero/homo-aggregation, agglomeration) in the investigated environmental media, which could explain the not linear variation of total Ag over time. To support leaching tests, colloidal stability of Ag and AgHEC NPs dispersed in environmental media has been investigated to assess the fate and behaviour of NPs. Results revealed that while ions in both AFW and AMW do not interact with Ag and AgHEC NPs, in the soil:water medium suspended particles interact with NPs

through heteroaggregation processes influencing the measurements and, depending on Ag NPs concentration, soil particles completely overlap the signals of the investigated NPs. However, at the concentrations of Ag close to those released from Ag-WDs, colloidal characterization could not provide any results because of the LOD of the adopted instruments.

The developed SbD procedure permits to highlight the importance to reduce Ag content (which was added in large excess in commercial Ag-WDs) while maintaining an effective antimicrobial efficacy.

However, as the presented procedure allowed a preliminary analysis for the selection of the safer alternatives among the investigated Ag-WDs, more in depth analysis on human health and environmental criteria need to be performed in the future on the most promising SbD alternatives.

Indeed, as the antimicrobial effect of Ag is exerted by Ag^+, Ag NPs and Reactive Oxygen Species (ROS) (Nešporová et al., 2020), an in depth analysis on the formation of ROS, stress response or cytotoxicity could be done to better scrutinize the safety assessment of Ag-WDs. Cytotoxicity of AgHEC has been investigated by Marassi et al., 2019 showing a lack of acute inflammatory response through the quantification of TNF-α, IL-6, IL-8 and IL-1β cytokines. However, further investigation of toxicological effects also of Ag Sigma NPs as well as of the entire Ag-WDs following standard protocols, such as ISO 10993-22: 2017, are needed. Moreover, as explained in the review of Mennini et al., 2016, the evaluation of efficacy and quality of wound dressings requires the use of specific test methods (e.g., testing of moisture vapour transmission rate, absorptive capacity, waterproofness) depending on the type of WDs (e.g., polyurethane foam, hydrogel, alginate) following the BS EN 13726-1:2002, BS EN 13726-2:2002, BS EN 13726-3:2003., and this type of assessment could be consider to complement the current study.

Considering additional environmental criteria, further physico-chemical characterization could help to study the behaviour of NPs and ionic Ag in environmental media (e.g., AgCl, AgS formation or Ag NPs interaction with Dissolved Organic Matter). Moreover, generating ecotoxicological data about the investigated Ag NPs (i.e., Ag Sigma and Ag coated with HEC polymer) could be useful to predict possible effects on aquatic and terrestrial organisms.

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Chapter 6

Using systemic stock-flow diagrams to visualize theranostic approaches to solid tumours in the context of benefit-risk analysis

Contents mostly included in:

Cazzagon V.*, Romano A., Gonella F., 2021. *Using stock-flow diagrams to visualize theranostic approaches to solid tumors in personalized nanomedicine.* Frontiers in Bioengineering and Biotechnology, Nanobiotechnology, 9:604, https://doi.org/10.3389/fbioe.2021.709727

Specific contribution of the PhD candidate:

The work performed within the PhD thesis included:
- a literature review on System Thinking approach and stock-flow diagrams, and also on the investigated material.
- the collection and selection of the information related to the investigated material needed for the development of the stock-flow diagram.
- the development of the stock-flow diagram.

The developed diagram was then further revised by the oncologist Alessandra Romano (adding clinical aspects) and by the professor Francesco Gonella (adding specific details on stocks, flows and feedbacks).

6.1 Introduction

The assessment of benefits and risks derived from the administration of a drug is a complex process required during the market authorization of a new drug (EMA, 2007), which should be based on a detailed understanding of the benefits of a treatment, in-depth knowledge of the associated safety profile and large availability of detailed clinical trial or observational data to support the decision-making (see paragraph 3.2.3).

Several methods and models are suggested to support and facilitate an effective and transparent benefit-risk analysis by the European Medicines Agency (EMA) such as Number

Needed to Treat/Number Needed to Harm (NNT/NNH), the “principles of three”, TURBO (Transparent Uniform Risk-Benefit Overview) model and Multi criteria decision analysis (MCDA) (EMA, 2007). However, also because of the difficulty of understanding what are the driving forces that can provoke adverse effects and how they act in the different biological systems, each model presents some limitations. Several approaches have been developed in recent years to facilitate the benefit-risk analysis and to better define the decision context, the drivers of the decision, and the associated uncertainty, such as the PrOACT-URL and Universal Methodology for Benefit Risk Assessment (UMBRA) frameworks developed by EMA. However, no single framework has been agreed upon among all regulators (Pignatti et al. 2015).

Moreover, while the authorisation of a medicine is based on an overall positive balance between the benefits and risks at population level, each patient is different and before a medicine is used, doctors should judge whether this is the right treatment option based on the information available on the biomedical products and on the patient’s specific situation (EMA 2019).

In the context of nanomedicine, difficulties in acquiring a comprehensive and detailed knowledge about the benefits and potential toxicity of a product could lead to a reduction of nanomedicines receiving marketing authorization. This is particularly true in the case of nanotheranostic agents (e.g., the combination of therapeutic and diagnostic capabilities using a single nano-based biomedical product) (Theek et al. 2014), where diagnostic and therapeutic procedures that operate at even slightly different temporal and spatial scales enhance the complexity of evaluating risks and benefits.

Some nanotheranostic agents are currently at Phase I and Phase II clinical trials (Singh et al. 2020; Verry et al. 2020) and have started demonstrating their efficacy in diagnosis but lack therapeutic competence or vice versa (Alshehri et al. 2021). Therefore, there is the urgent need to investigate not only their safety profile in both early and advanced phases of clinical trials (Singh et al. 2020), but also to understand how these innovative products can be personalized (so called “personalised nanomedicines”), considering inter-individual variability in therapy selection, treatment planning, objective response monitoring and follow-up therapy planning based on the specific characteristics of the tumour tissue (Degrauwe et al. 2019; Keek et al. 2018; Ryu et al. 2014). Indeed, as exhaustively explained in Bielekova et al. 2014, systems biology principles represent a unique opportunity to predict complex diseases

in comparatively small cohorts of patients through the identification of functional networks at the organism/patient-level. Moreover, as health systems are self-organizing and tightly linked, constantly changing and governed by positive or negative feedbacks (WHO, 2009), it is necessary to identify and represent the complexity of administration of personalised nanomedicines in a holistic perspective.

For this reason, the complexity of the choice of a patient-specific therapy of a nanotheranostic agent and its subsequent emerging benefits and/or adverse effects is investigated in this chapter through the adoption of a System Thinking (ST) perspective.

The ST approach shifts the attention from the study of local events, in terms of causes, effects, and mutual relationships, to the study of the systemic patterns from which they emerge, describing the change in the hierarchical feedbacks structure that gives access to the operational configurations of the system as a whole.

If we look at the ST origins, analytical tools based on stocks and flows representations have been developed since the 70s by Jay Forrester at the Massachusetts Institute of Technology mainly focused on Social Systems (Forrester 1971). Afterward, ST approaches have found application in several other fields, as reported, for example, for business (Sterman, 2002), energy and sustainability (Higgins 2015; Kutty et al. 2020), ecology (Assaraf and Orion 2005), biogeochemistry (Haraldsson and Sverdrup 2013), communication (Gonella et al. 2020) and medicine (Romano, Casazza, and Gonella 2021). Nevertheless, the use of ST approach in nanomedicine-related studies is still lacking.

In this chapter, in order to demonstrate the applicability of the ST approach in nanomedicine, a ST diagram was developed to visualize theranostic approaches of magnetite NPs to solid tumours in personalized nanomedicine.

In the oncologic context, magnetite (Fe_3O_4) NPs can be used as contrast agent in Magnetic Resonance Imaging (MRI) for diagnosis purposes, while in therapeutic nanomedicine they can be accumulated in cancer cells through the enhanced permeability and retention effect (Nuzhina et al. 2019), then generating heat upon the application of an alternate magnetic field in hyperthermia treatments (Vallabani and Singh 2018). The combination of therapeutic and diagnostic capabilities using a single nano-based biomedical product addresses the administration of magnetite NPs to i) obtain in vivo imaging of the tumour site, ii) treat the tumour site after the target drug delivery, iii) induce cancer cell death by hyperthermia.

6.2 System thinking approach and its elements

As extensively described in Odum and Odum (2000), the comprehensive ST approach includes the development of three scales of modelling:

1. Structural graphic model, in which the fundamental structure that determines the system dynamics is diagrammed in terms of stocks, flows, and processes.

2. Analytical model, in which formal relationships are established between the system's components, allowing to define a set of differential equations able to describe the systemic behaviour even for situations difficult to observe experimentally.

3. Computational model, which transforms the set of interconnected differential equations into a simulator, studying how the system dynamics is affected by a change in external parameters, driving forces, perturbations, or, in the case of disease systems, the application of specific therapies.

In this work, for the first time a structural graphic model for the representation of theranostic modalities of a nano-based biomedical product is presented. The development of this model is the first step for the other two, which will be made possible when clinical data on the administration of magnetite NPs containing anticancer drug used as theranostic agent start to become available. In the following sections, an introduction of the structural ST diagram and its application to the investigated case study is presented, to guide the reader through the final diagram.

The structural stock-flow representation of the system thinking-based approach is set up following the procedure:

1. Identification of a set of stocks.
2. Choice of a proper boundary.
3. Identification of the flows connecting the stocks, also with the external environment.
4. Identification of the processes occurring within a system.

Figure 25 shows the main symbols used in stock-flow diagrams based on the energy language (Odum and Odum 2000), where shields indicate the stocks, line arrows the flows, solid arrows the processes that are always activated or controlled by a stock inside or outside the system

(i.e., arrow coming from outside the system), and the smooth grey rectangle the system boundary. For the second principle of thermodynamics, energy is partially lost in any physical process, and this is represented by the flow going down to the earth symbol (heat sink).

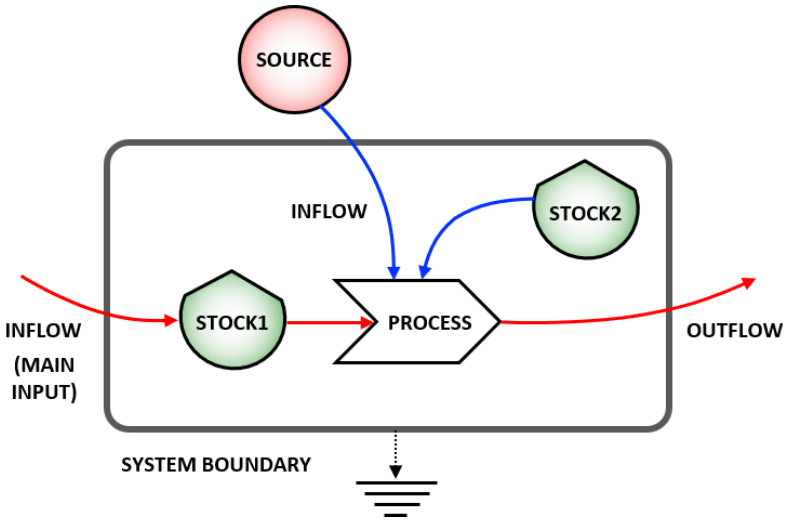

Figure 25. Representation of a stock-flow system. Stock 1 undergoes a process due to the action of both Stock 2 and an external source.

6.2.1 Stocks

Stocks are elements represented by an extensive variable (i.e., material, energy, information). A stock changes over time only through the action of flows (i.e., inflows and/or outflows), and may therefore act as delay or buffer or shock adsorber for the system (Sterman 2002). The stock content must be countable extensive state variables Q_i, $i=1, 2,...,n$, that constitute an n-tuple of numbers that at any time represents the state of the system. The choice of the set of variables depends on the hierarchical level of the desired description, as well as on the overall purpose of the study.

Stocks must be chosen respecting some requirements:

1. The number of the stocks must be as low as possible to describe the state of the system for the prescribed purposes.
2. It must be possible to describe any relevant macroscopic in terms of stocks interactions.

3. Any system change (either detectable from the external or not) must correspond to a change in the n-tuple of state variables.
4. Stocks should be measurables, or at least a set of plausible values at a certain time should be conceivable, to study their evolution.

A stock may represent either a physically located set of a variable, or a virtual set of elements that play a specific role in the system dynamics, even without having the corresponding location in the real space.

The choice of the stocks relevant for the case at issue is a fundamental step in the stock-flows approach. When clinical data are available, a value at t_0 must be assigned to each stock in order to make a quantitative analysis. The determination of these initial values can be performed by either directly measuring them or by determining a "plausibility interval". In this latter case, a sensitivity analysis is performed to validate the model testing the system response within the selected interval of values.

6.2.2 Boundary

A proper choice and definition of the systemic boundary is an important task since the boundary defines the objective of the systemic study depending on the main inflows and the system outputs. In ST, the boundary is an abstract element, possibly extended in both space and time, and has the main role of isolating the elements which are necessary to give an exhaustive description of the dynamics of the system at the chosen level of the study and to focus on the relationships between the internal elements (Brown, 2004). The choice of the boundary will reflect also the hierarchical level of the feedbacks that will depend on the time-span of the diagram description.

6.2.3 Flows

In a stationary state of the system, stocks values are constant and may change their values through inflows and/or outflows, represented by arrows entering or exiting stocks and expressed as *dQ/dt*.

In biological systems, flows can be flows of matter (energy), that constitute the mechanism by which a stock value may change in time, and flows of information, responsible for the control action exerted by stocks on the processes that in turn control the flows. The control flows network is a fundamental aspect in ST diagramming since their action is responsible for feedbacks and causal loop formation at different time scales (Haraldsson 2004). The pattern of feedbacks is therefore the feature that defines the system dynamics. For example, the unregulated proliferation of tumour cells in the human body can be represented by reinforced feedback, as represented in figure 26, when tumour cells growing and multiplying in an uncontrolled manner (Cooper 2000). An increase of tumour cells (TC) in the stock will then determine an exponential proliferation of TC at time t_1.

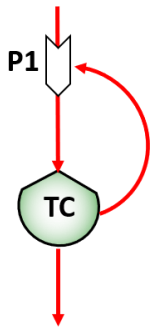

Figure 26. Reinforcing feedback of the tumour cells stock (TC) on the birth process (P1), giving rise to the proliferation.

Flows are described by phenomenological coefficients that represent how much of the contribution from one or more stocks will be effective in their interaction on the process. Therefore, these coefficients represent the dynamics of the system to point out the interconnection network between its operational elements. In fact, it is important to underline that ST approach is not interested in representing the physical mechanisms of feedback controls, but in drawing the interactions among them. A detailed description of the conceptual basis of the quantitative setting up of stock-flow diagrams may be found for example in Odum & Odum, 2000, where the counter-intuitive aspects of the approach are examined in many different systems.

6.2.4 Processes

Processes represent the interaction between the stocks and determine the dynamics of the system. Processes are capable to alter – either quantitatively or qualitatively – a flow, by the action of one or more system elements. Since the system state is a collection of stock values, and the only way to change the value of a stock is by acting on its in/outflows, processes are located along the flow lines. In general, the location of a process in the diagram does not have any correspondence in a physical location in real space. Moreover, a process must be activated by another driver as flows of information or matter control the occurring processes and thus the value and/or nature of the flows.

6.2.5 System thinking diagram of theranostic approach combined with personalized nanomedicine of solid tumours using magnetite NBMs

The stock-flow diagram is developed to represent the complexity of the use of Fe_3O_4 NBMs as a theranostic agent in solid tumours based on the personalized nanomedicine perspective. In particular, the magnetite case study consists of (1) a magnetic core of Fe_3O_4 NBMs coated with polyethylene glycol (PEG) and poly co-glycolic acid (PLGA), (2) a sustained released anticancer drug, and (3) immune system cell loading. This product can be classified as Advanced Therapy Medical Products (ATMPs), which constitute a class of innovative pharmaceuticals based on emerging cellular and molecular biotechnologies for somatic cell therapy (Regulation (EC) No 1394/2007) and patient-specific products.

Stocks, flows, and processes were selected based on information collected from the literature on the personalized nanomedicine and theranostics modalities of iron oxide NBMs. The descriptions of stocks and processes selected for the diagram on theranostic approach, combined with personalized nanomedicine of solid tumours using magnetite NBMs, are reported in table 18. All the stocks are countable variables. Immune system (IS) and the bloodstream (BS) are regarded as systems since their action involves different variables which are not essential for the overall description of the system at issue. The magnetic field (MF) and Magnetic Resonance Imaging (MRI) are regarded as sources of energy and represented by a circle.

Table 18. Description of the selected stocks, processes, sources, and systems and their abbreviations used in the final diagram.

Abbreviation	Type of element	Description	References
IC	Stock	Immune cells intravenously collected from the patient	Galli et al., 2021
NBMs	Stock	Magnetite NBMs coated with PEG and PLGA	Ghazanfari et al., 2016
TC	Stock	Tumour cells	Galli et al., 2021
D	Stock	Anticancer drug	Douziech-Eyrolles et al., 2007
ATMP	Stock	ATMP is formed when IC, NBMs and D are uptake by immune system	Regulation (EC) No 1394/2007
ATMP+TC	Stock	ATMP bonded with TC	Mura & Couvreur, 2012
i	Stock	Medical knowledge related to i) theranostic modalities and personalized nanomedicine, ii) information collected from diagnosis, iii) information needed for the selection of specific therapy	Comte et al., 2020; Bielekova et al., 2014
ROS	Stock	Reactive Oxygen Species generated by the hyperthermia process and outside the system	Aggarwal et al., 2019 Slimen et al., 2014
IS	System	Immune system	Le et al., 2019
BS	System	Bloodstream	Le et al., 2019
MF	Source	Magnetic Field	Mura & Couvreur, 2012
MRI	Source	Magnetic Resonance Imaging	Revia & Zhang, 2016; Mura & Couvreur, 2012
Injec.	Process	Injection administration	Mura & Couvreur, 2012
Upt.	Process	Uptake of NBMs from immune systems	Mura & Couvreur, 2012
Activ.	Process	Activation/targeting of the ATMP on the TC	Dadfar et al., 2019
Hyp.	Process	Hyperthermia	Ansari et al., 2018; Dadfar et al., 2019
Bioim.	Process	Bioimaging of the TC	Revia & Zhang, 2016; Mura & Couvreur, 2012
Apop.	Process	Apoptosis of the TC	Hou et al., 2014; Goya et al., 2008; Jagtap et al., 2020

6.3 Results

6.3.1 System Thinking diagram

In figure 27, the final diagram representing the theranostic approach combined with personalized nanomedicine of solid tumours using magnetite NBMs coated with PEG and PLGA is presented. In red flows of mass, in green flows of energy, and yellow flows of information are represented, where dashed lines indicate the controls exerted by the stocks on the processes.

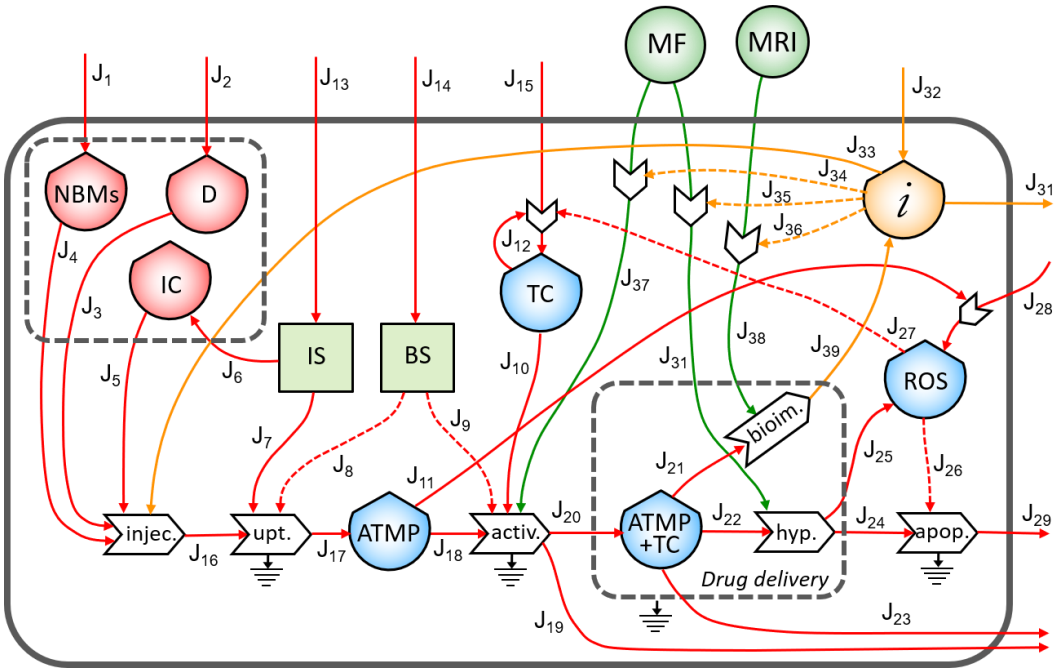

Figure 27. Representation of the theranostic approach of the magnetite NBMs to solid tumour.

NBMs (J1) and anticancer drug (J2) are the main inflows of the diagram. A specific quantity of NBMs (J4) and drug (J3) are intravenously administered together with immune cells previously sampled from the patient's blood (J5). In healthy people, the immune system plays important roles in controlling the growth of malignant cells while in cancer patients can even facilitate the growth of tumour cells (Le et al. 2019). For this reason, a quantity of immune cells needs to be carefully sampled (J6) to provide an efficient uptake process. The uptake process transforms the injected medicinal product (J16) into one outflow represented by the

ATMP bioavailable in the blood (J17), which is controlled and activated by both the immune system (J7) and the bloodstream (J8). Inflows of the bloodstream and immune system (J13 and J14) are coming from outside of the system. However, if the ATMP has low efficacy, the presence of the ATMP in the blood can activate the proliferation of ROS (J11) which may cause the activation of the proliferation of tumour cells (Aggarwal et al. 2019). The stock of tumour cells is formed by an inflow (J15) and a feedback loop (J12), which represents the uncontrolled proliferation of tumour cells (as mentioned in section 2.3). The accumulation of the ATMP (J18) at the tumour site (J10) is based on drawing it to the tumour site by using an external magnetic field (Revia and Zhang 2016) (J37) in the bloodstream, that activates the targeting/activation process (J9). Depending on the efficacy of this ATMP, a small quantity of this product may undergo the clearance process by the reticuloendothelial system without reaching the tumour site (J19) (Yu & Zheng, 2015).

During the formation of the stocks of ATMP and TC, the release of anticancer drug at the tumour site is represented by the small grey box (drug delivery). Then, under alternating MF (J38), magnetite NBMs on the tumour site (J22) can transform the electromagnetic energy into heat (hyperthermia process) causing localized heating of the tumour cells (J24) and thus triggering the commitment to apoptosis of cancer cells (Goya et al. 2008; Jagtap et al. 2020) and their death (J29). The apoptosis process of tumour cells can be generated not only as a consequence of heating of tumour site but also activated by ROS production (Hou et al. 2014) (J25). Indeed, in the diagram, the commitment to apoptosis of tumour cells is activated by the flow of ROS (J26). However, as some ROS can diffuse freely across cell membranes, they can mediate toxic effects far from the site of ROS production (Slimen et al. 2014), also activating the proliferation of tumour cells (J27) (Aggarwal et al. 2019).

The formation of the stock of ATMP and TC (J21) can also permit to perform imaging of the tumour site and real-time treatment monitoring of therapeutic drug delivery using MRI (J39), thereby adjusting treatment methods (Revia and Zhang 2016). Indeed, a flow of information is generated from the bioimaging process (J30) which constitutes, together with the medical knowledge of healthcare personnel (J32), the main inflow of the stock of information. All the collected information is then used i) to activate the hyperthermia process by setting the alternating MF properly (J35), depending on the morphological properties of the tumour tissues; ii) to activate the following bioimaging process (J36), and iii) to set the magnetic field

during the activation process of ATMP on TC (J34). Moreover, all the knowledge and considerations related to the inter and intra-patient variability are then used to define the quantity of the drug to be injected to increase the efficacy of the following therapy (Comte et al. 2020) (J33). Information collected during theranostic activities will be also used outside the system for further research (J31)

6.3.2 Feedback loops of the System Thinking diagram

In the ST diagram, five reinforced feedbacks were identified. The first is represented by the proliferation of the tumour cells as explained in Figure 26, while the other four are related to the personalized nanomedicine concept.

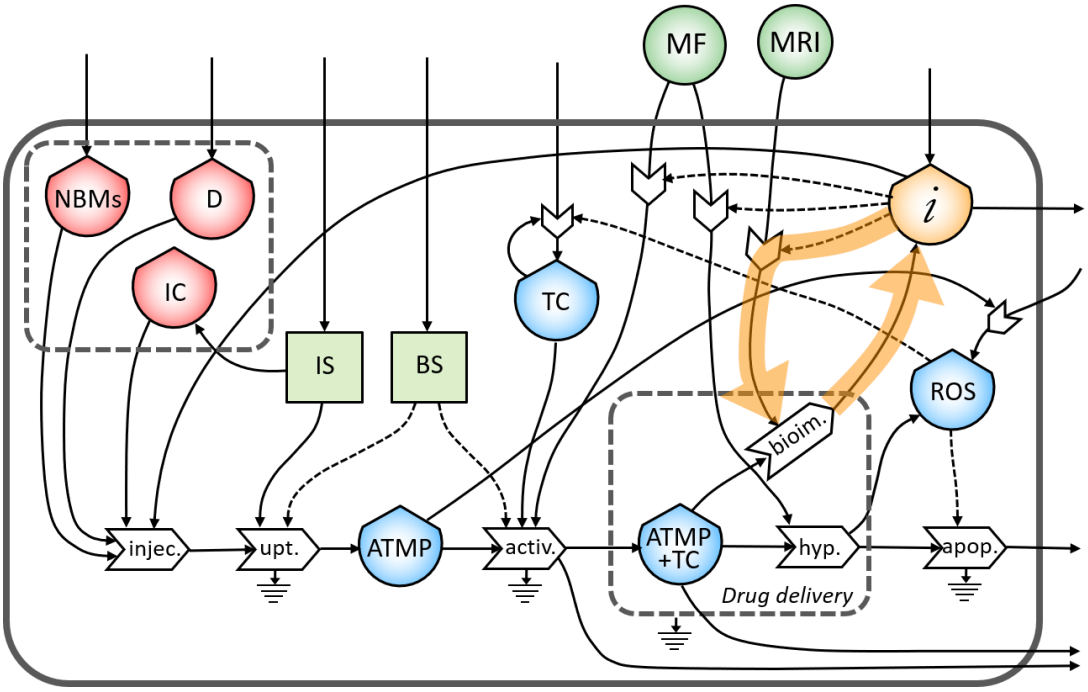

Figure 28. Representation of the feedback loop of the flow of information on the bioimaging process

As underlined in figure 28, the bioimaging process permits the generation of a flow of information related to the morphological characteristics of the tumour site. This flow creates a feedback loop of information necessary to tune the MRI operation itself.

The same flow of information coming from bioimaging process is useful also to tune a subsequent administration of the ATMP depending on the morphological characteristics of the tumour site (Figure 29).

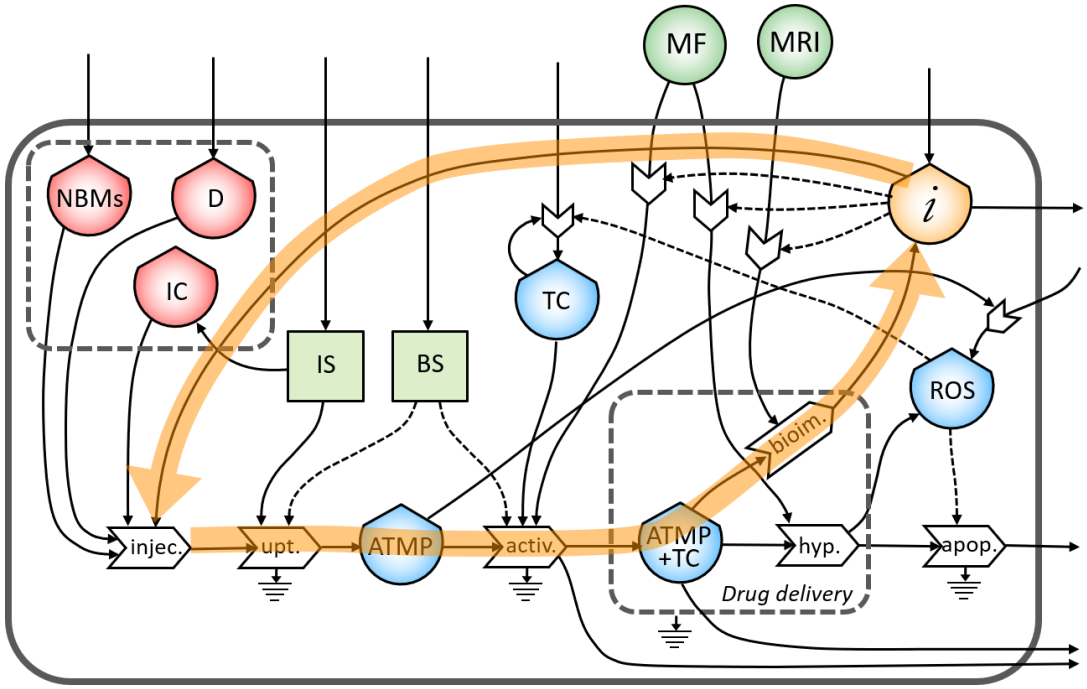

Figure 29. Representation of the feedback loop of the flow of information from the bioimaging to the injection process.

Moreover, information collected during the bioimaging is extremely useful also during the targeting of the magnetic NBMs to the tumour site through the application of a specific MF, as underlined in figure 30.

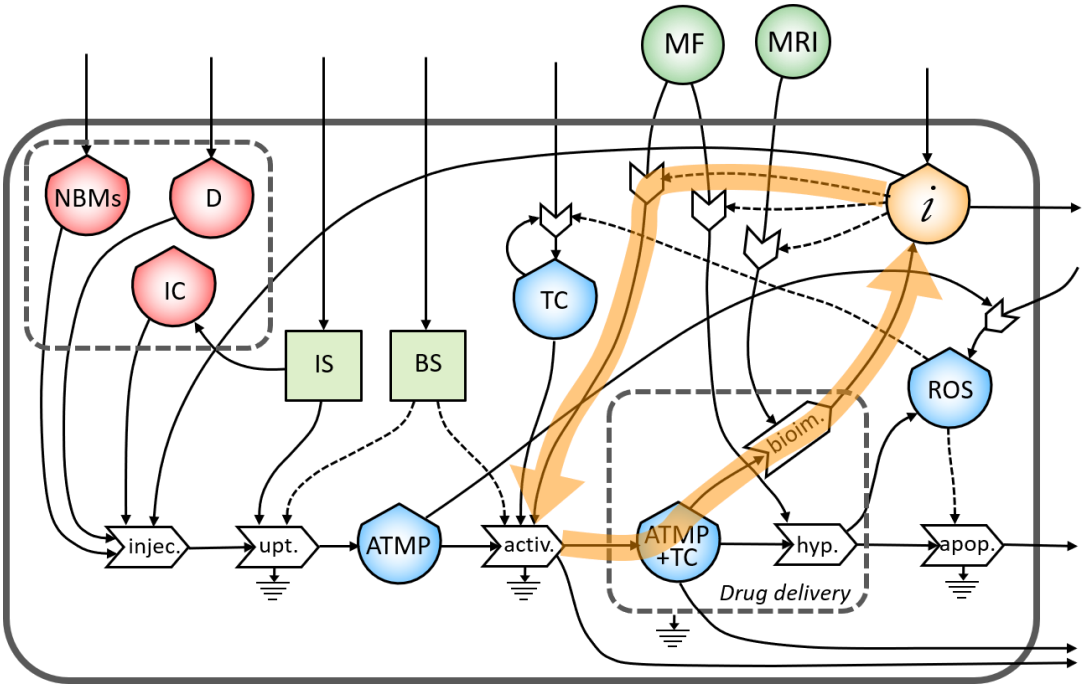

Figure 30. Representation of the feedback loop of the flow of information on the activation process

During the treatment of the tumour cells in the hyperthermia process, high levels of ROS are produced by the increased metabolic activity and mitochondrial dysfunction (Liou and Storz 2010), which can lead to the proliferation of tumour cells (figure 31). This feedback loop represents the theranostic activities of this ATMP. Indeed, the correct administration of this product permits the identification of the tumour site as well as treating it minimizing the proliferation of tumour cells.

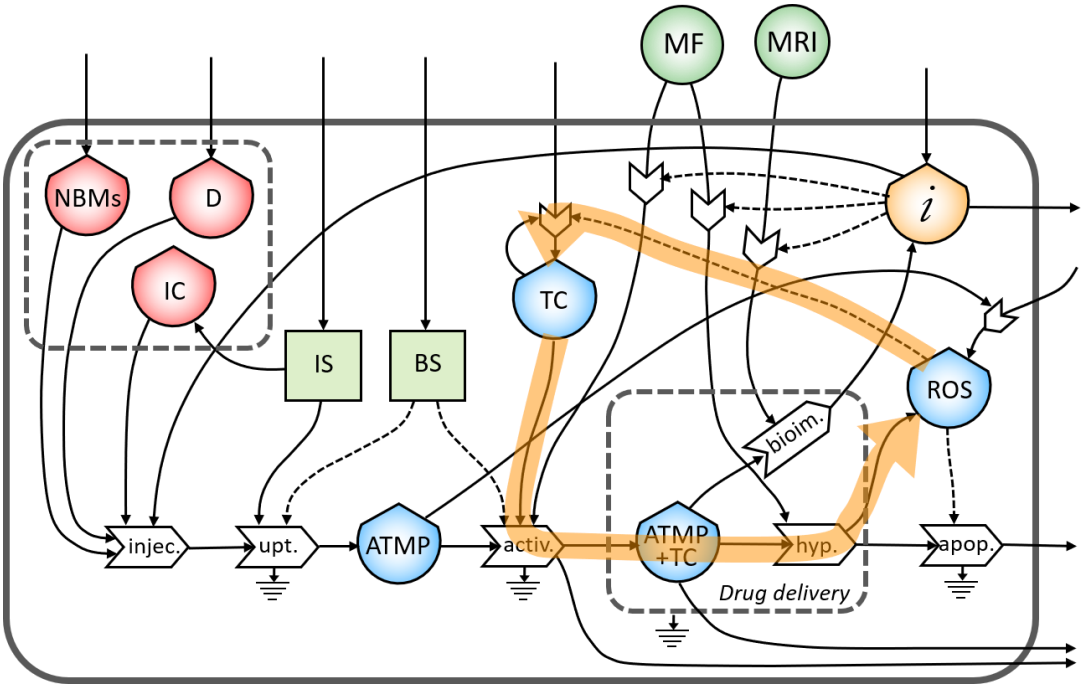

Figure 31. Representation of the feedback loop of the flow of Reactive Oxygen Species (ROS) in the proliferation of tumour cells (TC)

6.4 Discussion and conclusion

In this chapter, the application of a ST theory in personalized nanomedicine has been demonstrated as a support to benefit-risk analysis. More specifically, the ST approach has been considered to study the interconnection between diagnosis and therapy of solid tumours using a single nano-enabled biomedical product (so called nanotheranostic agent) (Theek et al. 2014) through the development of a stock-flow diagram. The investigated nano-product is a dispersion of Fe_3O_4 NPs coated with PEG and PLGA containing an anticancer drug, which is not yet commercialized medicinal product.

In the past, several methods have been developed to quantify biological networks, for example, flux balance analysis (FBA) (Lee et al., 2006), metabolic flux analysis (Lagziel, Lee, and Shlomi 2019), and quantitative systems pharmacology (QSP) (Balti et al. 2021; Chelliah et al. 2021; Wang et al. 2020). However, the dynamic systems are not sufficiently comprehensive for generating a large-scale model and could provide only a partial overview of the resulting benefits and risks. The whole complexity of anticancer nanomedicine was also suggested in the work by Sun et al., 2020, where authors underlined the need to carefully evaluate the

efficacy of nano-enabled anticancer drugs considering the tumour heterogeneity from a systemic point of view as, otherwise, benefits could not outweigh adverse effects.

For this reason, in the current study, a ST top-down approach has been followed to represent the self-organized system, through which the global dynamics of the systemic patterns may be obtained using an analytical representation of the stocks, flows, and processes in different systemic time-scales.

For the development of the presented diagram, no specific tools were used for the identification of flows and stocks. Indeed, the ST approach permits to develop several systems representing the same complex system but a different level of hierarchy. The structure of the presented ST diagram forces the system toward a limited set of possible configurations at the selected level of complexity, from which important feedback loops emerge, such as (i) how the personalized nanomedicine can help in the diagnosis and treatment of tumour sites, (ii) what could affect the proliferation of tumour cells, and (iii) how the obtained information can help in the choice of subsequent treatments and/or diagnosis.

Missing clinical data in the literature related to coefficients needed for the quantification of selected stocks, flows, and processes did not permit yet to simulate the dynamic behaviour of the system using a simulator, as analytical and computational models require the use of specific inventories on clinical data (Romano et al. 2021).

The strength of the presented ST diagram is its ability to clearly communicate the network of feedbacks. Indeed, the interconnections between stocks and flows may therefore shed some new light on how to manage the complexity of a disease, since a correct identification of the accessible dynamical patterns may allow finding the proper leverage points for intervening (Meadows 2008), especially in the oncologic context. Moreover, the ST diagram demonstrates the differences between personalized medicine and traditional medicine by the stock and flow of information. Indeed, the reinforced feedback of flows of information from the diagnosis to the therapy is the representation of the novelty of the personalized nanomedicine, which offers the opportunity to enhance the efficacy of a drug using patient-specific knowledge through the choice of the best temporal and spatial scales.

The presented ST diagram also allows to investigate system configurations in response to external driving forces (e.g., administration of other drugs), with the aim at understanding

and designing even safer personalized nanomedicine. The ST diagram reveals its applicability as a basis in benefit-risk analysis, especially in patient-specific treatment, where a comprehensive overview of driving forces and feedbacks is needed to obtain the balance between adverse effects and benefits.

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Chapter 7

Conclusions

Contents partially included in:

Giubilato E., **Cazzagon V.**, Amorim M. J. B., Blosi M., Bouillard J., Bouwmeester H., Costa A. L., Fadeel B., Fernandes T. F., Fito C., Hauser M., Marcomini A., Nowack B., Pizzol L., Powell L., Prina-Mello A., Sarimveis H., Scott-Fordsmand J. J., Semenzin E., Stahlmecke B., Stone V., Vignes A., Wilkins T., Zabeo A., Tran L. and Hristozov D.* (2021). *Risk Management Framework for Nano-Biomaterials used in Medical Devices and Advanced Therapy Medicinal Products.* 13(20):4532. https://doi.org/10.3390/ma13204532

This thesis was triggered by the need to develop specific approaches and methodologies for the assessment of risks of NBMs, with the aim of improving not only the safety of patients during the administration of an innovative nano-enabled product, as required by medicines regulatory agencies, but also the environmental and occupational protection along the entire life cycle of the product. For this purpose, a Risk Management Framework (RMF) has been developed, by adopting a life cycle approach for risk assessment and management not only for patients intentionally exposed to NBM-based medical devices/medicinal products in the use phase, but also for workers and healthcare professionals and the environment that may be accidentally exposed to NBMs released during the synthesis, product manufacturing, use and end-of-life stages.

The RMF provides a flexible and efficient approach to address different assessment goals depending on user needs, based on the principle of finding the optimal balance between compiling the data needed for a targeted and accurate risk assessment and for selecting adequate risk control measures, and the efforts and costs required to collect these data. The RMF addresses the differences in requirements in the chemicals and medical regulatory domains and facilitates cross-fertilisation for exchange of ideas, data and (standard) testing

methods and modelling tools between i) risk assessment and risk control, and ii) benefit-risk analysis.

To investigate the strengths and weaknesses of the RMF, this thesis has also focused on the application of the proposed methodologies to NBMs case studies. In order to do that, methodologies for NMs risk assessment developed, implemented and consolidated in recent years (e.g., (eco)toxicological tests, exposure monitoring techniques and modelling tools) have been critically evaluated and used as a basis to develop and apply the RMF to NBMs case studies, taking into account the peculiar characteristics and exposure scenarios of NBMs.

NBMs peculiarities were considered during the assessment of risks of NBMs used in medical devices and medicinal products, especially in the development of specific exposure scenarios for the use stage and the end-of-life, considering tasks performed by healthcare personnel and healthcare waste disposal personnel. In this regard, the work highlighted that exposure monitoring of these categories of workers remains a challenging task. Monitoring campaigns during medical working activities and during tasks performed by workers at waste incinerator facilities could not be realized within this thesis and only very few data are available from literature. Moreover, it should be noticed that also estimating workers exposure through predictive models present difficulties, in particular because specific tasks performed by healthcare workers are not considered and represented in exposure modeling tools for NMs (e.g., NanoSafer, iEAT).

However, as demonstrated in the current work, the use of the Decision Support System developed in the BIORIMA project can be considered as a powerful tool to guide the assessment and managements of risks of NBMs used in medical devices and medicinal products, because it can help in structuring the assessment procedure and guides the user in the collection, selection, and use of available data. It is advisable that the exposure models included in the DSS will be updated in the future considering specific tasks performed by waste disposal workers (e.g., handling of contaminated objects) and healthcare personnel (e.g., milling of a dental paste containing NBMs, administration of a nano-based drug or cleaning of contaminated surfaces in ambulatory rooms).

The generation and the collection of experimental data for the selected case studies highlighted how validated protocols for conducting the pysico-chemical characterization, exposure monitoring and (eco)toxicological tests of NBMs are still incomplete due to the

difficulty to develop standardized procedures suitable for such diverse medical applications. For example, once NBMs used for injection administration (e.g., iron oxide NPs developed to be stable in the blood) are dispersed in environmental media they become unstable, which could lead to misleading results about their ecotoxicity, while in exposure assessment, medical devices manufactured with the aim of inducing a constant release of NPs and ions during their application (e.g., wound dressing containing Ag NPs) could reveal a release of NPs also during their end-of-life. For this reason, another aspect to consider during the assessment and management of risks of NBMs is the medical purpose of the investigated nano-enabled product and its mode of action.

The testing of the proposed methodologies for NBMs used in medical applications different from what has been investigated in the current work (e.g., tissue engineering, diagnostics, dental applications) would enlarge the range of the applicability of the RMF. Moreover, the flexible structure of the RMF can facilitate the integration of new scientific insights to address the innovation and regulation needs of future NBMs generations.

Acknowledgements

I would firstly like to thank my supervisor, Prof. Elena Semenzin, for proposing to me to continue my academic training after my master's degree and for having supervised my PhD work, following with interest my research activities all in these three years.

I thank all my colleagues at the University Ca' Foscari of Venice (Dept. of Environmental Sciences, Informatics and Statistics) for sharing with me the difficulties and joys of everyday life in the office, in particular my tutors, Elisa Giubilato for her constant and patient support during all my PhD activities and Elena Badetti for her assistance in my lab activities with her pragmatic approach to solve issues.

I would also like to thank the revisors of this PhD thesis, Prof. Lang Tran and Prof. Gianluigi De Gennaro for their scientific contributions to this thesis.

I would like to acknowledge Danail Hristozov for inspiring my interest in risk assessment always with constructive advice, Lisa Pizzol and all the GreenDecision IT Team, in particular Alex Zabeo, Matteo Carisi and Gianpietro Basei for their support in resolving bugs of the Decision Support System as quickly as possible.

A special thanks also go to Prof. Francesco Gonella, for having inspired me and stimulated my interest even outside of the academic life, and the oncologist Alessandra Romano, with her clinical support in my research.

The work presented in this thesis was mainly developed within the H2020 BIORIMA project, offering me the great opportunity to develop my thesis in an international context and to work with leading experts in nanotechnology. In this regard, I would like to thank all the BIORIMA project partners, especially CNR-ISTEC, Colorobbia Industry, Heriot-Watt University, University of Tor Vergata, and ITENE Institute.

Then, I sincerely thank all my friends for supporting me, especially during the last two years of the pandemic emergency.

Last, but not least, my special thanks go to my family for their love and their presence in all the good and bad times, always giving me their support and advice, especially my sister Nora, with her suggestions on medical issues of my thesis: she is the best example of woman (physician) and researcher I could have at my side in this period.

Annexes

Annex 1. Physico-chemical characterization of magnetite NPs coated with PLGA-b-PEG-COOH

Fe_3O_4 PLGA-*b*-PEG-COOH NPs were characterized by Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), Electrophoretic Light Scattering (ELS) and volumetric centrifugation. The concentration of the stock solution of NPs is defined as 0.3 wt% by the product manufacturer.

TEM

For transmission electron microscopy analysis of NPs normal and ultra-thin plasma coated carbon film was used. TEM images were obtained by using a JEOL-JEM 1010 microscope operating at an acceleration voltage of 100 kV. The obtained TEM images are presented in figure 1 and then analysed using Image J software to obtain measurements of monodispersed and spherical NPs with a diameter of 23.5 ± 6 nm (Table 1).

Table 1. Results of TEM analysis of magnetite NPs

Count	Min (nm)	Max (nm)	Mean (nm)	St. Dev. (nm)
60	10.36	44.29	23.5	6

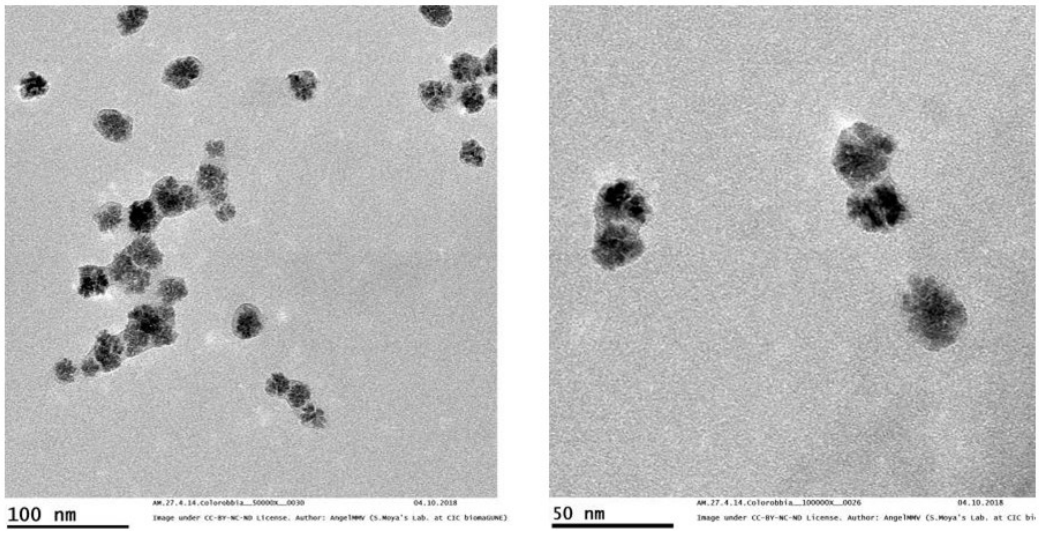

Figure 1. TEM images of magnetite NPs.

DLS and ELS

Dynamic Light Scattering measurements were carried out with a Zeta-Sizer Malvern Instrument (Zetasizer Nano-ZS) in backscattering mode. All studies were performed at a 173° scattering angle with temperature controlled at 25 °C in 1 mL polystyrene cuvettes. Nanoparticles were characterized in terms of hydrodynamic diameter and ζ -potential. The sample obtained from Colorobbia Consulting was diluted to 256 mg/L in deionized water before analysis. Three different aliquots were analysed for each sample and each aliquot was analysed in triplicate. The following instrument setting was applied: 10 replicates, delay time 0, equilibrium time 2 min, T = 25°C, dispersant refractive index and viscosity in water stock solution 1.330/0.8872 mPa s. Material refractive index and absorption: 1.329/20 (Fe_3O_4-PLGA-*b*-PEG-COOH). The results, referred to the intensity signal, represent the average of three independent measurements. The results show a hydrodynamic diameter in ultrapure water of 51 ± 1 nm with a polydispersion index (PDI) of 0.150 ± 0.010 and a ζ-potential of -53.1 ± 2.1 mV.

Volumetric centrifugation

1 mL sample suspension of Fe_3O_4- PLGA-*b*-PEG-COOH (0.2% wt, 2000 ppm) diluted 1:4 in deionized water was dispersed into TPP PCV tubes (Techno Plastic Products, Trasadingen, Switzerland) and centrifuged at 12500 rpm for 30 min. Agglomerate sediment volume, V_{sed}, was measured using a slide rule-like easy-measure device also obtained from the PCV tube manufacturer.

Effective density (ρ^e_A) was calculated from V_{sed} using the Equation (1)

$$\rho_A^e = \frac{m_p^A}{V_{sed} SF}\left(1 - \frac{\rho_L}{\rho_p}\right) + \rho_L \qquad (1)$$

where m^A_P is the mass of NPs in agglomerates (mg); V_{sed} is the volume of sediment (µL), measured with volumetric centrifugation method (VCM); ρ_L is the liquid density (gcm^{-3}) and ρ_P is the NPs density (4.8-5.1 gcm^{-3}); SF is the stacking fraction, i.e., fraction of agglomerates in the sediment that depends on the efficiency of agglomerate stacking. In this work, we consider SF values to approximate the theoretical value for random close stacking (i.e., 0.634) (Song et al. 2008).

The resulting effective density (ρ^e_A) of Fe_3O_4 NPs coated with PLGA-*b*-PEG-COOH is calculated as follow in Equation 2:

$$\rho_A^e = \frac{0.5\ mg}{5.1\ \mu L * 0.634}\left(1 - \frac{1}{4.95\ g/cm3}\right) + 1 = \mathbf{1.12 g/cm^3}$$

Song C., Wang P., Makse H. A., 2008. A phase diagram for jammed matter. Nature 453:7195, 629-632. 10.1038/nature06981.

Annex 2. Questionnaire on activities performed by healthcare personnel

A questionnaire in Word form (see below) was designed and implemented to elicit expert feedback on specific aspects regarding the activities performed during the administration of contrast agent. Nine experts from the University Hospital of Padova were contacted in October 2020 with a request to participate on the survey: 3 medical radiologists, 3 radiology technicians and 3 nurses.

Questionnaire on activities performed by healthcare personnel during the administration of biomedical products containing nano-biomaterials

Date of compilation:

Please select your role in the department:

☐ Physician (ER doctor, surgeon, hospitalist)

☐ Technician (i.e., radiology or surgical)

☐ Nurse or assistant of nurses

☐ Healthcare assistant

The following descriptions of your daily activities is focusing on:

☐ Injection of contrast agent for MRI

☐ Injection of anticancer drug

☐ Other, please specify:

Please specify if the biomedical product previously selected containing or not nano-biomaterials:

☐ YES

☐ NO

☐ I don’t know

If YES, please specify the type of nano-biomaterials:

Please describe shortly maintenance and cleaning operations (i.e. category of workers who provide this service, duration of the activity, frequency per day/week):

Are you aware of protocols on the exposure monitoring of workers who manage medical devices or medicinal products containing nano-biomaterials?

☐ YES

☐ NO

If YES, please specify name of protocols:

Are you aware of protocols on the use of risk management measures (e.g. personal protective equipment) for healthcare personnel who manage medical devices or medicinal products containing nano-biomaterials?

☐ YES

☐ NO

If YES, please specify name of protocols:

Description of activities performed considering the application previously selected:

1st activity (short description):

Duration activity:

Number of repeated work cycles daily (N/day):

Materials used (please indicate in brackets if containing or contaminated with NBMs):

Amount of materials used in one cycle of treatment:

Please specify the substance form:

☐ Powder

☐ Liquid

☐ Mixture

Volume of the room:

Type of air ventilation in the room:

☐ Mechanical ventilation system

☐ Chemical hood

☐ Laminar flow cabin

☐ Others, please specify:

Personal Protective Equipment used:

☐ Gloves

Please specify the type:

☐ Masks

Please specify the type:

☐ Glasses

Please specify the type:

☐ Lab coat

Please specify the type:

☐ Other, please specify:

Can the release of dust and/or aerosols into this workplace air be reasonably excluded during this activity?

☐ YES

☐ NO

If NO, please specify the reason:

Can the ingestion of nanoscale particles by workers be reasonably excluded during this activity?

☐ YES

☐ NO

If NO, please specify the reason:

Can the dermal contact of nanoscale particles by workers be reasonably excluded during this activity?

☐ YES

☐ NO

If NO, please specify the reason:

Annex 3. Analytical techniques and their settings used in the monitoring campaign

Measurements were performed by measuring particle concentration (#/cm^3) in air using NanoTracer and Optical Particle Sizer (OPS) and by collecting particles through peristaltic pumps connected with a filter. Monitoring was conducted during all the activities performed by the worker in the product manufacturing stage except during filtration and packaging in glass bottles (CES4) (so called 'nano-activities'). For more information on CES, see table SI5.

Calibration of the NanoTracer, OPS and pumps was performed before the monitoring began. Activities in CES1, CES2, CES3 and CES5 were then performed by the worker without using NPs in order to determine possible release of magnetite NPs (so called 'non-nano activities'). The duration of each monitoring activity reflects the time to perform the activity by the worker, i.e., 2 min for CES1, 3 min for CES2, 1 h 2 min for CES3, and 3 min for CES5.

NanoTracer (Aerasense/Oxility) was settled in Advanced Mode, measuring particle concentration every 16 sec with a diameter from 10 to 300 nm and up to 10^6 #/cm^3.

Size channels of the Optical Particle Sizer (OPS) Model 3330 (TSI) were settled at ranges of 300-500 nm, 500 nm-1 µm, 1-2 µm, 2-5 µm, 5-8 µm, 8-10 µm in order to determine both number concentration and particle size distribution of NPs and their agglomerates.

Flow rate of the two peristaltic pumps (Casella, model APEX) was settled at 3 L/min collected particles for 2 h for a total air volume of 240 L during nano and not nano activities. Pumps were connected with Tygon tubes of 1 m length and at the end of the tube a 3-piece type cassette containing a polycarbonate HEPA filter (thickness of 0.4 µm and diameter of 37 mm) was added in order to analyse particles with FESEM and EDS. Filters and the control filter were coated with a thin layer of gold to enhance surface conductivity and imaging resolution, then positioned on aluminium stubs (diameter 12.7 mm) and fixed by means of a conductive adhesive tape. The particles morphology was investigated by a Field Emission Scanning Electron Microscope, FESEM (Carl Zeiss Sigma NTS, Germany) coupled to an energy dispersive X-ray micro-analyser (EDS, mod. INCA) applied to map the elemental composition. Dimensional analysis of the collected samples was performed by using ImageJ software and measuring length and width of all the particles of the 13 images obtained for each filter. FESEM images and EDS spectra were collected at low, medium, and high magnifications to best represent size distribution, shape and composition of the particles captured.

Annex 4. Transformation of particle concentration (#/cm^3) in mass concentration (mg/m^3)

If the obtained values of inhalation exposure are expressed in particle concentration (#/cm^3), results need to be transformed in the same mass concentration (mg/m^3) of the DNEL. The transformation can be made by following a simplified version of the approach proposed in Bekker et al., 2016 (Equation 3).

$$C_m = C_n * \frac{\pi}{6} * d^3 * \rho * 10^{-18} \quad (3)$$

where C_m is the mass concentration (mg/m^3), C_n is the particle number concentration (#/cm^3), ρ the particle density (g/cm^3) and d the particle diameter (nm).

It should be noted that equation 3 can be properly applied under the following assumptions: i) all the detected particles belong to the NBM under assessment and have spherical shapes and the same density, ii) no aggregation or agglomeration processes occurred, thus the diameter value is fixed. Moreover, as the obtained value represents the total amount of particles in air, background measurements are subtracted from Near Field measurements in order to obtain particle concentration of the investigated NBM.

Bekker, C., Voogd, E., Fransman, W., & Vermeulen, R. (2016). The validity and applicability of using a generic exposure assessment model for occupational exposure to nano-objects and their aggregates and agglomerates. *Annals of Occupational Hygiene*, *60*(9), 1039–1048. https://doi.org/10.1093/annhyg/mew048

Annex 5. Information collected for each Contributing Exposure Scenarios.

Table 2. Information related to the work cycle, investigated substance and risk management measures used for each Contributing Exposure Scenarios (CES).

Life cycle stage	CES	Brief description of the activity	Duration activity (min)	Exposure duration (min)	Number of repeated work cycle daily	Substance used	Amount used	Type of packing received	Product type of substance	Room conditions (T, hum., air velocity)	Volume of the room	Type of process	Targets potentially exposed	Route of exposure
Product manufacturing	**CES1. Weighing, solution preparation and mixing**	Weighting of substances, preparation and mixing of 1) magnetite NPs suspension and 2) THF solution with PLGA-b-PEG-COOH	15	15	1	Suspension of 0.5% w/w magnetite NPs	500 g	Glass bottle	Mixture	Temperature 24.3 °C; humidity 57%; air velocity 0.09 m/s	75 m^3	Manual	1 lab worker	Inhalation, dermal, ingestion
						Solution of PLGA-b-PEG-COOH and THF	3 g of PLGA-b-PEG-COOH in pellet form	Glass bottle	Mixture					
	CES2. Formation of magnetite NPs coated with PLGA-b-PEG-COOH	The two solutions are mixed trough two different tubes in the mixing chamber where the formation of stable NPs takes place	10	2	1	Aqueous solution	5000 mL	Glass bottle	Liquid			Semi-automatic	1 lab worker	Inhalation, dermal, ingestion
						Suspension of Fe_3O_4 NBMs with PLGA-b-PEG-COOH in THF	500 mL	Glass bottle	Mixture					
	CES3. Dialysis and concentration	Elimination of THF in a semi-automatic closed	10	2	1	Suspension of Fe_3O_4 coated with PLGA-b-	5500 mL	Glass bottle	Mixture			Semi-automatic	1 lab worker	Inhalation, dermal, ingestion

		reactor and an increase of the NPs concentration				PEG-COOH in aqueous solution								
						Buffered solution	30 L	Glass bottle	Liquid					
	CES4. Filtration and packaging in glass bottles	Manual packaging by feeding glass bottles of 10-1000ml of the final product using syringe containing porous septum	10	10	1	Suspension of Fe_3O_4 coated with PLGA-b-PEG-COOH in aqueous solution	10- 1000 ml for each bottle	Glass bottle	Mixture			Manual	1 lab worker	Dermal, ingestion
	CES5. Cleaning and maintenance	Cleaning of table and lab objects used	15	15	1	Water to clean all the surfaces and objects used during product manufacturing	Contaminated objects	Glass bottles and lab surfaces to clean	Mixture			Manual	1 lab worker	Inhalation, dermal, ingestion
Use	**CES6: Injection administration**	Contrast agent administration for MRI using venflon which automatically administer CA and physiological water maintaining a constant blood pressure	20	2	10-15	general contrast agent, physiological water, venflon, wounds dressing, disinfectant	10-20 mL	Small glass bottle	Liquid	N.A.	150	Semi-automatic	Radiologist, nurse, technician	Dermal and ingestion

	CES7: Cleaning and waste disposal	Contaminated materials disposal and cleaning of the surfaces	5	5	10-15	Water and cleaner products to clean all the surfaces and objects used during injection	5	Glass bottles and lab surfaces to clean	Liquid	N.A.	150	Manual	Nurse, cleaning staff	Dermal and ingestion
End-Of-Life	**CES8: Incineration**	Handling of bags containing healthcare waste on the thermal treatment plants based on fluidized bed technology	10	5	5-10	All the contaminated objects in a waste bag	1kg	Specific bags containing	Solid	N.A.	6000	Manual	Waste management workers	Dermal, ingestion

N.A.: information not available

Annex 6. Summary of results obtained from the questionnaire on activities performed by healthcare personnel during the administration of biomedical products containing nano-biomaterials

This section reports the summary of the results collected from the questionnaire, which has been developed to support the development of exposure scenarios related to the use stage. Three nurses, three technicians and three physicians filled in the questionnaire. Results shown a division between activities performed by different categories of workers: administration of contrast agent is performed by nurses, technicians, and radiologists, while cleaning activities only by nurses. Then, all the workers agree on the duration activity (10 min for each patient) and the quantity of the product used (10-20 mL). 7 workers to 9 excluded an exposure during their daily activities, and only 3 workers affirmed that a possible dermal and ingestion exposure could occurred during administration and cleaning activities.

A key point on the results obtained from the questionnaire is related to the different activities performed by the different categories of workers. Indeed, while physicians describe the administration of the contrast agent to the patient, nurses also describe cleaning activities performed after the injection through cleaning all the surfaces and removing all the contaminated objects. For this reason, we decided to consider the activities performed by nurses calculating risks through a precautionary approach, which produce more conservative risk estimations but can represent all the possible scenarios performed by workers during the use stage.

Annex 7. Results of the monitoring campaign of activities performed by workers during product manufacturing stage

Measurements of particle concentration in the air for 10 min after placing NanoTracer and OPS on the table prior to weighing of reagents and mixing activities were performed. A value of 7817 ± 1073 #/cm^3 in Advanced Mode was obtained from the NanoTracer, and a concentration of 155 ± 33 #/cm^3 for particles at range of 300-500 nm was measured with OPS. As these values could represent powder coming from other rooms of the industry through the general ventilation system, background measurements were considered by measuring the same activity performed by the worker but without using the magnetite NPs (so called 'nano-activities'). Measurements of particles released during 'not nano activities' were performed, and after 30 minutes, OPS and NanoTracer measurements during 'nano activities' were conducted.

Results obtained from NanoTracer operated in Advanced Mode are shown in figure 2. As at the beginning and at the end of the monitoring campaign turning, drilling, and milling of ceramics were performed next to the lab where we performed the monitoring, particle concentration during nano-activities in CES1 and CES5 are higher than during CES2 and CES3. Similar results were obtained from OPS measurements, as during nano-activities in CES1 and CES5 higher particle concentration values were analysed compared to CES2 and CES3.

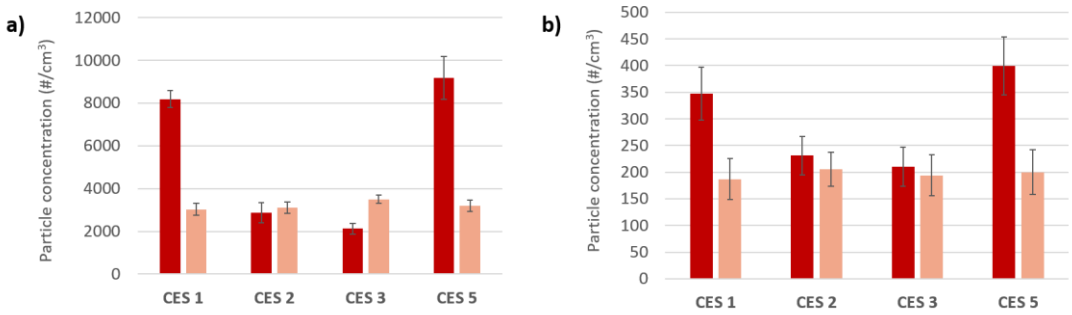

Figure 2. Particle concentration in each CES during nano (red) and not nano (pink) activities obtained with a) NanoTracer and with b) Optical Particle Sizer.

Table 3 summarizes the maximum (peak) and average (geometric mean (GM)) of the particle number concentration (#/cm^3) reached near the breathing zone of the worker for all four CES during nano-activity (i.e., using magnetite NPs) and not nano-activity (i.e., without using

magnetite NPs). Results acquired during the use of magnetite NPs both from the NanoTracer and OPS for particles range 300-500 nm show higher values in CES1 and CES5 (Figure 2 and Table 3) than CES2 and CES3, while during activities performed without using NPs, no huge differences are present in the release of particles among the different CESs.

Table 3. Summary of maximum (peak) concentration and the geometric mean of the steady state concentration for each scenario measured with the NanoTracer (#/cm^3), and the Optical Particle Sizer (#/cm^3) for particles range 300-500 nm.

	CES	**NanoTracer**		**Optical Particle Sizer**	
		Max. peak	**GM ± St. Dev.**	**Max. peak**	**GM ± St. Dev.**
Nano activity (A)	CES1	8515	8183 ± 388	469	347 ± 50
	CES2	3541	2879 ± 467	311	231 ± 36
	CES3	2877	2125 ± 250	339	210 ± 37
	CES5	12073	9171 ± 1004	581	399 ± 54
Not nano activity (B)	CES1	3488	3021 ± 277	284	187 ± 38
	CES2	3569	3107 ± 265	318	200 ± 42
	CES3	3813	3502 ± 194	300	194 ± 38
	CES5	3674	3199 ± 271	289	205 ± 32

GM: Geometric Mean, NT: NanoTracer, OPS: Optical Particle Sizer

The ratio between nano (A) and not-nano (B) activities was then calculated (Table 4). For each CES, the ratio obtained from OPS measurements is always >1 which suggests a release of particles during the monitoring campaign. Considering the A/B ratio calculated from NanoTracer results, only in CES2 and CES3 the ratio is <1. For this reason, considering these ratios it is not possible to exclude a release of particles during activities performed by workers in CES1, CES2, CES3 and CES5.

Table 4. Geometric Mean ratio between nano (A) and not-nano (B) activities of NanoTracer and OPS results.

Contributing Exposure Scenario	GM ratio A/B (NanoTracer)	GM ratio A/B (OPS)
CES1	**2.71**	**1.86**
CES2	0.93	**1.15**
CES3	0.61	**1.08**
CES5	**2.87**	**1.95**

GM: Geometric Mean

Morphological analysis of FESEM images revealed the presence of NPs and their agglomerates on filters. Lengths and widths of the particles are reported in Table 5. Results revealed no significative differences between particles captured during activities performed with and without NPs and all the particles analysed has an irregular shape (Figure 3a and 3c).

Table 5. Results of length and width of the analysed particles from TEM images.

	N	Length (nm)			Width (nm)		
		Min	Max	Median	Min	Max	Median
Filters with NMs	28	1.61E+02	3.29E+06	4.82E+02	1.35E+02	7.35E+05	3.05E+02
Filters without NMs	28	1.74E+02	1.40E+04	1.68E+03	2.56E+02	6.73E+03	1.24E+03

Moreover, EDS analysis revealed ceramics content and the absence of iron in the filter (Figure 3b and 3d). Therefore, it is possible to exclude the release of magnetite NPs during the investigated activities performed by manufacturers.

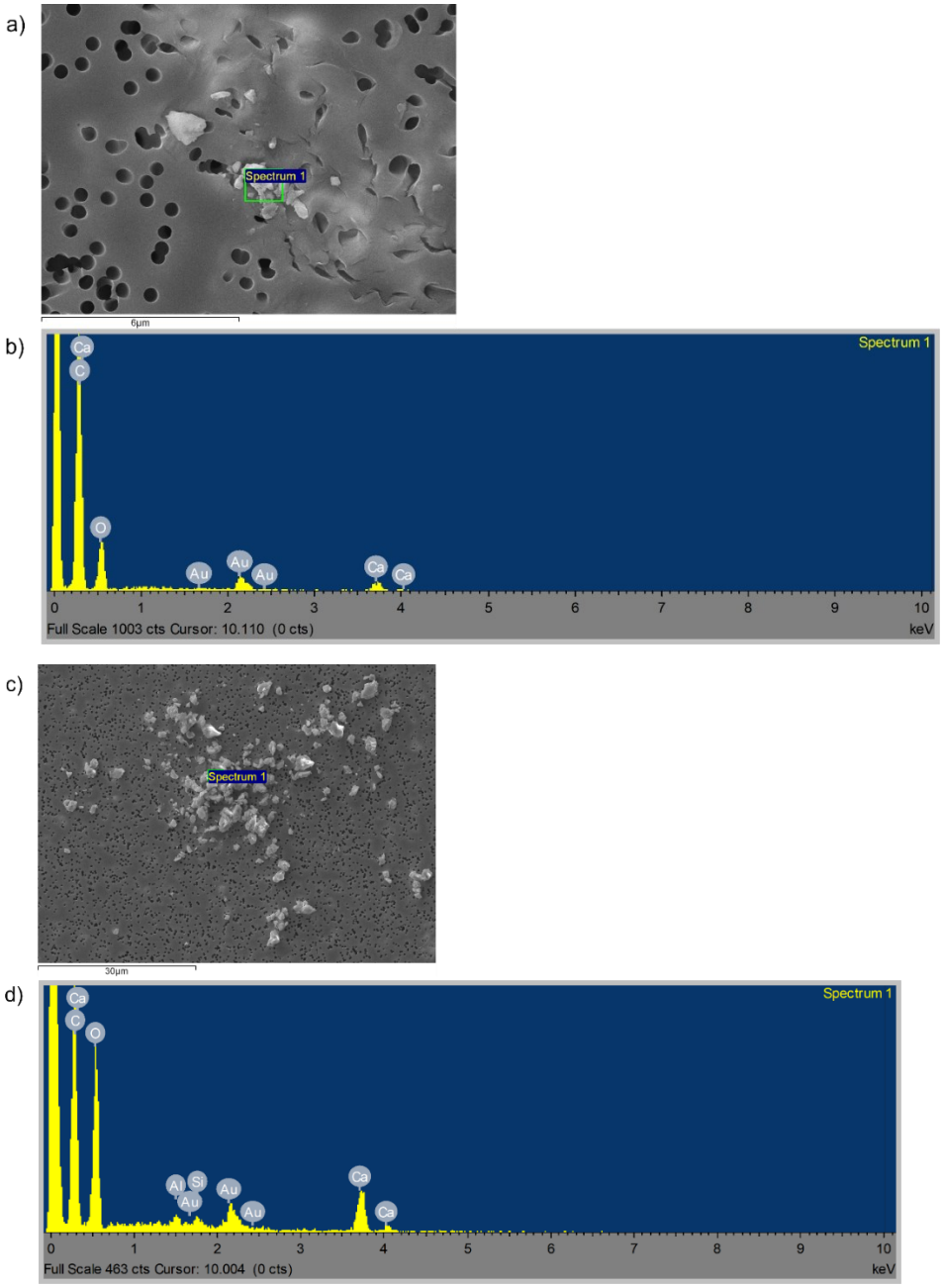

Figure 3. a) FESEM image and b) its corresponding EDS analysis of the filter when activities are performed with magnetite NPs and c) and d) without the use of magnetite NPs.

Annex 8. Input parameters for inhalation model

Since the monitoring campaign results as well as interviews with healthcare personnel and waste management workers revealed a negligible inhalation exposure for CES1, CES2, CES3, CES5, CES6, CES7, CES8, the inhalation model was used to determine exposure value for CES4. Input of the model and the corresponding values in brackets are listed below:

- Particles size diameter (23.5 nm)
- Density (1.12 g/cm^3)
- Percentage of pure NM (0.5%)
- Quantity of used mass (10 g)
- Task duration (480 min)
- Duration of the generation phase (10 min)
- Number of repetitions per day (1)
- Volume of the room (75 m^3)
- Number of air changes per hour (6 ACH)
- Activity generating the release rate: Packaging

Where task duration should consider the time spent on that activity along the entire working day and duration of the generation phase is the fraction of task duration when the release occurs.

Values obtained are 6.15E-06 mg/m^3 for the Near Field and 2.46 E-06 mg/m^3 for the Far Field which correspond to average exposure values of the worker during his/her working day.

Annex 9. Dermal exposure quantification using dART tool equations

As no dermal exposure tools are available in literature for NMs, equations presented in the dermal Advanced REACH tool (dART) were applied to quantify exposure in the present work. dART is an extension of the higher tier exposure assessment ART tool for inhalation exposure based on a source receptor approach by applying a two-compartment model (near field and far field). Indeed, after the quantification of dermal exposure through the deposition from the air (ART tool), the transfer from contaminated surfaces as well as from the direct contact of the substance is added in dART.

In our work, deposition (D), transfer (T) and emission (E) are calculated using equations 4, 5, 6 and 7 presented in Goede et al. 2018 and here reported. All the parameters are described in table 7.

$$C_{hands} = w_f \cdot (DP_{hands} + E_{hands} + T_{hands}) \text{ x glove protection factor} \quad (4)$$

$$DP_{hands} = (C_{nf} + C_{ff} + S_u)\ ESA_{dp} \quad (5)$$

$$E_{hands} = (E_e \cdot H_e \cdot LC_e)\ ESA_e \quad (6)$$

$$T_{hands} = E_t \cdot H_t\ ESA_t \quad (7)$$

Firstly, for each CES, the contribution of the different type of exposure (i.e., through deposition, transfer, and emission), are reported in table 6, where D, E and T excluded in this analysis are represented in black. As from the monitoring campaign and the results obtained from the questionnaire the inhalation exposure is considered negligible for each CESs, deposition from the air can be considered not relevant (represented in black in table 6). In order to define if an emission or transfer occurred during the activities performed by workers, we selected a possible emission if a direct emission (e.g., splashes, overspray, hand immersion) and/or a transfer from a contaminated surface take place during the different

CES. As in CES2 and CES3, product manufacturing activities are performed in semi-automatic reactors, no emission and transfer occurred. For this reason, dermal exposure can be considered negligible for both CES2 and CES3.

Table 6. Deposition (D), Emission (E) and Transfer (T) considered (in white) or not (in black) for each CES.

Contributing Exposure Scenario	**D**	**E**	**T**
CES 1: Weighting, solution preparation and mixing			
CES 2: Formation of coated NPs in a mixing chamber			
CES 3: Dialysis and concentration			
CES 4: Filtration and packaging in glass bottles			
CES 5: Cleaning and maintenance			
CES 6: Semi-automatic injection administration			
CES 7: Cleaning and waste disposal			
CES 8: Incineration			

Then, each CES was associated to an Activity Class presented in SI of Goede et al. 2018 and values of the different parameters was selected based on the peculiarities of the investigated CES. Below are reported the selected values for each CES.

<u>CES 1: Weighting, solution preparation and mixing</u>

AC: Activities with open liquid surfaces and open reservoirs (AC2) - Activities with relatively undisturbed surface (e.g., dipping) (AC2.1)

Table 7. Parameters and their corresponding values selected for the application of dART equations for CES1.

Parameter	**Description of the parameter**	**Value**	**Reference**
W_f	Weight fraction of substance in the product	0.05	Colorobbia
ESA_{dp}	Exposed surface area of the hands	1	Considering both hands exposed
Lce e Ee	Not described	-	-
S_u	Surface Contamination	negligible for low vol. liq.	Goede et al. 2018
E_t	Substance transfer potential	0.7	Low viscosity
H_t	Activity transfer potential	3, 0.1, 3, 0.1	Short hand tool <0.5 m, infrequent control panels, dipping or mixing/agitation for treatment applications, infrequent contact
He	Activity direct emission & contact potential (He)	0.3; 0.1; 1; 1; 1	Short hand tool <0.5 m; Repeated or almost constant use; open surface <1 m^2; low agitation; downward orientation of work
ESAe	Exposed surface area of hands	0.25	Handling of small objects with fingers or one hand palm or less
ESA_t	Exposed surface area of hands	0.25	Handling of small objects with fingers or one hand palm or less

CES 4: Filtration and packaging in glass bottles

AC: Transfer of liquid products- falling liquids, top loading

Table 8. Parameters and their corresponding values selected for the application of dART equations for CES4.

Parameter	**Description of the parameter**	**Value**	**Reference**
W_f	Weight fraction of substance in the product	0.05	Colorobbia
ESA_{dp}	Exposed surface area of the hands	1	Considering both hands exposed
Lce e Ee	Not described	-	-
S_u	Surface Contamination	negligible for low vol. liq.	Goede et al. 2018
E_t	Substance transfer potential	0.7	Low viscosity
H_t	Activity transfer potential	3; 1; 1; 0.1	Manual transfer using a single small/medium container; Infrequent, occasional use of control panels; infrequent occasional use of receiving containers; infrequent contact at surfaces at source when using automated equipment
He	Activity direct emission & contact potential (He)	1; 0.01: 0.3; 1; 0.1	Manual transfer using a single small/ medium containers; Infrequent or occasional use of control panels; Transfer of liquid product with flow of < 0.1 L/min; Submerged loading; Handling that reduces contact between product and adjacent air
ESAe	Exposed surface area of hand	0.25	Handling of small equipment (<0.1 L/min)
ESA_t	Exposed surface area of hands	0.25	Handling of small equipment (<0.1 L/min)

CES 5: Cleaning and maintenance

AC3: handling of contaminated objects

Table 9. Parameters and their corresponding values selected for the application of dART equations for CES5.

Parameter	**Description of the parameter**	**Value**	**Reference**
W_f	Weight fraction of substance in the product	0.05	Colorobbia
ESA_{dp}	Exposed surface area of the hands	1	Both hands
E_t	Substance transfer potential	0.7	Low viscosity
H_t	Activity transfer potential	1; 3; 1; 0.1; 1	Long hand tool or extended tools (≥0.5 m); Infrequent | occasional use of control panels; Objects contaminated with treatment products or general deposits; Occasional | repeated contact; infrequent contact on surfaces at source when using automated equipment; Objects are smooth surfaces, e.g. like glass
ESA_t	Exposed surface area of hands	0.25	Handling small objects with fingers or one hand palm or less

CES 6: Semi-automatic injection administration

AC: Transfer of liquid products- falling liquids, top loading

Table 10. Parameters and their corresponding values selected for the application of dART equations for CES6.

Parameter	**Description of the parameter**	**Value**	**Reference**
W_f	Weight fraction of substance in the product	0.05	Colorobbia
ESA_{dp}	Exposed surface area of the hands	Both hands	1
Lce e Ee	Not described		
S_u	Surface Contamination	negligible for low vol. liq.	Goede et al. 2018
E_t	Substance transfer potential	0.7	Low viscosity
H_t	Activity transfer potential	3; 0.1; 0.1; 0	Manual transfer using a single small/ medium container; Infrequent, occasional use of control panels; infrequent occasional use of receiving containers; no contact at surfaces at source when using automated equipment
He	Activity direct emission & contact potential (He)	1; 0.01: 0.3; 1; 0.1	Manual transfer using a single small/ medium containers; Infrequent or occasional use of control panels; Transfer of liquid product with flow of < 0.1 L/min; Submerged loading; Handling that reduces contact between product and adjacent air
ESAe	Exposed surface area of hand	0.25	Handling of small equipment (<0.1 L/min)
ESA_t	Exposed surface area of hands	0.25	Handling of small equipment (<0.1 L/min)

CES 7: waste disposal

AC3: handling of contaminated objects

Table 11. Parameters and their corresponding values selected for the application of dART equations for CES7.

Parameter	**Description of the parameter**	**Value**	**Reference**
W_f	Weight fraction of substance in the product	0.05	Colorobbia
ESA_{dp}	Exposed surface area of the hands	1	Both hands
E_t	Substance transfer potential	0.7	Low viscosity
H_t	Activity transfer potential	1; 1; 1; 0.1; 1	Long hand tool or extended tools (≥0.5 m); Infrequent | occasional use of control panels; Objects contaminated with treatment products or general deposits; Occasional | repeated contact; infrequent contact on surfaces at source when using automated equipment; Objects are smooth surfaces, e.g. like glass
ESA_t	Exposed surface area of hands	0.25	Handling small objects with fingers or one hand palm or less

CES 8: Incineration

AC3 handling of contaminated objects

Table 12. Parameters and their corresponding values selected for the application of dART equations for CES8.

Parameter	**Description of the parameter**	**Value**	**Reference**
W_f	Weight fraction of substance in the product	0.05	Colorobbia
ESA_{dp}	Exposed surface area of the hands	1	Both hands
E_t	Substance transfer potential	0.7	Low viscosity
H_t	Activity transfer potential	1; 1; 1; 0; 1	Long hand tool or extended tools (≥0.5 m); Infrequent | occasional use of control panels; Objects contaminated with treatment products or general deposits; Occasional | repeated contact; no contact on surfaces at source when using automated equipment; Objects are smooth surfaces, e.g., like glass
ESA_t	Exposed surface area of hands	0.25	Handling small objects with fingers or one hand palm or less

Once dART score was obtained using equations, the application of a fixed factor of 1.14 converts the dimensionless dART score to the exposure in mg/min (Table 13). Finally, as this value needs to be integrated with DNEL expressed in mg/cm^2/d, the unit was converted by multiplying the concentration in mg/min to the duration activity and the number of repetitions daily and dividing per the standardized value of hands surface (i.e., 820 cm^2) (ECHA 2017).

Table 13. dART score obtained from dART equations, dART exposure concentration and its corresponding value considering hand exposure mg/min, and the hand exposure concentration in mg/cm^2/d for each CES.

CES	dART score	dART exposure concentration (mg/min)	Hand exposure concentration (mg/min)	Hand exposure concentration (mg/cm^2/d)
CES 1	1.41	1.61	0.08	1.47E-03
CES 4	4.59	5.23	0.26	3.19 E-03
CES 5	4.59	5.23	0.26	2.36 E-03
CES 6	0.0004	4.29 E-03	0.21 E-06	0.80 E-03
CES 7	1.53	1.74	0.09	7.97 E-03
CES 8	0	0	0	0

European Chemicals Agency (ECHA), 2017. Default human factor values for use in exposure assessments for biocidal products. Helsinki, Finland.

Goede, H. A., McNally, K., Gorce, J. P., Marquart, H., Warren, N. D., Fransman, W., Tischer, M., Schinkel, J., 2018. Dermal Advanced REACH Tool (dART)- Development of a Dermal Exposure Model for low-volatile liquids. Annals of Work Exposures and Health. 63:6, 624-636. 10.1093/annweh/wxy106.

Annex 10. Input parameters for iEAT model

Table 14. Inputs used in iEAT model for each CES.

	Body weight (kg)	Surface contact area hand-mouth (cm^2)	Geometric mean finger moisture (µs)	Geometric standard deviation finger moisture (µs)	Minimum value of hand loading (ng)	Maximum value of hand loading (ng)	Estimate of N of hand-to-mouth contacts per hour	Gloves worn	Glovers worn for more than 75% of shift	RPE worn for more than 50% of shift	Job profile 1 or 2
CES 1: Weighting, solution preparation and mixing	70	10	688	166	100	5000	NO	YES	YES	NO	1
CES 2: Formation of coated NPs in a mixing chamber	70	10	518	168	100	5000	NO	YES	YES	NO	1
CES 3: Dialysis and concentration	70	10	518	168	100	5000	NO	YES	YES	NO	1
CES 4: Filtration and packaging in glass bottles	70	10	518	168	100	5000	NO	YES	YES	NO	1
CES 5: Cleaning and maintenance	70	10	518	168	100	5000	NO	YES	YES	NO	1
CES 6: Semi-automatic injection administration	70	10	518	168	100	5000	NO	YES	YES	NO	1
CES 7: Cleaning and waste disposal	70	10	518	168	100	5000	NO	YES	YES	NO	1
CES 8: Incineration	70	10	518	168	5000	150000	NO	YES	YES	NO	1

Surface contact area hand-mouth was selected as 10 as it indicates the value of surface contact for the whole hand in cm^2. As no specific values for magnetite NPs are available from literature, values of finger moisture calculated in Gorman, 2013 insoluble powder were considered for CES1, while for CES2-CES8, finger moisture calculated for a liquid were selected. For hand loading, a value of 100 ng/hand indicates a 'very clean and regularly decontaminated workplace', 5000 ng/hand a 'clean industrial environment', while 150000 ng/hand a 'dirty industrial environment with visible contamination'. For all the CESs, a job profile 1 was selected as workers spends 80% or more time of manual activities and the rest of time at meetings or working at pc, while job profile 2 represents a worker who spends 20% or less time of manual activities and the rest of time at meetings or working at pc.

Estratto per riassunto della tesi di dottorato

L'estratto (max. 1000 battute) deve essere redatto sia in lingua italiana che in lingua inglese e nella lingua straniera eventualmente indicata dal Collegio dei docenti.

L'estratto va firmato e rilegato come ultimo foglio della tesi.

Studente: Virginia Cazzagon _____ matricola: 839247 _____

Dottorato: Scienze Ambientali _____

Ciclo: 34 _____

Titolo della tesi[1] : Development and application of a Risk Management Framework for nano-biomaterials used in medical devices and medicinal products

Abstract:

The objectives of the PhD thesis are the development of a Risk Management Framework (RMF) for the assessment and management of nano-biomaterials used in medical devices and medicinal products and its application to real case studies. The proposed RMF is based on two main pillars: occupational and environmental risk assessment, and benefit-risk analysis for patients. An occupational risk assessment of magnetite nanoparticles (NPs) used as contrast agent was conducted by applying a Decision Support System. Moreover, a Safe-By-Design approach was developed for wound dressings containing silver NPs to select the safer solution among five nano-based wound dressing alternatives based on human health and environmental safety criteria. Considering the benefit-risk analysis, the complexity of the use of a theranostic agent containing magnetite NPs and its subsequent emerging benefits and/or adverse effects was investigated using a System Thinking perspective.

Gli obiettivi della tesi di dottorato sono lo sviluppo di un framework per la valutazione e gestione dei rischi di nano-biomateriali utilizzati in dispositivi medici e prodotti medicinali e la sua applicazione in casi studio reali. Il framework si compone di due pilastri principali: l'analisi di rischio occupazionale/ambientale, e l'analisi rischi-benefici per i pazienti. È stata quindi svolta un'analisi di rischio occupazionale per nanoparticelle di magnetite utilizzate in un mezzo di contrasto applicando un sistema di supporto alle decisioni. Inoltre, è stato sviluppato un approccio Safe-By-Design per garze contenenti nanoparticelle di argento per selezionare l'alternativa migliore tra cinque garze basandosi su criteri di sicurezza per la salute umana e ambientale. Riguardo all'analisi rischi-benefici, è stata valutata la complessità dell'utilizzo di nanoparticelle di magnetite come agente teranostico e i suoi effetti avversi e/o benefici attraverso un approccio System Thinking.

Firma dello studente

[1] Il titolo deve essere quello definitivo, uguale a quello che risulta stampato sulla copertina dell'elaborato consegnato.