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Assessment of the biological and toxicological effects of silica-based nanoparticles: contribution to grouping of nanomaterials

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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ASSESSMENT OF THE BIOLOGICAL AND TOXICOLOGICAL EFFECTS OF SILICA-BASED NANOPARTICLES: CONTRIBUTION TO GROUPING OF NANOMATERIALS

Thesis for application to the Doctoral degree in Biomedical Sciences; Doctorate of University of Porto (Institute of Biomedical Sciences Abel Salazar)

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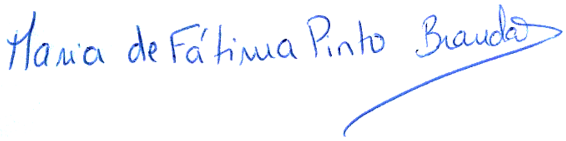
Declaration of Honor

I declare that this thesis is my own and has not been previously submitted to another course or curricular unit, in ICBAS-UP or another institution. References to other authors (statements, ideas, thoughts) scrupulously respect the rules of attribution and are duly indicated in the text and bibliographic references, according to the rules of reference. I am aware that the practice of plagiarism and self-plagiarism is an academic illicit.

Porto, 16 November 2024



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Funding

This work received financial support from the Portuguese Foundation for Science and Technology (FCT) through projects ERA-NET SIINN NanoToxClass (SIINN/0001/2013) and NanoBioBarriers (POCI-01-0145-FEDER-031162) co-financed by Operational Program for Competitiveness and Internationalisation (POCI) through European Regional Development Funds (FEDER/FNR). Moreover, this work was supported by the Doctorate Scholarship No. SFRH/BD/101060/2014 awarded by the FCT through the Human Capital Operating Program (POCH) and European Union (EU) funding. Additional financial support was provided by the Doctorate in Biomedical Sciences of the Institute of Biomedical Sciences Abel Salazar of the University of Porto (ICBAS-UP), as well as by the Portuguese National Institute of Health (INSA), Toxicology Unit of University A Coruna, and by the Epidemiology Research Unit (EPIUnit; UIDB/04750/2020; DOI https://doi.org/10.54499/UIDB/04750/2020) and the Associated Laboratory for Integrative and Translational Research in Population Health (ITR; LA/P/0064/2020; DOI https://doi.org/10.54499/LA/P/0064/2020) through FCT/ Ministry of Science, Technology and Higher Education (MCTES).

Legal Details

This thesis describes research conducted at the Dept. of Environmental Health from the National Institute of Health Dr. Ricardo Jorge (INSA; Porto, Portugal), the Epidemiology Research Unit (EPIUnit), the Toxicology Unit of the University of A Coruña (A Coruña, Spain), the Unité de Recherche en Biologie Cellulaire (URBC), Namur Research Institute for Life Sciences (Namur, Belgium), the StratiCELL Laboratories, Research and Development (Les Isnes, Belgium), Namur Nanosafety Centre, Depart. of Pharmacy, Namur Research Institute for Life Sciences of University of Namur (Namur, Belgium), the Dept. of Biochemistry and Molecular Biology, University of Bucharest (Bucharest, Romania), the Dept. of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR) (Berlin, Germany) and at the Institute of Energy and Environmental Technology (IUTA) (Duisburg, Germany).

In compliance with what is stated in Regulation 707/2018 (Diário da Républica 2ª série, nº 204 of 23.10.2024) it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results shown, as well as in their interpretation and writing of the respective manuscripts. The manuscripts in this thesis were not previously included in other theses and correspond to their integral versions. Under the current legislation the reproduction of any part of this thesis is not allowed.

Publications

Complying with the terms of the relevant national legislation [n.º 2, alínea a) do artigo 31.º do Decreto -Lei n.º 230/2009], the author declares that the work developed in this doctoral thesis is published in peer-reviewed scientific journals (detailed below). The author participated actively in the conceptualisation and execution of the work that originated that data, as well as in their interpretation, discussion, and writing.

Original research articles published in international peer-reviewed journals

1. **Brandão F.**, Costa C., Bessa M.J., Valdiglesias V., Hellack B., Haase A., Fraga S., Teixeira J.P. (2023). Multiparametric in vitro genotoxicity assessment of different variants of amorphous silica nanomaterials in rat alveolar epithelial cells. *Nanotoxicology* 17(6-7), 511-52. https://doi.org/10.1080/17435390.2023.2265481.
2. **Brandão F.**, Costa C., Bessa M.J., Dumortier E., Debacq-Chainiaux F., Hubaux R., Salmon M., Laloy J., Stan M. S, Hermenean A., Gharbia S., Dinischiotu A., Bannuscher, A., Hellack B., Haase A., Fraga S., Teixeira J.P. (2021) Genotoxicity and Gene Expression in the Rat Lung Tissue following Instillation and Inhalation of Different Variants of Amorphous Silica Nanomaterials (aSiO_2 NM). *Nanomaterials* 11(6), 1502. https://doi.org/10.3390/nano11061502.
3. Fernández-Bertólez N., **Brandão F.**, Costa C., Pásaro E., Teixeira, J.P., Laffon B., Valdiglesias V. (2021). Suitability of the In Vitro Cytokinesis-Block Micronucleus Test for Genotoxicity Assessment of TiO_2 Nanoparticles on SH-SY5Y Cells. *International Journal of Molecular Sciences*, 22(16), 8558; https://doi.org/10.3390/ijms22168558.
4. **Brandão F.**, Fernández-Bertólez N., Rosario F., Bessa M.J., Fraga S., Pásaro E., Teixeira J.P., Laffon B., Valdiglesias V., Costa C. (2020). Genotoxicity of TiO_2 Nanoparticles in Four Different Human Cell Lines (A549, HEPG2, A172, and SH-SY5Y). *Nanomaterials* 10(3), 412; https://doi.org/10.3390/nano10030412.

Abstracts published in international peer-reviewed journals

1. **Brandão F.**, Costa C., Bessa M.J., Fraga S., Haase A., Teixeira J.P. (2019). Evaluation of DNA damage in the rat lung after inhalation exposure to TiO_2 and SiO_2 nanoparticles. *Toxicology Letters*. 314S1 (2019) S197. https://doi.org/10.1016/j.toxlet.2019.09.002.

2. **Brandão M.F.P.**, Bessa M.J., Costa C., Fraga S., Haase A., Teixeira, J.P. (2018). Insights into the role of the physicochemical properties in the toxicity of SiO_2 nanoparticles in rat alveolar epithelial RLE-6TN cells. *Toxicology Letters,* 295 S1, S212-S213. https://doi.org/10.1016/j.toxlet.2018.06.921.
3. **Brandão F.**, Bessa M.J., Costa C., Fraga S., Haase A., Teixeira J.P. (2017). Role of surface capping on the cytotoxicity of silica nanoparticles in rat alveolar epithelial cells. *Toxicology Letters,* 280 S1, S187. https://doi.org/10.1016/j.toxlet.2017.07.526.
4. **Brandão, F.**, Costa, C., Valdiglesias, V., N., Fernández-Bertólez, N., Pásaro, E., Laffon, B., Teixeira, J. (2017). Genotoxicity Evaluation of Nanomaterials: What Is the Most Suitable Option of In Vitro Cytokinesis-Block Micronucleus Cytome Assay?. *The Toxicologist,* PS 1540, 127. https://www.toxicology.org/pubs/docs/Tox/2017Tox.pdf.

Awards

Under the frame of the work developed in the current thesis, the following awards were received:

- **2023** IACOBUS award for the best paper: **Brandão F.**, Costa C., Bessa M.J., Valdiglesias V., Hellack B., Haase A., Fraga S., Teixeira J.P. 2023. Multiparametric in vitro genotoxicity assessment of different variants of amorphous silica nanomaterials in rat alveolar epithelial cells. *Nanotoxicology*, 1-18. https://doi.org/10.1080/17435390.2023.2265481
- **2021** IACOBUS award for the best paper: Fernández-Bertólez N., **Brandão F.**, Costa C., Pásaro E., Teixeira J.P., Laffon B., Valdiglesias V. 2021. Suitability of the In Vitro Cytokinesis-Block Micronucleus Test for Genotoxicity Assessment of TiO_2 Nanoparticles on SH-SY5Y Cells. *International Journal of Molecular Sciences* 22. https://doi.org/10.3390/ijms22168558
- **2021** IACOBUS award for the best paper: **Brandão F.**, Fernández-Bertólez N., Rosario F., Bessa M.J., Fraga S., Pásaro E., Teixeira J.P., Laffon B., Valdiglesias V., Costa C. (2020) Genotoxicity of TiO_2 Nanoparticles in Four Different Human Cell Lines (A549, HEPG2, A172 and SH-SY5Y). *Nanomaterials*.10 3. https://doi.org/10.3390/nano10030412.
- **2019** - Mutagenesis (Oxford University Press) – Best Poster presentation award as presenting author at the 13rd International Comet Assay Workshop (ICAW), Pushchino (Russia). **<u>Brandão F.</u>**, Bessa MJ., Costa C., Fraga S., Haase A., Teixeira JP. Assessment of DNA damage in the lung tissue of rats exposed by inhalation to TiO_2 and SiO_2 nanoparticles.
- **2018** - Taylor and Francis Group – Best Oral Communication award as presenting author at the 4th International Congress on Occupational and Environmental Toxicology (ICOETox). Matosinhos (Portugal). **<u>Brandão F.</u>**, Fraga S., Costa C., Silva S., Bessa M.J., Haase A., Teixeira J.P. Toxicity of different classes of manufactured nanomaterials in rat alveolar epithelial RLE-6TN cells.

Resumo

Os nanomateriais de sílica amorfa (aSiO_2 NM) são cada vez mais utilizados numa vasta gama de aplicações, como biossensores, tintas, cosméticos, aditivos alimentares, sistemas de transporte e entrega de fármacos e material genético, produtos dentários e agentes de bioimagem. Com esta utilização crescente, a exposição humana a estes materiais, particularmente por inalação em ambientes profissionais, também aumentou. O presente trabalho teve como objetivo preencher lacunas de conhecimento existentes ao avaliar sistematicamente a toxicidade de aSiO_2 NM em modelos *in vitro* e *in vivo* com foco na via inalatória e em como variações de tamanho e química de superfície influenciam a sua citotoxicidade e genotoxicidade ao nível pulmonar.

Assim, foram testadas sete variantes de aSiO_2 NM, com diferentes tamanhos (7, 15 e 40 nm) e química de superfície (não modificadas ou revestidas com grupos fosfonato, amino e trimetilsilil). Estudos *in vitro* em células epiteliais alveolares de rato (RLE-6TN) avaliaram os efeitos citotóxicos e genotóxicos de todas as variantes de aSiO_2 NM, incluindo alterações na integridade da membrana, atividade metabólica e danos no DNA (quebras de cadeia simples (SSB) e dupla (DSB)) através do ensaio do cometa. As variantes com maior potencial citotóxico e genotóxico (danos no DNA) (SiO_2_7, SiO_2_15_Unmod (não modificado), SiO_2_15_Amino e SiO_2_40) foram ainda analisadas quanto aos seus efeitos na progressão do ciclo celular e nos níveis de histona H2AX fosforilada (γ-H2AX), um biomarcador de resposta ao dano no DNA, por citometria de fluxo.

Os resultados revelaram que as variantes não revestidas, particularmente as SiO_2_7 e SiO_2_15_Unmod, induziram efeitos citotóxicos significativos, enquanto as variantes com revestimento demonstraram um menor potencial citotóxico. É interessante notar que, apesar do elevado potencial citotóxico, as variantes SiO_2_7, SiO_2_15_Unmod, e a variante SiO_2_15_Amino não induziram danos significativos no DNA em concentrações não citotóxicas, ao passo que exposição à variante SiO_2_40 aumentou significativamente o dano no DNA das células epiteliais alveolares, sem, no entanto, afetar a sua atividade metabólica. Deste modo, a modificação da superfície com grupos hidrofóbicos de organosilanos e, em menor grau, com grupos fosfonatos, parece ser uma estratégia eficaz para atenuar a toxicidade dos aSiO_2 NM nas células RLE-6TN. Por outro lado, todas as variantes testadas (SiO_2_7, SiO_2_15_Unmod, SiO_2_15_Amino e SiO_2_40) aumentaram significativamente os níveis totais de γ-H2AX. Na concentração mais elevada (28 μg/cm^2), a exposição a todos os materiais testados levou a uma diminuição da subpopulação G_0/G_1, acompanhada de um aumento significativo das subpopulações S e G_2/M, exceto no caso da variante SiO_2_40, que não afetou a progressão do ciclo celular. Com base nos dados obtidos, as variantes de aSiO_2 NM podem ser classificadas quanto ao seu potencial

genotóxico de dano no DNA da seguinte forma: SiO_2_7 = SiO_2_15_Unmod = SiO_2_40 > SiO_2_15_Amino.

Foi realizado um estudo *in vivo* onde as variantes com maior toxicidade *in vitro* (SiO_2_7, SiO_2_15_Unmod, SiO2_15_Amino e SiO_2_40), foram administradas por instilação intratraqueal no rato, para avaliação dos danos primários e oxidativos no DNA e das alterações da expressão génica no tecido pulmonar. Além disso, foi efetuado um estudo de inalação a curto prazo (STIS) no rato, com a variante de menor tamanho (SiO_2_7) e com o TiO_2-NM105 como NM de referência. Nos estudos de instilação, o SiO_2_7 ou o SiO_2_40 causaram lesões oxidativas significativas no DNA, embora estas não tenham sido observadas nos grupos de recuperação, o que sugere que estas lesões tenham sido reparadas. No STIS, detetámos um aumento significativo das quebras de cadeia de DNA no tecido pulmonar de animais expostos a 0,5 mg/m³ de SiO_2_7 ou 50 mg/m³ de TiO_2_NM105, em ambos os grupos (grupo exposto e grupo de recuperação). As alterações observadas na expressão génica sugerem que as vias do stress oxidativo e/ou inflamatórias estão provavelmente envolvidas na indução dos danos oxidativos no DNA observados. De um modo geral, *in vivo* não se detetou uma toxicidade significativa dos aSiO_2 NM testados quer no estudo de instilação intratraqueal, quer no STIS. Os efeitos genotóxicos da variante SiO_2_7, foram concordantes em ambos os estudos *in vivo* realizados, no entanto, as alterações observadas nos animais do STIS foram mais permanentes, mais difíceis de reverter.

Os resultados dos estudos *in vivo* alinharam-se com os resultados dos estudos *in vitro*, indicando que a via de síntese desempenha um papel na determinação do modo de ação (MoA) do SiO_2 NM, com as variantes pirogénicas (SiO_2_7 e SiO_2_40) a causarem maior toxicidade do que as variantes sintetizadas por precipitação (SiO_2_15_Unmod e SiO_2 _15_Amino). Consequentemente, as células RLE-6TN parecem ser um modelo preditivo da toxicidade pulmonar *in vivo* dos SiO_2 NM.

Os nossos resultados contribuíram para o desenvolvimento de uma estratégia de agrupamento de nanomateriais de engenharia (ENM) no âmbito do projeto NanoToxClass, baseada na abordagem DF4nanoGrouping, com objetivo de agilizar o processo de avaliação de risco dos nanomateriais. Esta classificação sistemática revelou que o revestimento da superfície e a via de síntese influenciam significativamente o MoA dos aSiO_2 NM de, revelando que as variantes pirogénicas são mais ativas do que as variantes sintetizadas por precipitação, enquanto as variantes revestidas apresentam uma menor toxicidade comparativamente às não revestidas. Além disso, os nossos resultados apoiam a utilidade de abordagens multiparamétricas para melhorar a compreensão do MoA dos ENM e a previsão do seu perigo. Eles salientam também o potencial destas abordagens e estratégias de agrupamento para se alinharem com os 3R (Substituição, Redução e

Refinamento), reduzindo a necessidade de testes *in vivo* e promovendo um desenvolvimento de ENM seguros por conceção (safe-by-design).

Por último, o presente trabalho sublinha a necessidade de protocolos rápidos, robustos e reprodutíveis para a avaliação dos perigos dos ENM, defendendo a adaptação dos ensaios clássicos de genotoxicidade e a utilização de técnicas de elevado rendimento. Estes conhecimentos são cruciais para o avanço da nanotoxicologia, promovendo a conceção segura de ENM e facilitando o desenvolvimento eficiente de produtos com recurso à nanotecnologia novos e sustentáveis.

Palavras-chave: nanomateriais de sílica amorfa; toxicidade pulmonar; ensaios de genotoxicidade; classificação de perigo; estratégias de agrupamento e classificação de perigo.

Abstract

Amorphous silica nanomaterials (aSiO_2 NM) are increasingly utilized in a wide range of products, such as biosensors, paints, cosmetics, food additives, carriers for gene and drug delivery, dentistry and bioimaging agents. Consequently, human exposure to aSiO_2 NM, particularly through inhalation in occupational settings, has also increased. The present study aimed to fill in knowledge gaps regarding the hazards of aSiO_2 NM by systematically assessing their pulmonary toxicity *in vitro* and *in vivo*, focusing on the influence of particle size and surface chemistry on cyto- and genotoxicity.

Variants of aSiO_2 NM differing in size (7, 15, and 40 nm) and surface capping (unmodified, phosphonate-, amino-, and trimethylsilyl-modified) were tested. *In vitro* studies in assessed the cytotoxic and genotoxic effects of all aSiO_2 NM variants rat alveolar epithelial cells (RLE-6TN), including changes in membrane integrity, metabolic activity, and DNA damage (single- (SSB) and double-strand breaks (DSB)) by the comet assay. The variants with higher cytotoxic and DNA-damaging potential (SiO_2_7, SiO_2_15_Unmod (unmodified), SiO_2_15_Amino and SiO_2_40) were further analysed for effects on cell cycle progression and levels of phosphorylated histone H2AX (γ-H2AX), a biomarker of DNA damage response, by flow cytometry.

The results revealed that unmodified variants, particularly SiO_2_7 and SiO_2_15_Unmod, exhibited significant cytotoxicity, while surface-modified variants demonstrated lower cytotoxicity. Interestingly, despite high cytotoxic potential, the variants SiO_2_7, SiO_2_15_Unmod, and SiO_2_15_Amino did not induce significant DNA damage at non-cytotoxic concentrations, whereas SiO_2_40 caused notable DNA damage without affecting metabolic activity of RLE-6TN cells. Thus, surface modification with hydrophobic organosilanes, and in a lesser extent with phosphonate groups, seems to be an effective strategy to mitigate the toxicity of aSiO_2 NM in RLE-6TN cells. On the other hand, all variants tested (SiO_2_7, SiO_2_15_Unmod, SiO_2_15_Amino, and SiO_2_40) significantly increased total γ-H2AX levels. At high concentrations (28 mg/cm^2), exposure to all tested materials led to a decrease in the G_0/G_1 subpopulation, accompanied by a significant increase in the S and G_2/M subpopulations, except for SiO2_40, which did not affect cell cycle progression. Based on the obtained data, aSiO_2 NM variants can be ranked for its genotoxic DNA damage potential as follows: SiO_2_7 = SiO_2_15_Unmod = SiO_2_40 > SiO_2_15_Amino.

In vivo studies included intratracheal instillation in rats of the SiO_2 NM variants with higher *in vitro* toxicity (SiO_2_7, SiO_2_15_Unmod, SiO_2_15_Amino and SiO_2_40), evaluating primary and oxidative DNA damage and gene expression changes in the lung tissue. Additionally, a short-term inhalation study (STIS) with the smallest size variant of SiO_2 NM (7 nm), and using TiO_2-NM105 as a reference NM, was conducted. In the instillation studies,

SiO_2_7 or SiO_2_40 caused significant oxidative DNA lesions in the lung tissue of the exposed animals, although those seem to be repaired as no significant changes have been detected in recovery groups. In the STIS, a significant increase in DNA strand breaks in the lung tissue of rats exposed to 0.5 mg/m³ of SiO_2_7 or 50 mg/m³ of TiO_2_NM105 was detected in both groups (i.e. in the exposure and recovery groups). The observed changes in gene expression suggest that oxidative stress and/or inflammatory pathways are likely implicated in the induction of oxidative DNA damage. Overall, the $aSiO_2$ NM tested were not associated with significant *in vivo* toxicity following either instillation or STIS. The genotoxicity findings for SiO_2_7 were concordant in both studies, however, the changes observed in the STIS animals were more permanent, more difficult to revert.

The *in vivo* results are aligned with the *in vitro* findings, indicating that the synthesis route plays a role in determining the mode of action (MoA) of $aSiO_2$ NM, with pyrogenic $aSiO_2$ NM variants (SiO_2_7 and SiO_2_40) exhibiting greater toxicity than precipitated $aSiO_2$ NM variants (SiO_2_15_Unmod and SiO_2_15_Amino). Consequently, RLE-6TN cells seem to be a predictive model of $aSiO_2$ NM pulmonary toxicity *in vivo*.

Our findings contributed to the development of a grouping strategy for engineered nanomaterials (ENM) within the NanoToxClass project, based on the DF4nanoGrouping approach, to support ENM risk assessment. This systematic classification revealed that surface coating and synthesis route significantly influence the MoA of $aSiO_2$ NM, supporting that pyrogenic $aSiO_2$ variants are more active than the variants synthesized by precipitation, while the surface-capped variants exhibit reduced toxicity compared to the uncoated ones. Additionally, our findings support the usefulness of multiparametric approaches to improve understanding of NM MoA and hazard prediction. They also emphasize the potential of these approaches and grouping strategies to align with the 3R (Replacement, Reduction, and Refinement) by reducing dependence on *in vivo* testing and promoting safer-by-design of ENM.

Finally, the present work underscores the need for rapid, robust, and reproducible protocols for ENM hazard assessment, advocating for the adaptation of the classical genotoxicity assays and the use of high-throughput techniques. These insights are crucial for advancing nanotoxicology, promoting the safe design of ENM, and facilitating the efficient development of new and sustainable nano-based products.

Keywords: amorphous silica nanomaterials; pulmonary toxicity; genotoxicity testing; hazard ranking; grouping and hazard classification strategies.