Biocompatibility evaluation of innovative antimicrobial bioactive coatings for metal surfaces in a novel 3D cell culture system

Gori M.[1], Laudizi D.[2], Vigato G.[1], Cappelletti M.[3], Balbo A.[4], Chiavari C.[5], Bernardi E.[6], Bertoldo M. [7], Gallo F.[7], Farè S.[2], Graziani G.[2]

[1]Institute of Biochemistry and Cell Biology (IBBC) – National Research Council (CNR), Monterotondo Scalo (Rome), Italy; [2]Dept of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Italy; [3]Dept of Pharmacy and Biotechnology, University of Bologna (UNIBO), Italy ; [4]Dept of Engineering, Corrosion and Metallurgy Study Centre "A. Daccò", University of Ferrara, Italy; [5]Department of Cultural Heritage, University of Bologna (UNIBO), Italy; [6]Dept of Industrial Chemistry "Toso Montanari", University of Bologna (UNIBO), Italy; [7]Department of Chemical, Pharmaceutical and Agricultural Sciences, University of Ferrara, Italy.

Email: manuele.gori@cnr.it

Finanziato dall'Unione europea NextGenerationEU

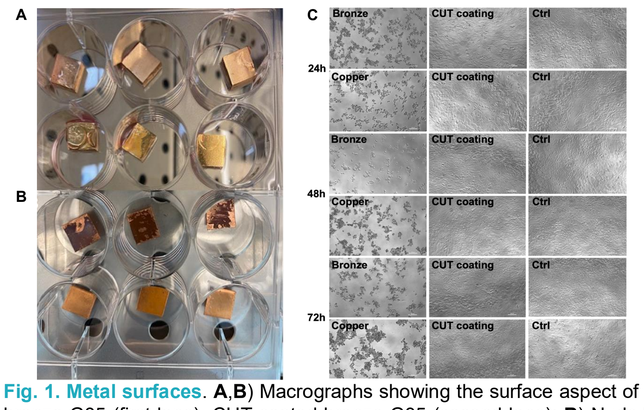
Italiadomani – Piano Nazionale di Ripresa e Resilienza

Ministero dell'Università e della Ricerca

ABSTRACT

In modern society, engineering novel sustainable biomaterials with antibacterial and antibiofilm activities, aiming at preventing microbial colonization of metal surfaces in healthcare environments and in cultural heritage, is a matter of urgent importance [1, 2]. Thus, in the present work, funded by the European Union - NextGeneratonEU (PRIN 2022 PNRR), we aim to develop and characterize novel sustainable bioactive coatings for protecting metallic surfaces from bacteria colonization, corrosion and deterioration due to external environmental factors. Notably, here we evaluate the biocompatibility levels of an innovative bio-based protective coating (*i.e.,* a cutin/silane hybrid coating derived from tomato peel - CUT/PSH) for metal surfaces using a novel gelatin-based 3D cell culture system to closely mimic the physiological state of tissue architecture.

1 Metal substrates of bronze and copper



Metal substrates and coatings

Metal Substrates:

- **Bronze G85 (Br) for Cultural Heritage applications**
- **PHC Copper (Cu) for Healthcare safety applications**

Coatings:

1. **Cutin/silane hybrid coating (CUT/PSH) from food waste (tomato peel)**
2. **CUT/PSH functionalized with Ag-nanoparticles (NPs) embedded at 2.400 ppm**

Fig. 1. Metal surfaces. **A**,**B**) Macrographs showing the surface aspect of the metal substrates after 24h incubation with cell culture medium: nude bronze G85 (first lane), CUT-coated bronze G85 (second lane); **B**) Nude PHC copper (Cu, first lane), CUT-coated PHC copper (second lane); all surfaces of the metal substrates, except those that were tested, were protected with a semi-permanent and biocompatible nail polish. **C**) Phase contrast images of mouse fibroblasts (*i.e.,* BALB/3T3 cells, 10X magnification) cultured in 2D in the 24h-conditioned media from nude bronze, copper and CUT-based coatings *vs.* control medium (Ctrl) for 24h, 48h and 72h showing different morphology based on the surface, with growth characteristics similar to Ctrl on the CUT-based coatings that prevent the detachment and stress observed on nude substrates, scale bars: 100um.

2 3D cell culture system

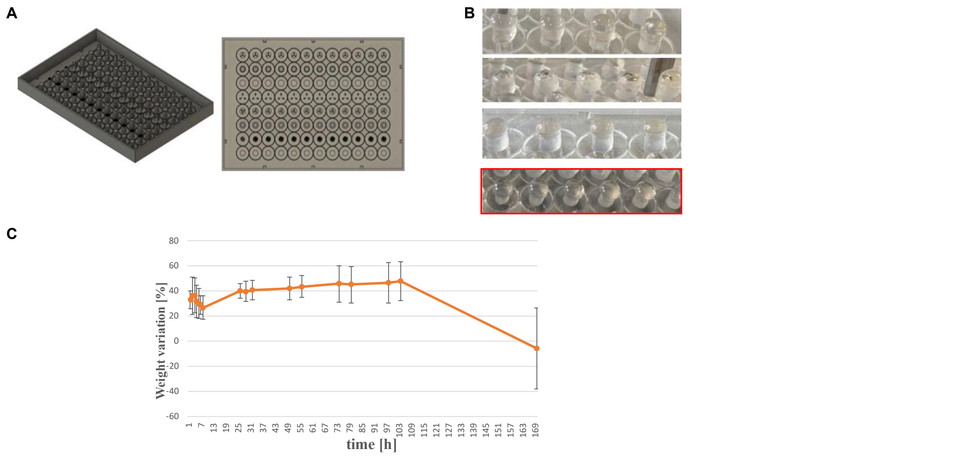


Fig. 2. Customized gelatin-based 3D cell culture system. A) Customized top plate, with different geometries of the pillars, designed to guarantee anchoring of cellularised gel beads. **B**) Bovine gelatin type B beads formed on pillars having various geometries show different shape fidelity. In the bottom image (highlighted in red), the optimized geometry permits correct formation of gelatin beads without detachment or defects. **C**) Results of the swelling and stability test for the pillar + bead system show moderate swelling in the first 24h, with minor modifications in the beads size, and optimal adhesion at all time points analysed (*i.e.,* 24-48-72-96h and 1 week).

3 Biocompatibility of CUT/PSH coatings for the PHC Cu surface on mouse cells

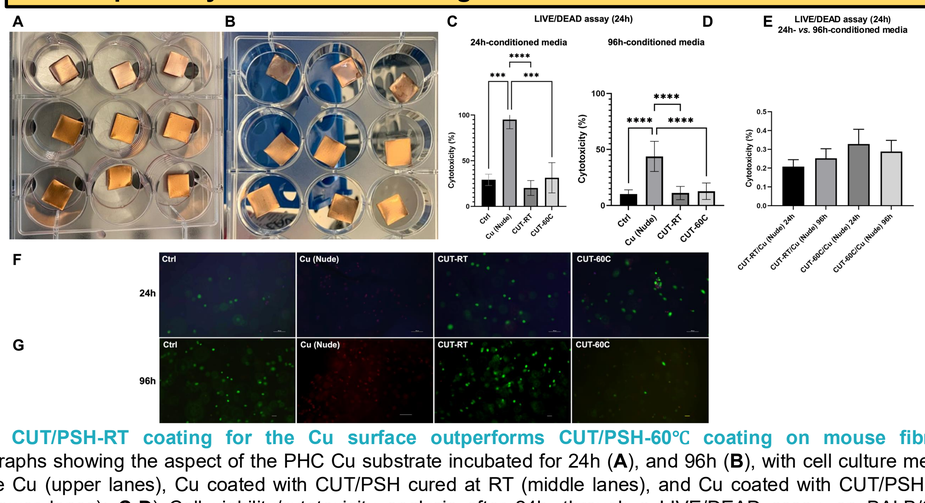


Fig. 3. CUT/PSH-RT coating for the Cu surface outperforms CUT/PSH-60℃ coating on mouse fibroblasts. Macrographs showing the aspect of the PHC Cu substrate incubated for 24h (**A**), and 96h (**B**), with cell culture medium: **A-B**) nude Cu (upper lanes), Cu coated with CUT/PSH cured at RT (middle lanes), and Cu coated with CUT/PSH cured at 60°C (lower lanes); **C-D**) Cell viability/cytotoxicity analysis after 24h, through a LIVE/DEAD assay, on BALB/3T3 cells cultured into 3D gelatin pillars incubated with the 24h-conditioned media (**C**) and the 96h-conditioned media (**D**), ***p<0.001, ****p<0.0001, with representative confocal images (**F** and **G**, respectively, 10X magnification) of live (green) and dead (red) cells, nuclei (blue) were counterstained with Hoechst 33258, scale bars: 100um; **E**) Comparison of the protective effect (cytotoxicity ratio *vs.* Cu Nude) between 24h- and 96h-conditioned media from CUT-RT and CUT-60C incubated for 24h showing no statistically significant differences: the 24h time point has been selected as standard incubation period.

4 Biocompatibility of CUT/PSH-RT coating for the PHC Cu and bronze G85 surfaces on human cells (HDFs)

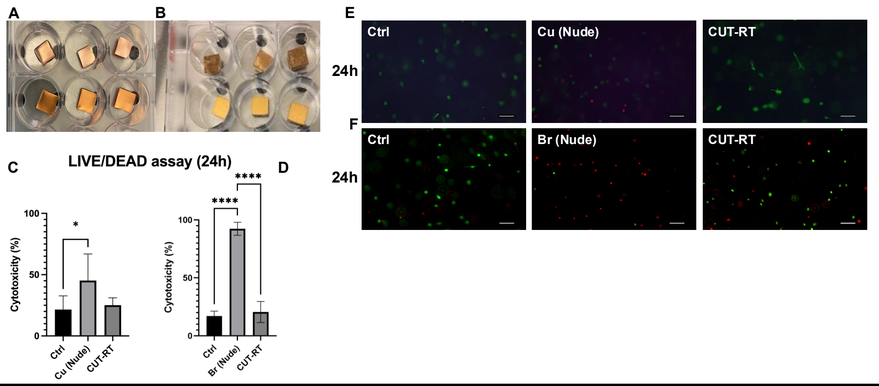


Fig. 4. Biocompatibility of CUT/PSH-RT coating for the Cu and bronze surfaces on HDFs. Macrographs showing the aspect of the PHC Cu substrate (**A**) and the bronze G85 substrate (**B**) incubated for 24h with cell culture medium: **A**) nude Cu (upper lane), Cu coated with CUT/PSH cured at RT (lower lane), **B**) nude bronze (upper lane) and bronze coated with CUT cured at RT (lower lane); **C-D**) Cell viability/cytotoxicity analysis after 24h, through a LIVE/DEAD assay, on HDFs cultured into 3D gelatin pillars incubated with the 24h-conditioned media from the CUT/PSH-RT coating for PHC Cu (**C**) and bronze G85 (**D**), *p<0.05, ****p<0.0001, with representative confocal images (**E** and **F**, respectively, 10X magnification) of live (green) and dead (red) cells, nuclei (blue) were counterstained with Hoechst 33258, scale bars: 100um.

5 Oxidative stress protection of CUT/PSH-RT coating for the PHC Cu and bronze G85 surfaces on HDFs

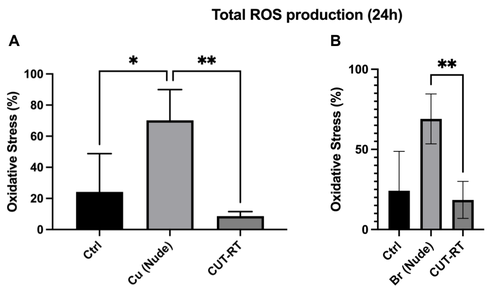


Fig. 5. CUT/PSH-RT coating for the Cu and bronze surfaces is protective against oxidative stress on HDFs. Total ROS production analysis, through a CellROX assay, on HDFs cultured into 3D gelatin pillars incubated with the 24h-conditioned media from the CUT/PSH-RT coating (in Figure 4A-B) on Cu (**A**) and bronze (**B**) for 24h; *p<0.05, **p<0.01.

6 Biocompatibility of CUT/PSH-RT coating for the PHC Cu and bronze G85 surfaces on human cells (A549)

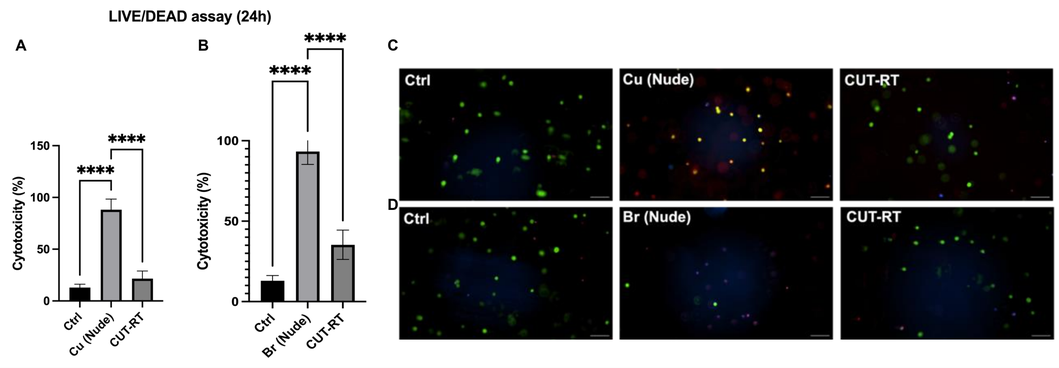


Fig. 6. Biocompatibility of CUT/PSH-RT coating for the Cu and bronze surfaces on A549 cells. A-B) Cell viability/cytotoxicity analysis after 24h, through a LIVE/DEAD assay, on A549 cells cultured into 3D gelatin pillars incubated with the 24h-conditioned media from the CUT/PSH-RT coating for PHC Cu (**A**) and bronze G85 (**B**), ****p<0.0001, with representative confocal images (**C** and **D**, respectively, 10X magnification) of live (green) and dead (red) cells, nuclei (blue) were counterstained with Hoechst 33258, scale bars: 100um.

7 CUT/PSH-RT coating functionalized with Ag-NPs for the PHC Cu and bronze G85 surfaces

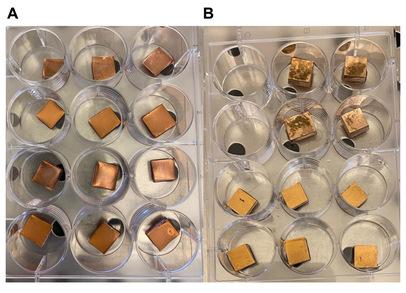


Fig. 7. Functionalized CUT/PSH coating for the Cu and bronze surfaces. Macrographs showing the aspect of the PHC Cu (**A**) and bronze G85 (**B**) substrates after 24h-incubation with cell culture media: **A**) nude Cu incubated with DMEM (first upper lane) and RPMI (second lane) media, Cu coated with CUT/PSH cured at RT + Ag-NPs in DMEM and RPMI, (third lane and lower lane, respectively); **B**) nude bronze G85 incubated with DMEM (first upper lane) and RPMI (second lane), bronze coated with CUT/PSH cured at RT + Ag-NPs incubated with DMEM and RPMI media (third and lower lane, respectively).

8 Biocompatibility of CUT/PSH+Ag-NPs coating for the PHC Cu and bronze G85 surfaces on HDFs and A549

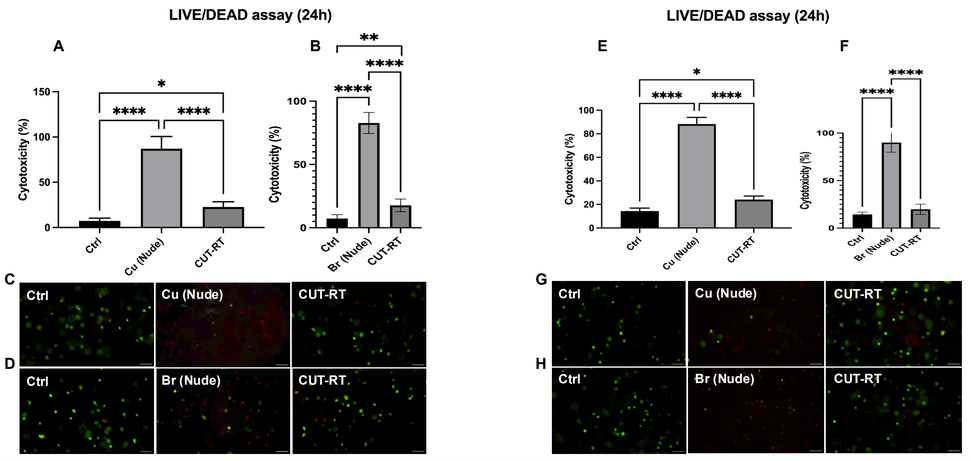


Fig. 8. Biocompatibility of CUT/PSH-RT+Ag-NPs coating for the Cu and bronze surfaces. Cell viability/cytotoxicity analysis, through a LIVE/DEAD assay, on HDFs (**A-D**) and A549 cells (**E-H**) cultured into 3D gelatin pillars incubated with the 24h-conditioned media from Cu (**A,C,E,G**) and bronze (**B,D,F,H**) surfaces coated with CUT/PSH coating cured at RT + Ag-NPs in DMEM for 24h (from Figure 7A-B); *p<0.05, **p<0.01, ****p<0.0001. **C-D**, **G-H**) Representative confocal images (10X magnification) of live (green) and dead (red) cells, nuclei (blue) were counterstained with Hoechst 33258, scale bars: 100um.

9 Anticorrosion activity of the CUT/PSH-RT coatings

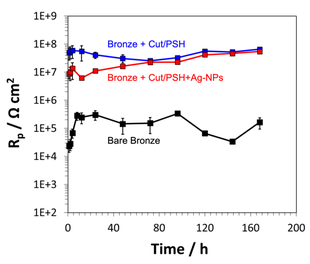


Fig. 9. Representative anticorrosive properties of the CUT/PSH-RT coatings for the bronze surface. Rp, Polarization Resistance values (inversely proportional to corrosion rate) measured over a 7-day exposure to synthetic rain. Values exceeding 10^6 $\Omega \cdot cm^2$ indicate a protective coating.

10 Antibacterial activity of the CUT/PSH-RT coatings

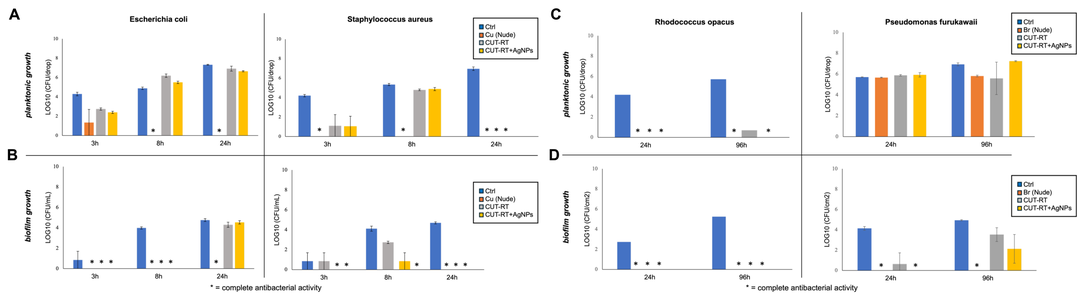


Fig. 10. Antibactrial activities of the CUT/PSH-RT coatings for the Cu and bronze surfaces. Analysis of *Escherichia coli* colonization of the CUT/PSH-RT and CUT/PSH-RT+Ag-NPs coatings for the PHC Cu surface *via* planktonic growth (**A** left) and biofilm growth (**B** left) evaluation and *Staphylococcus aureus* (**A** and **B** right, respectively) after 3, 8 and 24h; **C**,**D**) Same analysis described above for colonization of the same coatings for the bronze G85 surface by *Rhodococcus opacus* (left) and *Pseudomonas furukawaii* (right) after 24 and 96h; *p<0.05.

CONCLUSIONS and OUTLOOKS

Collectively, the innovative CUT/PSH-based coating developed and characterized by our group turned out to be safe and protective against the cytotoxicity induced by the bare metal surfaces. Therefore, the herein described surface coating could represent a good candidate as a new generation of protective coating for metal surfaces that are used in cultural heritage and healthcare institutions, showing excellent biocompatibility, anticorrosion properties, and antibacterial activity together with environmental sustainability.
Additional biocompatibility experiments for the same functionalized CUT/PSH coating will be carried out on human cells for the analysis of oxidative stress, apoptosis and inflammation before and after artificial ageing for a comprehensive evaluation of the coating biocompatibility.

References and Acknowledgments

[1] Alt, V., 2017, 10.1016/j.injury.2016.12.011
[2] Branysova, T. et al., 2022, 10.1016/j.culher.2022.03.013

The authors acknowledge the financial support from the European Union – Next Generation EU, in the framework of PRIN 2022 PNRR, Project title: «BIO-COATCH».