University of Salento
NANOTECHNOLOGY

Ibtikar
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«Nano-structured electrochemical sensors as devices for the measurement of oxidative potential (OP) of atmospheric particulate (PM) and correlation with cellular endpoints»

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PON RICERCA E INNOVAZIONE

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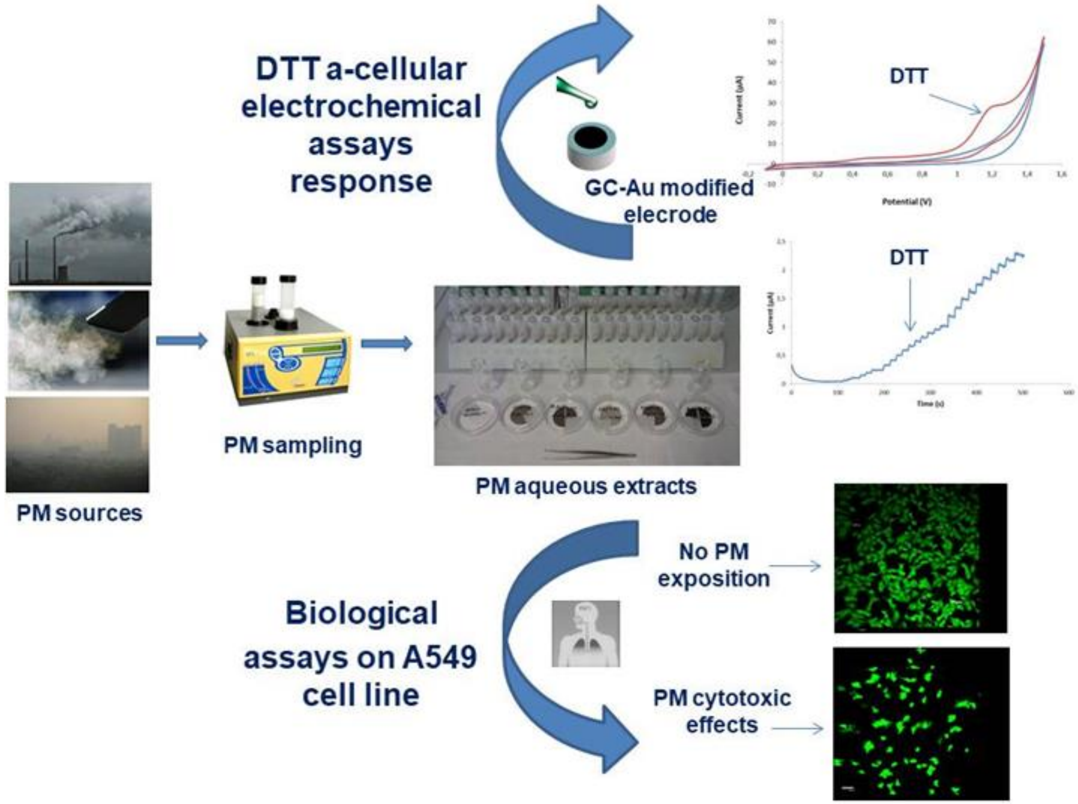
The interdisciplinary study

Research Focus on **PM toxicity** and its **oxidative potential**

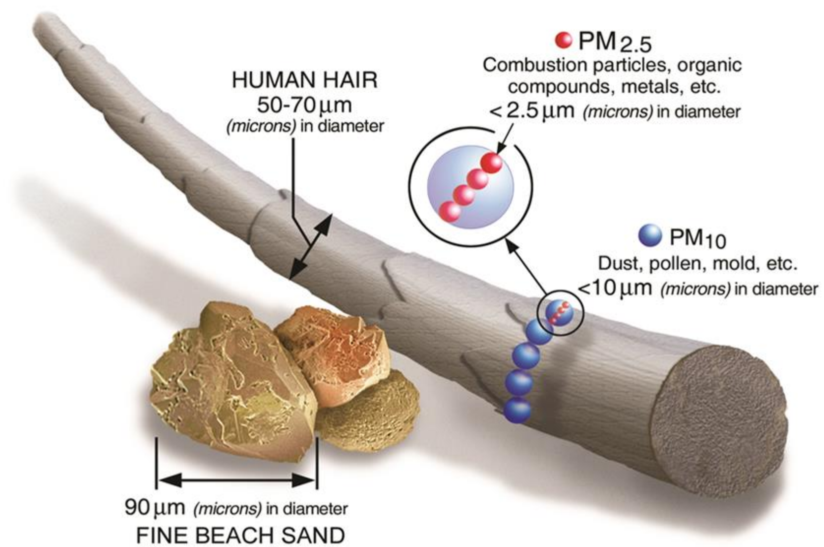


Reliable tools are needed for estimating the oxidant generating capacity of PM at high temporal resolution [1].

PM is a prime candidate for the generation of ***cellular oxidative stress****. Concerning the mechanisms by which PM impacts tissues, the evidence suggests that PM induces cellular oxidative stress through the generation of reactive oxygen species (ROS). [2]*

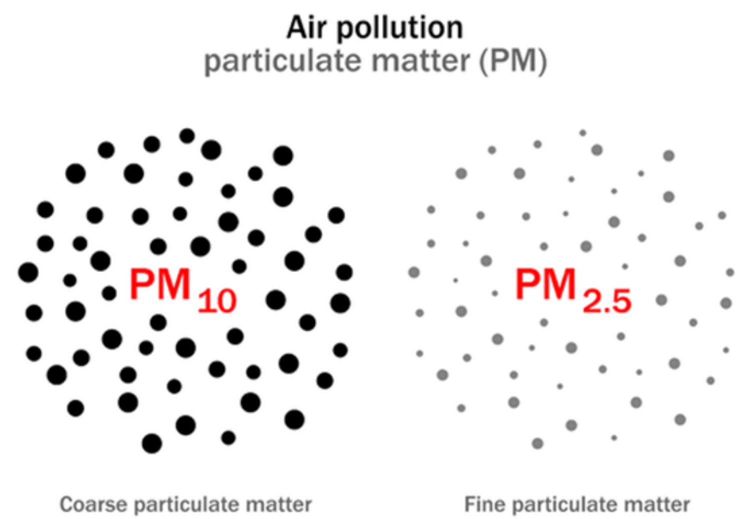
[1] Koehler et al. "Aerosol Sci Technol. " 48, 489-497, 2014; [2] Cho et all., 2005. "Environ. Res. " 99, 40-47

Introduction



The term "particulate" refers to the solid and liquid particles dispersed in the atmosphere and it is responsible for the generation of the biological oxidative stress.

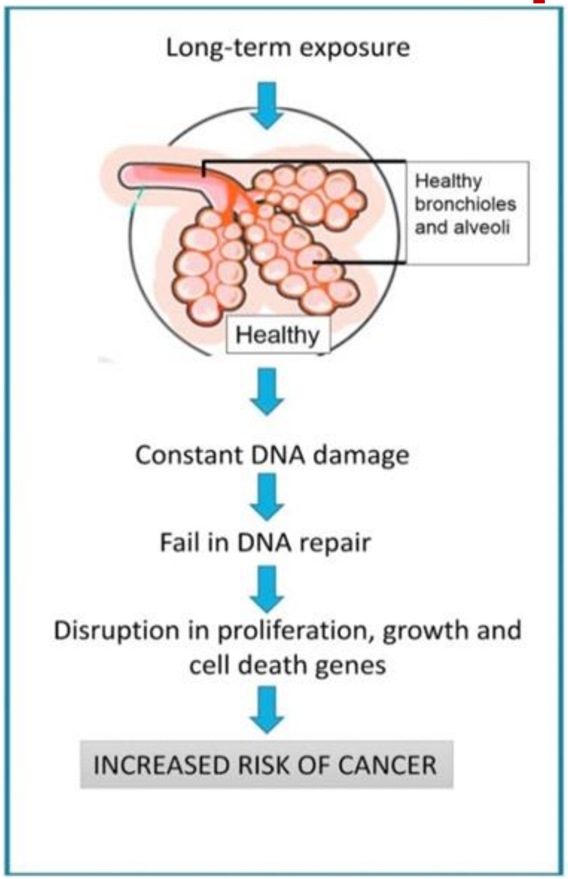
Long and short-term exposure to atmospheric **particulate matter (PM)** has detrimental effects on human health being associated with morbidity and mortality. [3]

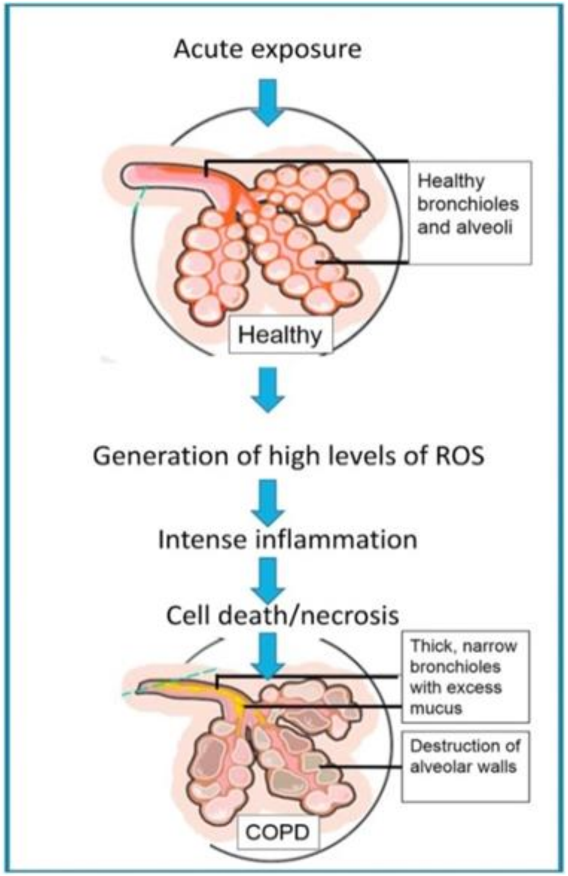


[3] Lionetto et al., 2021 Atmosphere, 12, 464

Health effect of exposure to PM

Airborne PM induces harm by generating **ROS** in human tissues, leading to **oxidative stress.** [4]



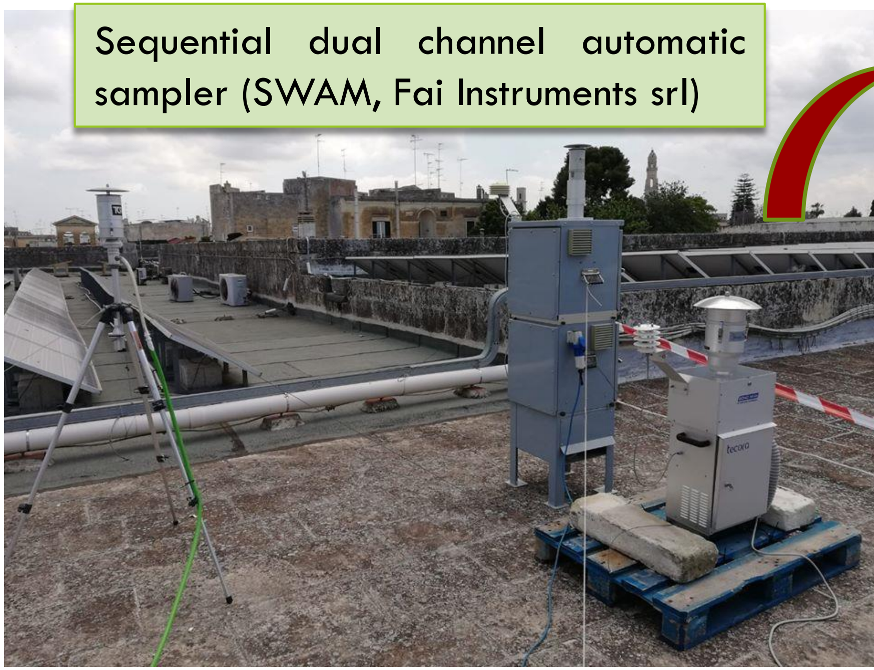


Air pollution has been associated with harmful effects on human health. Small particles can penetrate deep into the lungs (the first sites of exposure to PM) and cause severe problems: DNA damage and mutations. [5]

[4] Yupaporn Sameenoi et al., JACS 2012.; [5] Peixoto MS et al, Elsevier 2017.

The equipment: PM sampling

Sequential dual channel automatic sampler (SWAM, Fai Instruments srl)



PM filters

PM aqueous extracts for cellular and acellular tests

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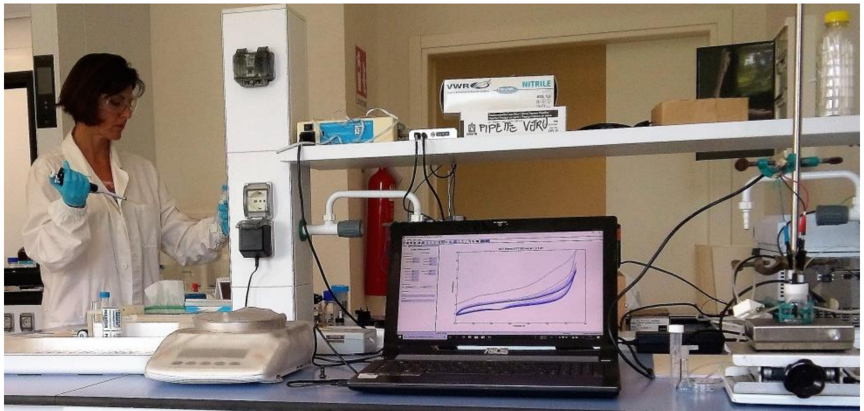
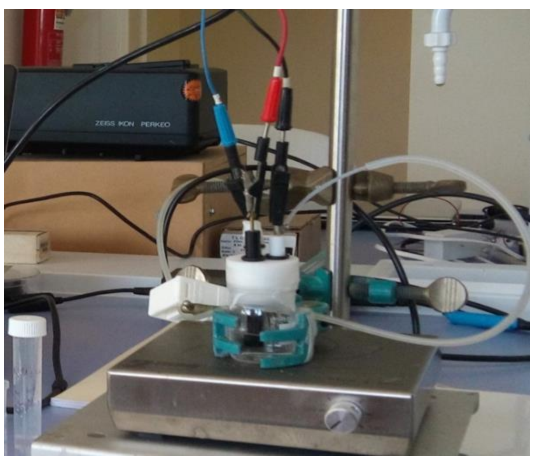
The equipment for a-cellular tests

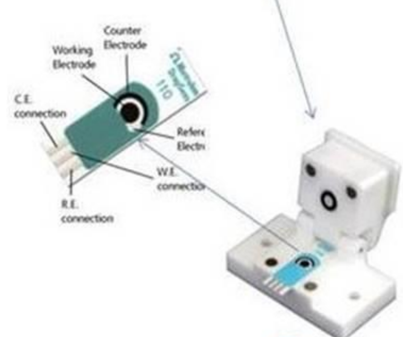


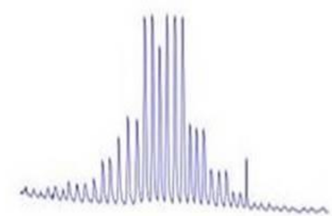


Batch experiments: the conventional three-electrode cell, Pt wire counter electrode and Ag/AgCl reference electrode.

DropSens Flow-Cell (FLWCL) with an innovative open-close system (no screws needed) and an easy sensors replacement

Microfluidic cell



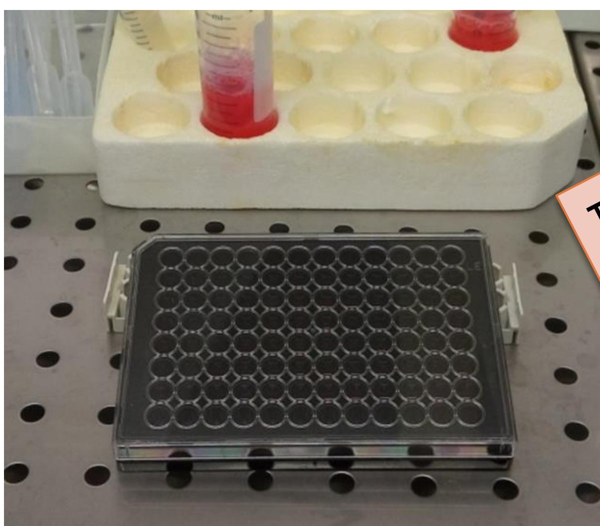


FIA analysis

The equipment for cellular assays

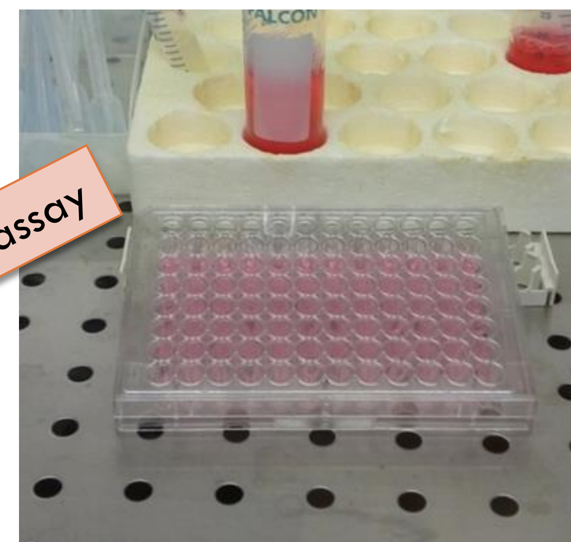
The cells detachment

The manipulation of the cell cultures took place under sterile conditions, under a laminar flow hood



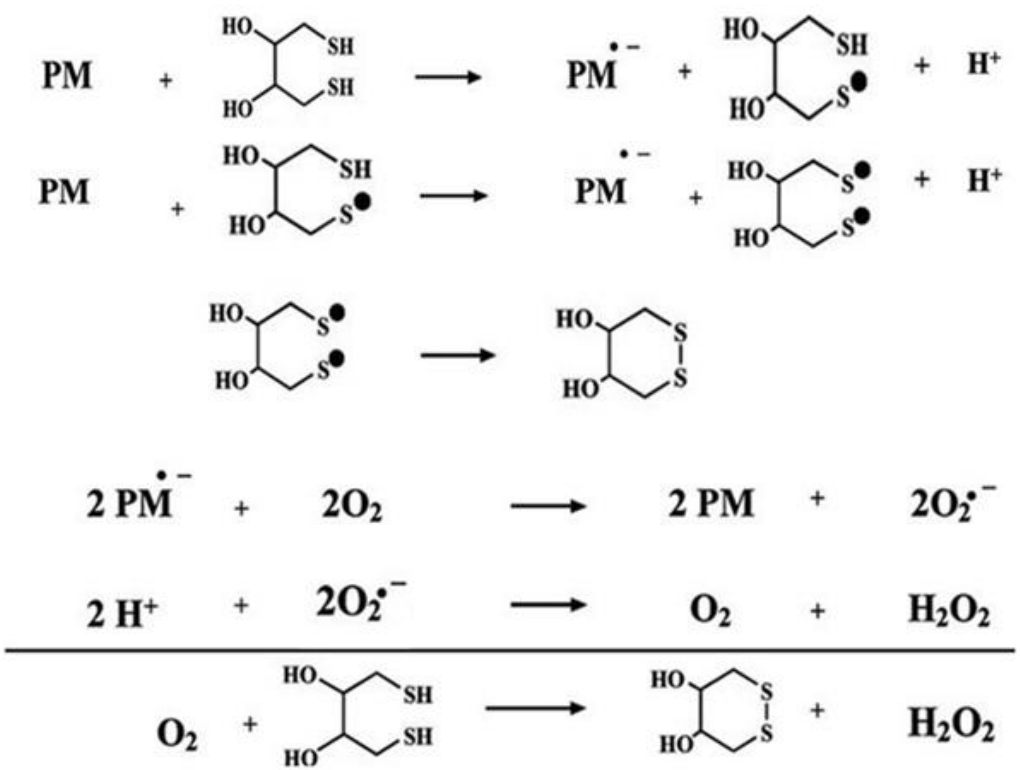
The ROS-sensitive fluorescent probe

The MTT assay



The spectrophotometric DTT assays

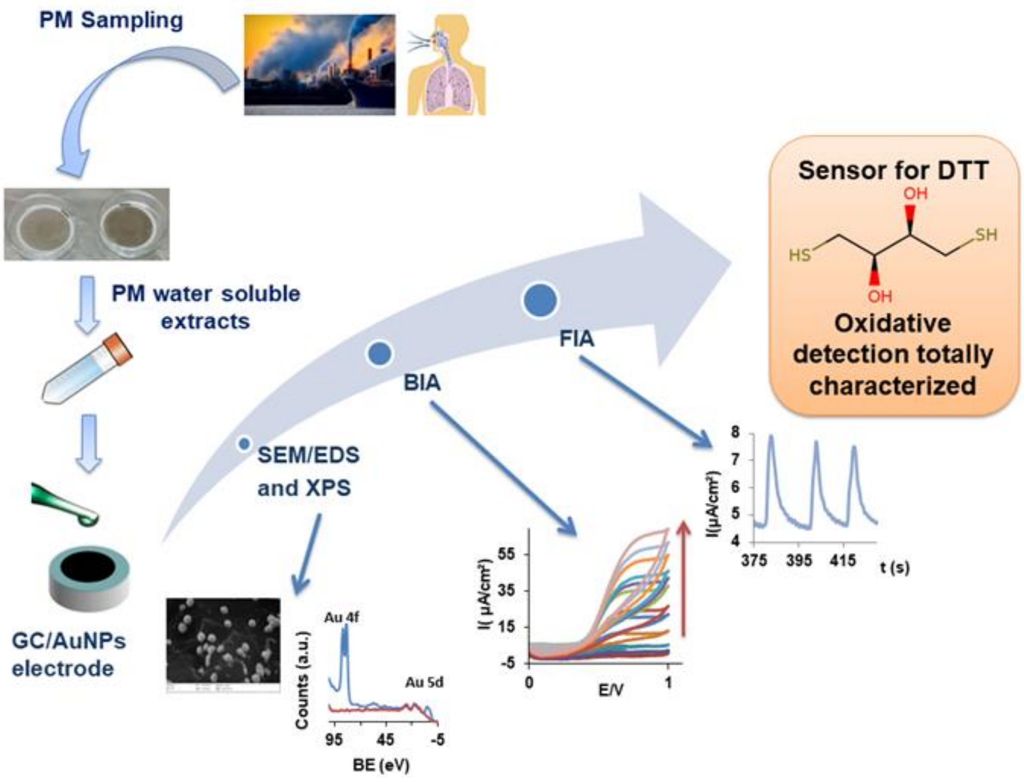
The **thiols** are important molecules involved in biological and environmental processes. Among the thiols, the dithiothreitol DTT, known as Cleland reagent.



DTT assay, the reduced DTT in presence of ROS, generated by PM, is oxidized to its disulfide, the remaining reduced DTT reacts quantitatively with Ellman's reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) (DNTB) to form 2-nitro-5-thiobenzoic acid (TNB).

Aim of the study

The main aim → **The development of an innovative electrochemical sensor for thiols' detection.**



The sensor is a gold nanostructured electrochemical one based on modified **Glassy Carbon** (GC) electrode. It is a suitable device for the measurement of the oxidative potential (OP) of the atmospheric particulate matter (PM), considered a global indicator of adverse health effects of PM.

The specific objectives

- The **implementation of an innovative sensor strategy in dithiotreitol (DTT) detection** based on a gold nanoparticles glassy carbon modified electrode. The operating principle of the sensor is the determination of the OP, through the measurement of the consumption of DTT content, **as an alternative to the classic DTT spectrophotometric methods.**

- The application of the developed sensor to the measurement of OP for some **PM real samples** as proof of concept and its validation by using the classic DTT spectrophotometric method.

- The study, deepened through the **chemical and toxicological characterization** of the water-soluble fraction of particulate matter, in order to reach an extensive knowledge of the **relationship** between the oxidative potential of PM_{10} assessed by the use of the DTT oxidation principle and the effect of PM_{10} at the cellular level.

The modified electrode

The sensor is a **Glassy Carbon electrode modified by electrochemical deposition with Gold Nanoparticles (GC/AuNPs)**, useful for the dithiothreitol (DTT) detection, in order to evaluate the OP.

THE STEPS:

- **Direct drop casting** of the $AuCl_3$ solution (15μL) 0,137 mM on a bare **Glassy Carbon** electrode.
- The Au electrodeposition had been performed by **chronoamperometry** at the applied potential of -0.9V *vs* Ag/AgCl for t=200s.
- **Ciclic Voltammetry** between -1.0V and 1.5V was performed until the steady state (20 cycles).

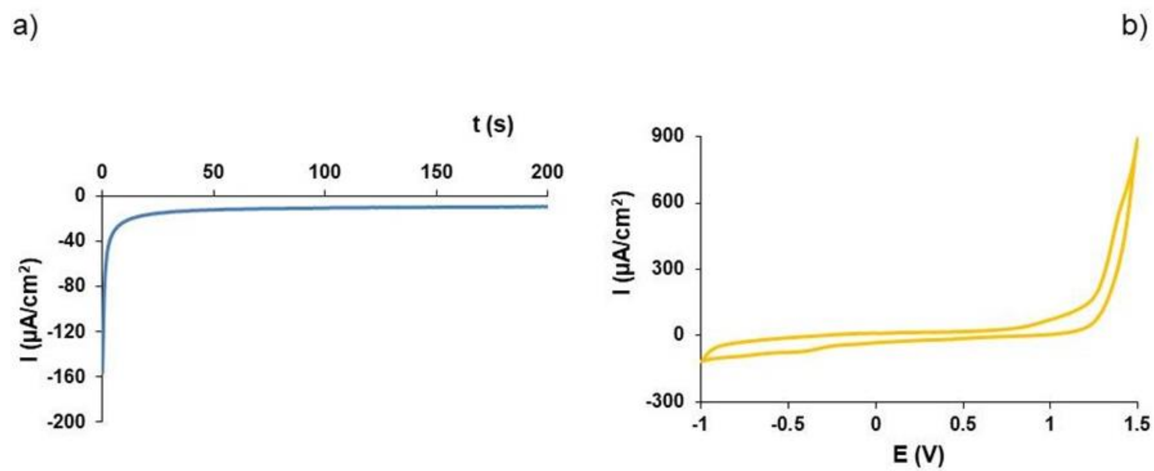
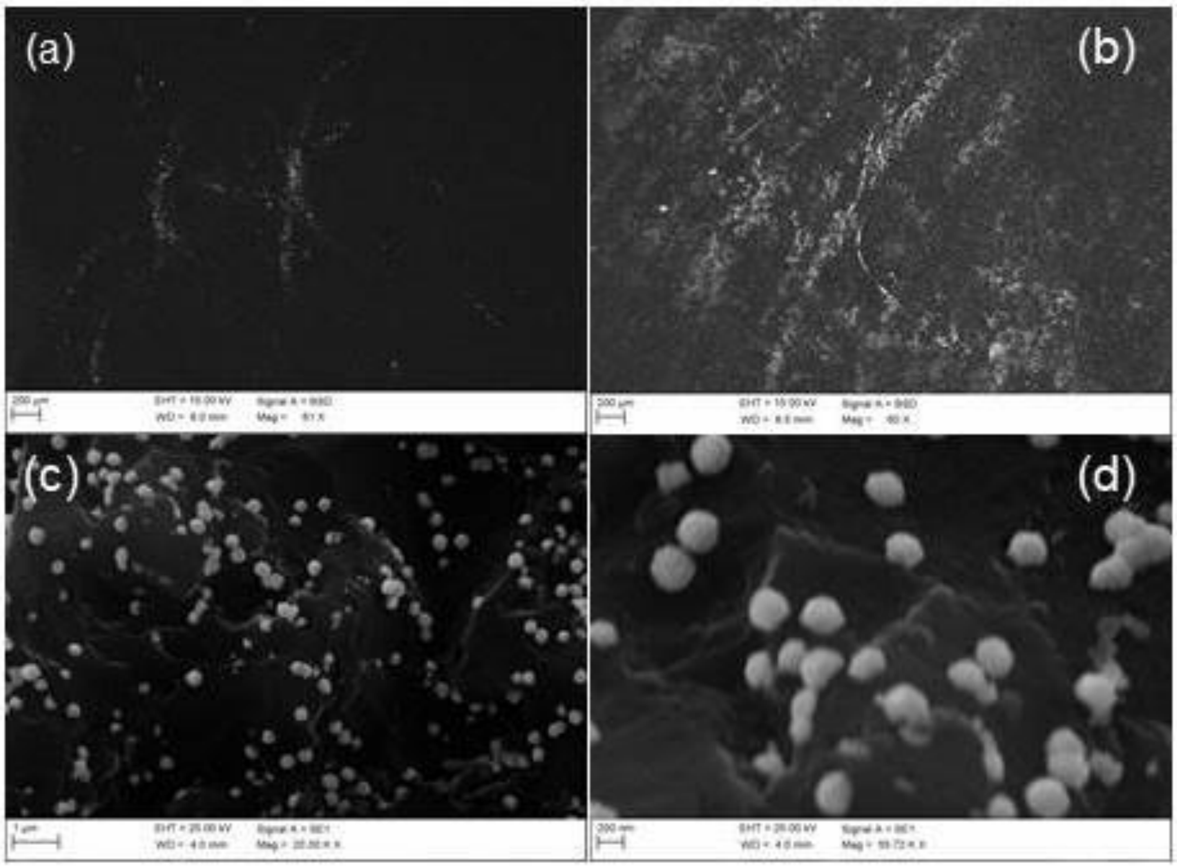


FIG. Electrodeposition of gold by chrono-amperometry at -0.9V vs Ag/AgCl, deposition time t=200s, Q= 2200 ±100 μCcm-2; (b). CV in clean phosphate buffer solution (pH 7.4) between -1.000V and 1.500V of the GC gold modified electrode (yellow curve).

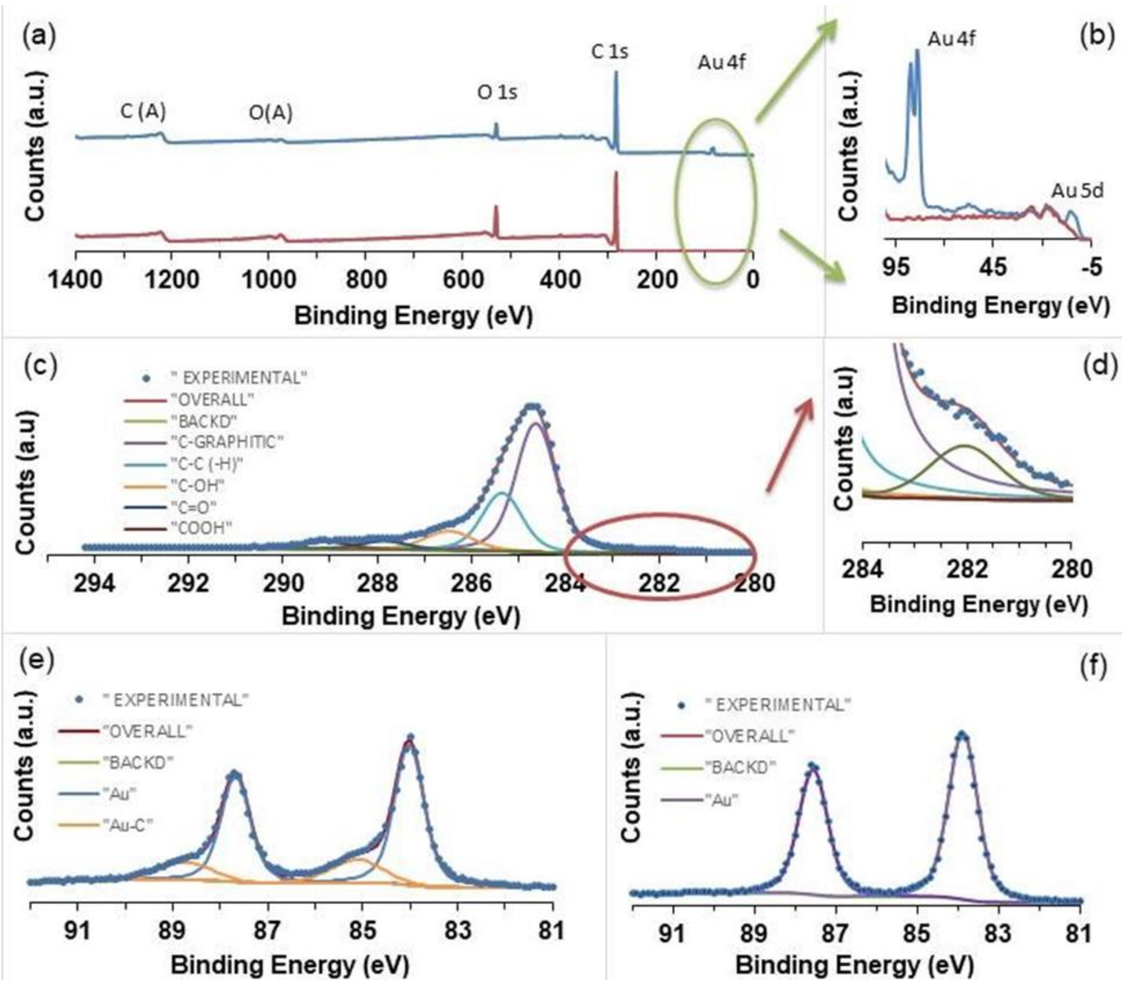
SEM-EDS analysis

The morphology of the chemically modified GC/AuNPs electrodes was examined by SEM microscopy.



The SEM image of the bare GC substrate is shown (Fig.5a). The images related to the modified surfaces of GC/Au deposition are observable (Fig.5 b,c,d). High-resolution SEM observations clearly showed the presence of **randomly distributed nanoparticles** (NPs) on the GC surface with a typically **spherical shape** and uniform diameter distribution (average 250 nm).

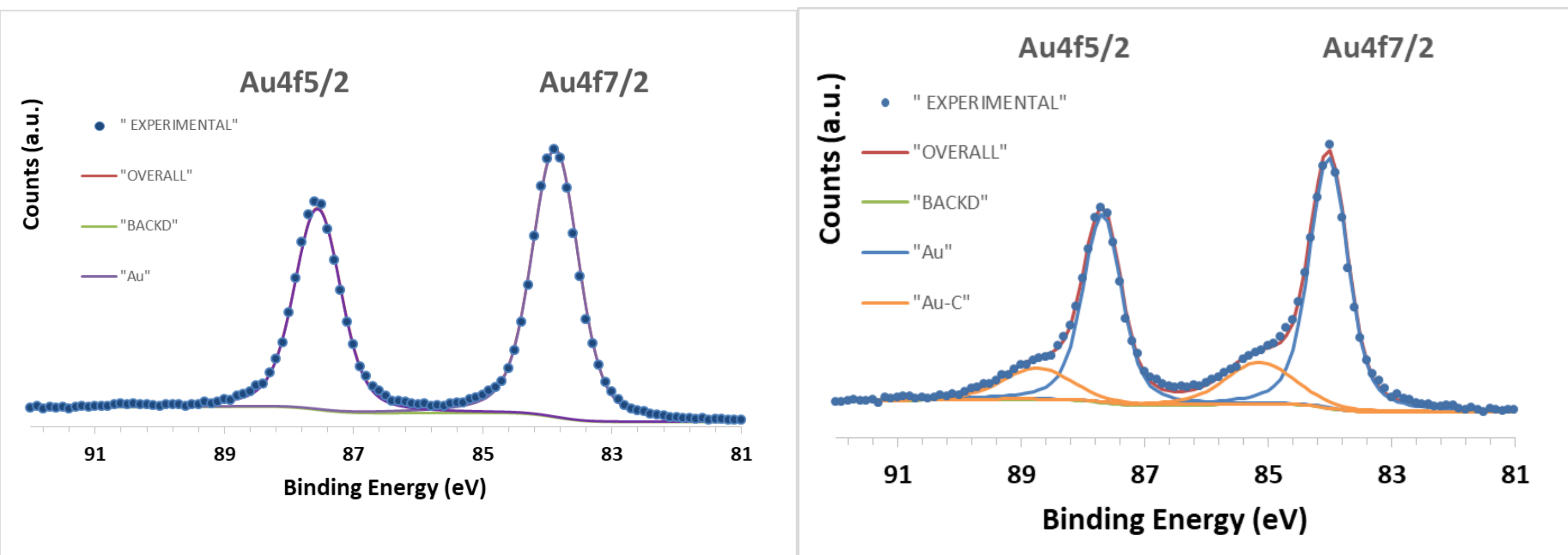
XPS analysis



The XPS (KRATOS Instrument) spectra have been useful for the chemical characterization of GC/AuNPs modified electrodes' surface. For comparison, results relevant to bare GC have been also acquired. As expected the C1s and O1s peak signals are the most intense on both sample typologies, moreover the Au 4d, Au 4f and Au 5d peak signals related to gold nanoparticles are present only on GC AuNPs modified. The atomic ratio between Au and C in average is almost 0.8 %, as obtained on **10 different area spots**.

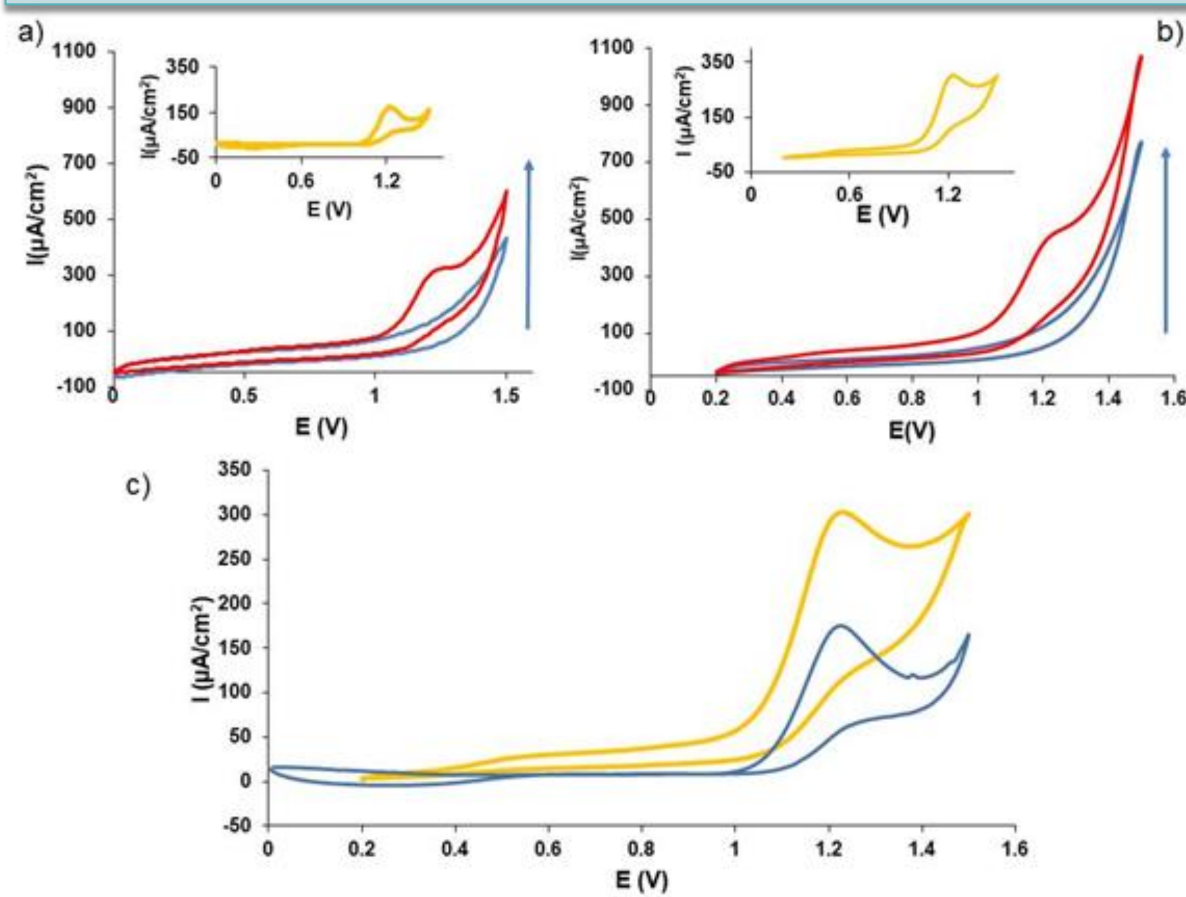
XPS analysis

Au4f peak present on GCAuNPs has been fitted by using two spin–orbit split doublets. The first doublet, related to the most intense component, shows well separated spin-orbit splitting peaks, Au4f7/2 and Au4f5/2, at 83.9 ±0.1s eV BE and 87.6 ±0.1 eV BE respectively, is assigned to **Au(0)**. Near the Au(0) components, **a different form of gold is also observed** (peak pair Au4f7/2 at 84.9 ± 0.1 eV and Au4f5/2 at 88.4 ± 0.1 eV), always correlated to the presence of carbide C1s peak signal at 283.1 ±0.1 eV. This Au peak component is **attributed to Au(I), likely originated from a surface gold carbide species.**



Electrochemical *characterization*

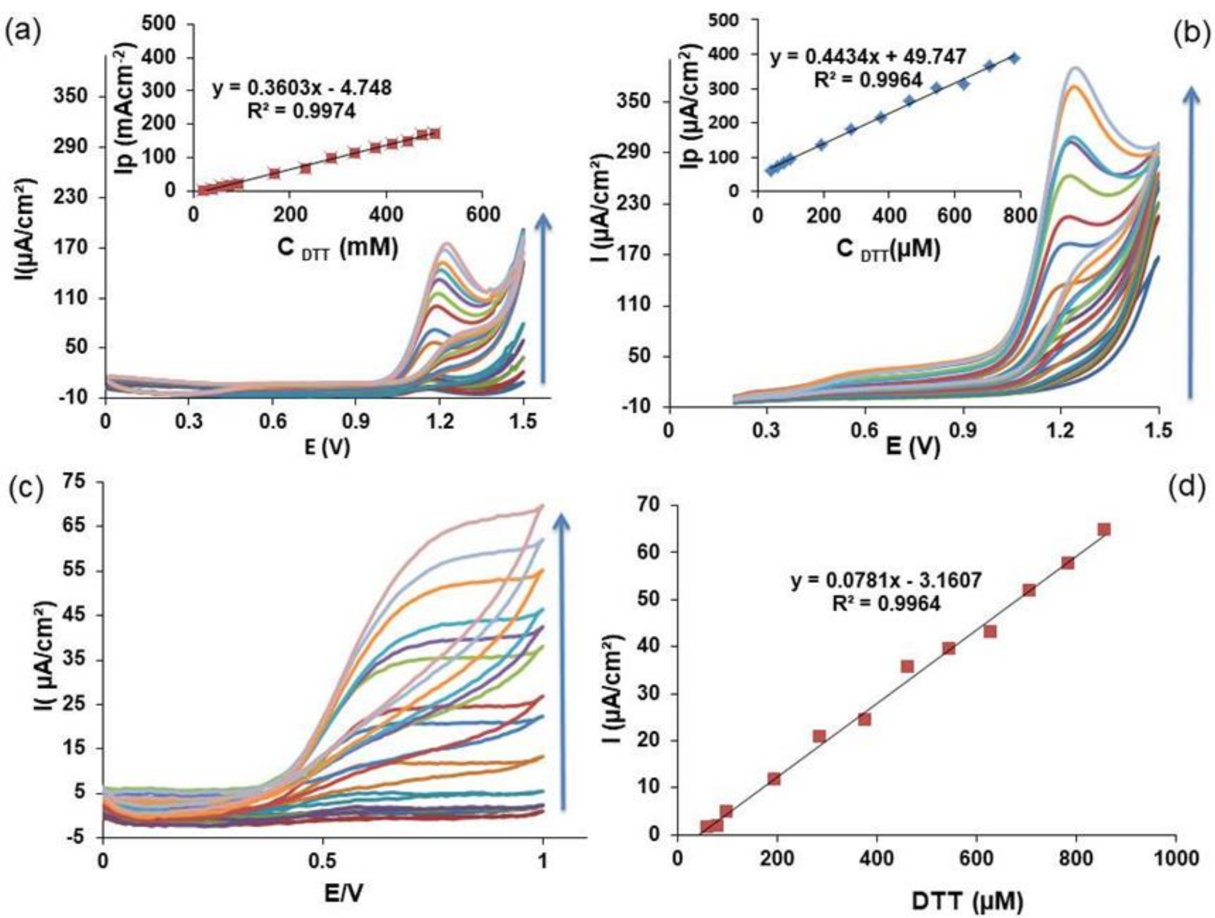
The GC/AuNPs reactivity towards the DTT electro-oxidation in phosphate buffer has been studied in order to demonstrate that the electrochemical properties of the deposited gold nanoparticles can improve the well known GC electroactive properties.



(a)CVs recorded on GC electrode bare in absence (blue trace) and presence of DTT 500 μM (red trace). Inset: curve difference between CVs recorded in presence of DTT 500 μM and related base line. (b) CVs recorded on GC/AuNPs electrode in absence (blue trace) and presence of DTT 500 μM (red trace). Inset: curve difference between CV recorded in presence of DTT 500 μM and related base line. (c) Direct comparison between curves differences (GC, blue trace; GC/AuNPs, yellow trace). Scan rate 50 mVs-1.

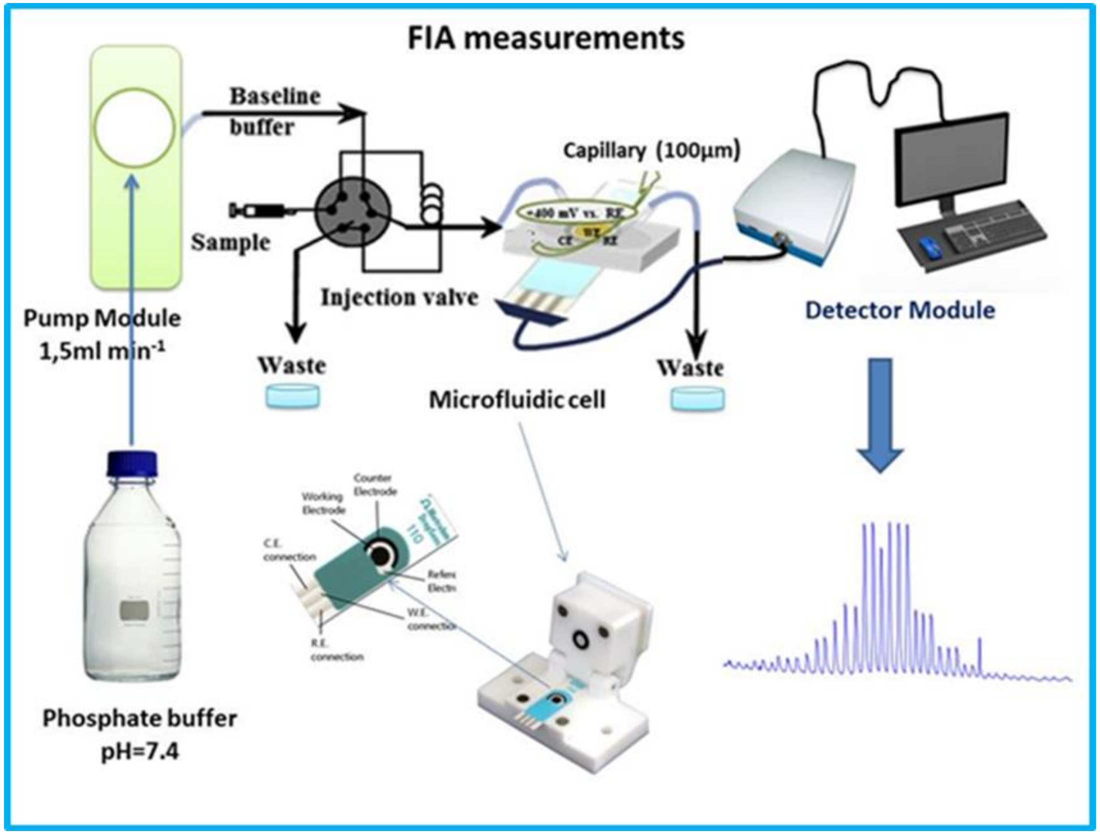
Electrochemical *characterization*

The progressive addition of DTT in CV experiments, from the initial concentration of 19.6 μM until the final ones of 500.0 μM and 783.0 μM, has been performed on the bare GC and on the GC/AuNPs electrodes.



(a) CVs recorded on bare GC electrode; Inset: plot of the anodic peak current Ip_{aGC} in function of DTT concentrations; (b) CVs recorded on GC/AuNPs; Inset: plot of the anodic peak current $Ip_{aGC/AuNPs}$ in function of DTT concentrations. (c) CVs recorded on GC/AuNPs between 19.6 μM and 880.0 μM, potential range 0.000 mV and 1000 mV. (d) Plot of anodic current $I_{LGC/AuNPs}$ in function of DTT concentrations, measured at +800 mV. Scan rate 50 mVs^{-1}.

FIA: apparatus and methods

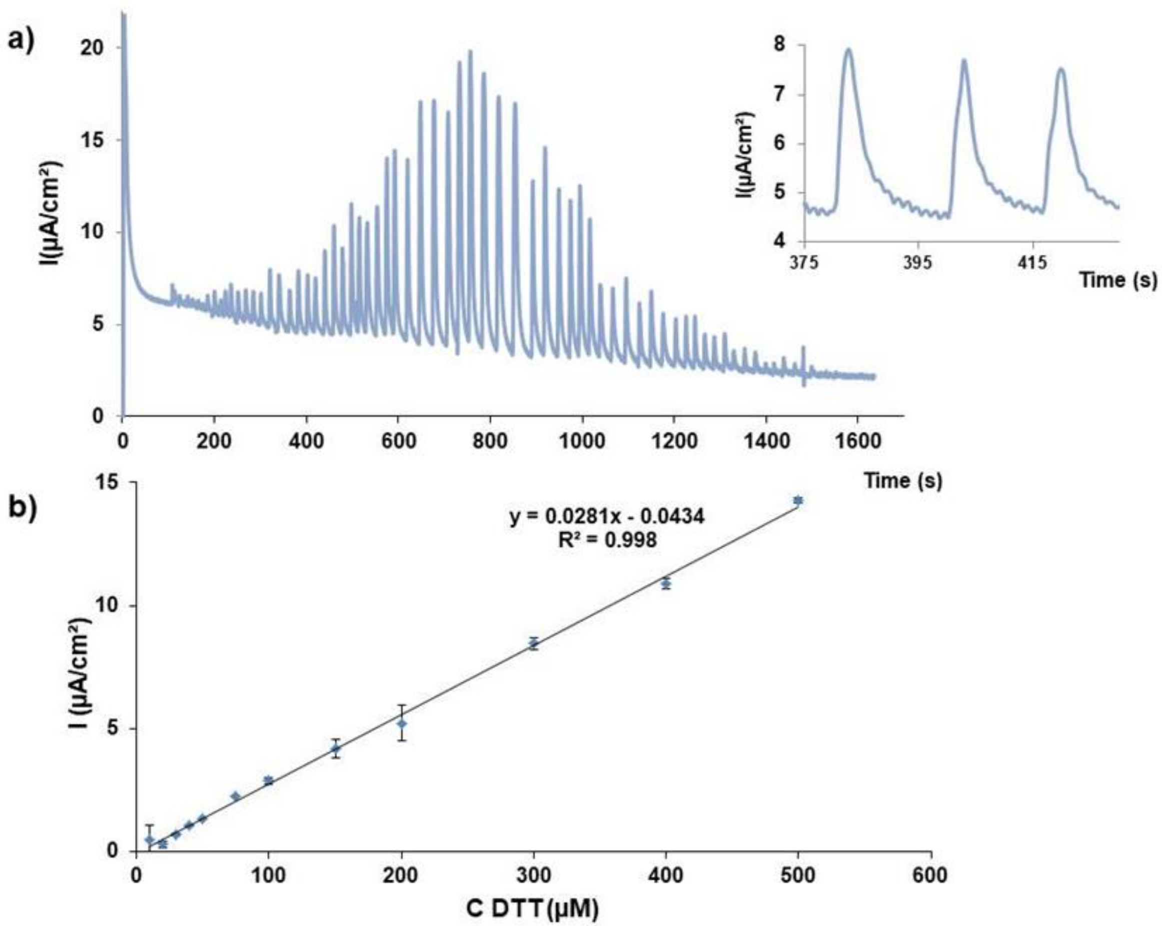


The flow rate of 1,5mL/min had been considered the best compromise to obtain more reproducible signals in a short time. After many tests, 600mV has been selected as the potential value to be applied for PAD. (E1 0,8V for t1 0,01s; E2 -1V for t2 0,02s; Ei 0,6V for t 0,5s).

- **PAD (pulsed amperometric detection)** had been very useful in order to reduce fouling of the electrode surface, just to **avoid a loss of electroactivity** over time and also to enhance the performance of electrodes in the AD (a pulsed potential is often applied during amperometric measurements).

FIA: the results

Typical FIA peaks for triplicate injections of different DTT concentration, between 10 and 500 μM, and relevant calibration curve.

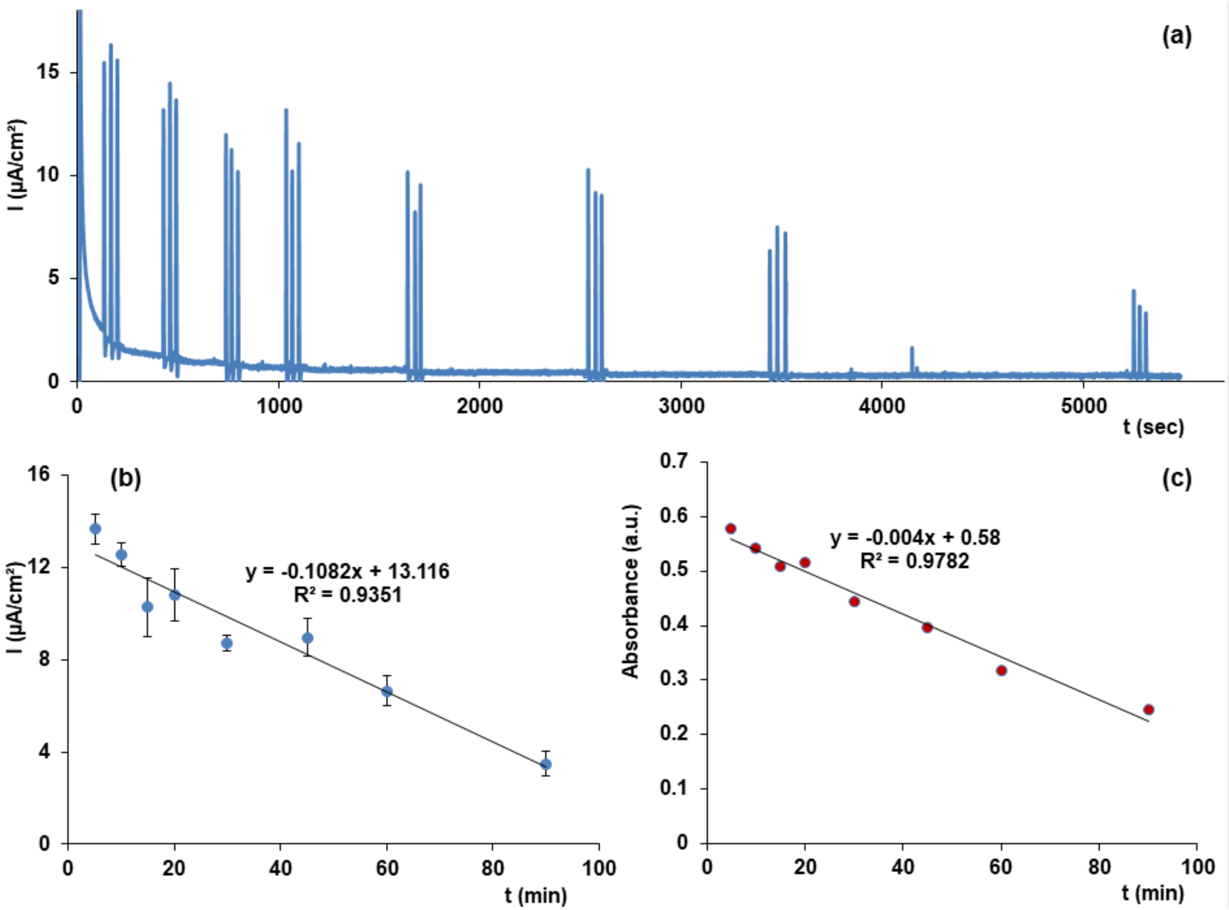


PAD experiments in FIA analysis.

(a) Peaks recorded for six injections of DTT and inset with peaks for injections of DTT 100μM

(b) relevant calibration curve. Carrier: phosphate buffer solution (pH = 7.4, I=0.2); flow rate: 1.5mL min^{-1}.

The proof of concept

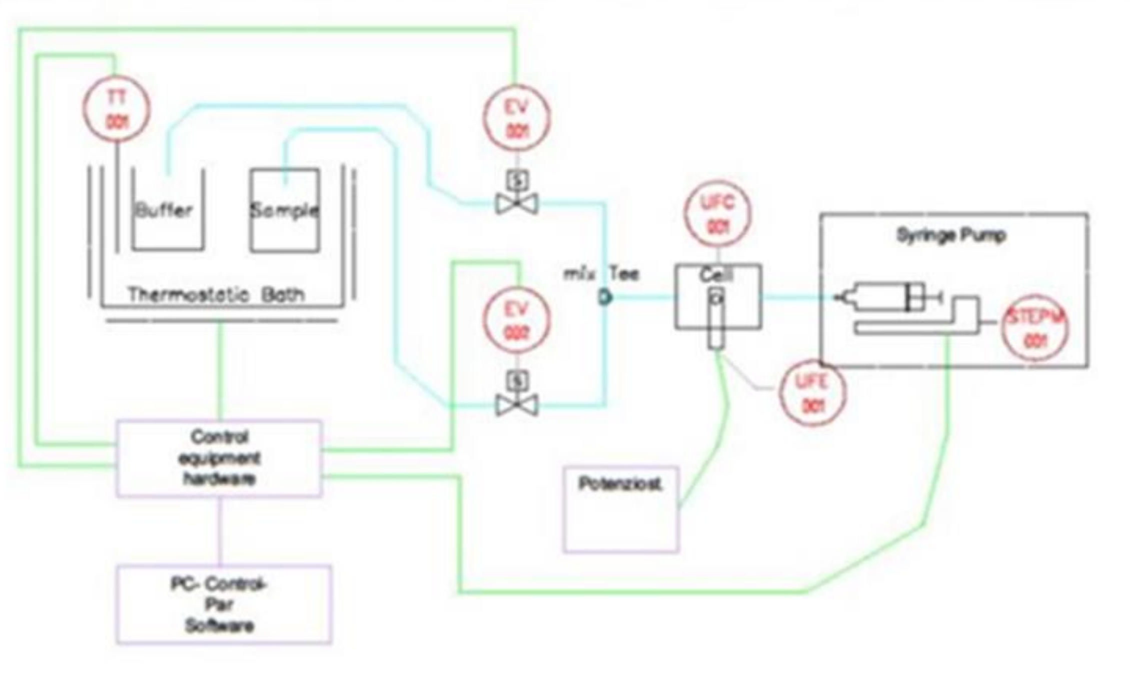


The suitability of the electrochemical DTT assay for measuring the OP in **real samples:** FIA experiments addressed to measure the **DTT (100 µM) rate consumption,** $\delta_{DTT\text{-}Electrochemical}$ activity, in PM_{10} aqueous extracts . To validate the method, the obtained results were compared with the data obtained on the same PM samples using the traditional spectrophotometric method based on the Ellman's reactive use (DTT assay).

(a) Peak currents related to DTT (100 µM) electrochemical detection in FIA analysis at reaction times of 5, 10, 15, 20, 30, 45, 60 and 90 min, in in PM aqueous extract (Filter 9). (b) The related linear regression (c) DTT absorbance decrement versus time at reaction times of 5, 10, 15, 2, 30, 45, 60 and 90 min obtained in parallel on the same extract used in electrochemical detection.

The industrial development with LPT Measure

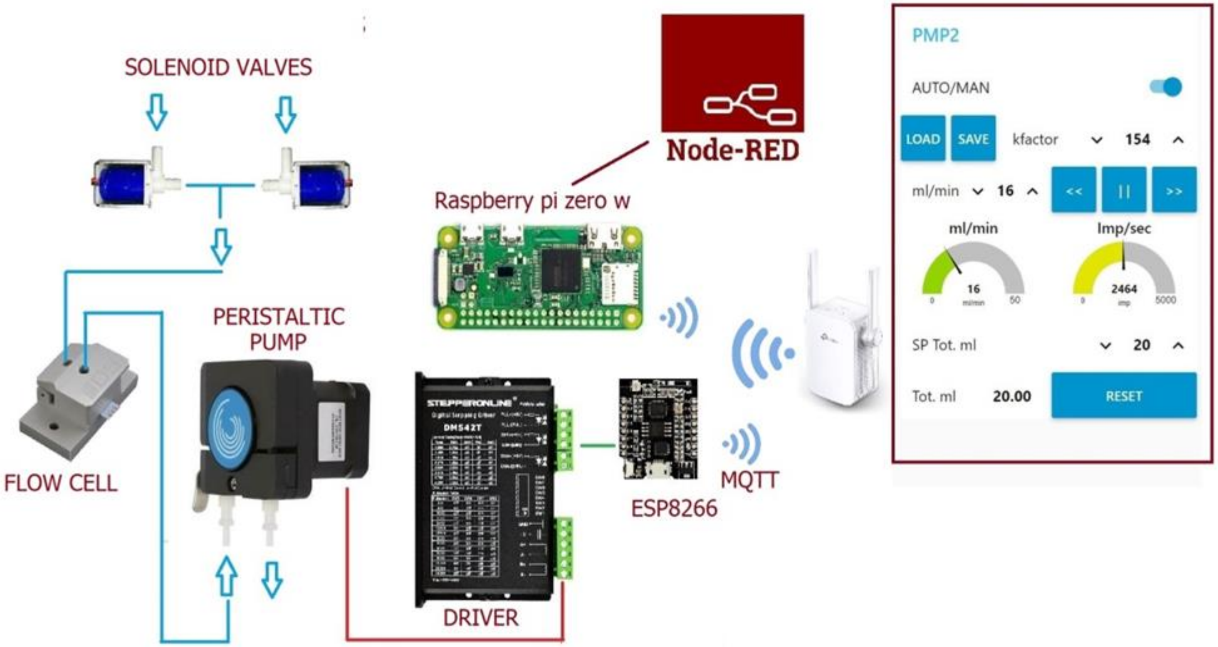
LPT Measure s.r.l., based in Martina Franca (TA), develops the process control systems and integrates both hardware and software technologies, the smart sensors capable of detecting process parameters and of making them flow into logistics and traceability management systems. **LPT Measure s.r.l.** develops also some "ad hoc" interfaces, that are specific to the application process.



Starting from the flow system, a technological solution was studied for the automation and the interfacing of the electrochemical sensor with an external read-out system.

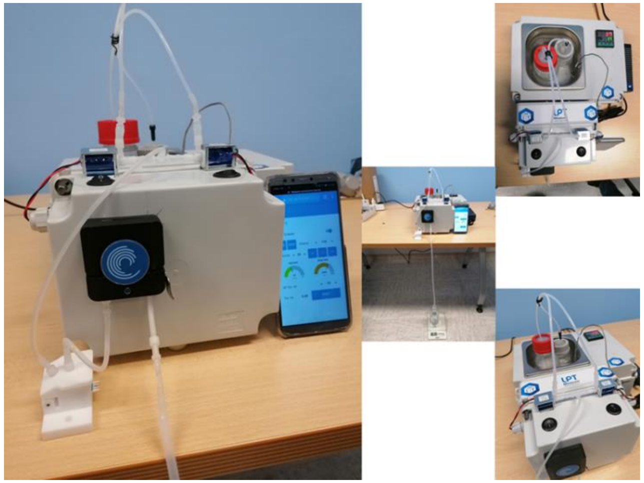
The technological integration for the prototype

A special program has been created for the ESP8266 microcontroller that allows it to connect to the Wi-Fi network using a password and to the MQTT broker, to receive data from the supervision system using the MQTT protocol, and to generate the appropriate control signals for the motor driver. step-by-step and in turn send the operating data to the supervisory.



The automation and interfacing of the electrochemical sensors with an external read-out system.

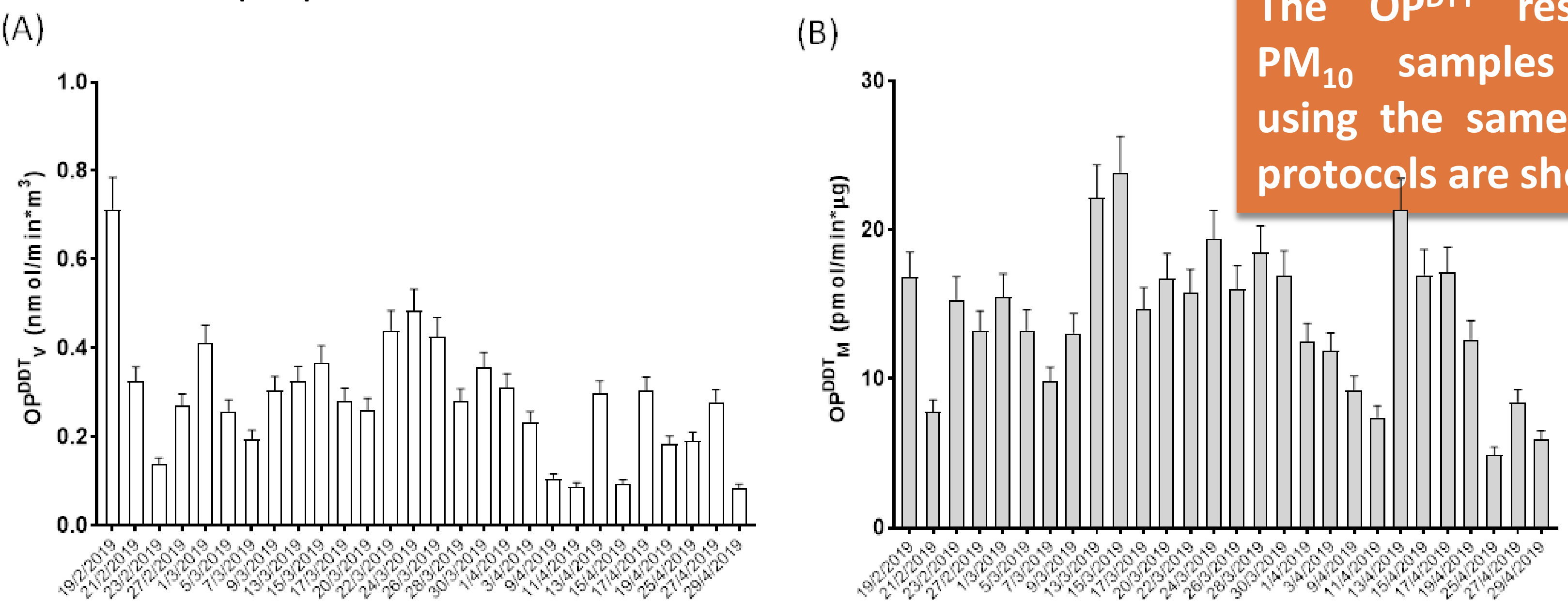
The solution studied for the automation: a prototype of a measurement system



PM Oxidative Potential

The analysis of **OP** of some water-soluble fraction of PM_{10} was performed using the **DTT assay**. The OP values are expressed as either $\mathbf{OP^{DTT}_M}$ (the OP value normalized by the sampled PM_{10} mass) or $\mathbf{OP^{DTT}_V}$ (the OP value normalized by the volume of the sampled air). OP^{DTT}_V rapresents an exposure metric referring to the inhaled air, important ho human exposure, while OP^{DTT}_M is an intrinsic propertied of PM and it is related to the sources.

The OP^{DTT} responses of PM_{10} samples measured using the same DTT assay protocols are shown.



The study was carried out on the water-soluble fraction of 28 PM samples collected on **teflon filters**, as part of a greater sampling campaign performed by the **Daniele Contini**'s Team, **ISAC-CNR** Lecce, in 2019, from February to April at an urban background site (Environmental-Climate Observatory of ISAC-CNR in Lecce)

The MTT assay (cell vitality test)

In parallel to the OP measurement, the cytotoxicity of PM_{10} was measured by the exposure (24h) of A549 cells (representative of the alveolar II type human pneumocytes) to the water-soluble fraction of the same PM_{10} samples and assessment of cell vitality reduction by MTT test, which measure the cell metabolic activity.

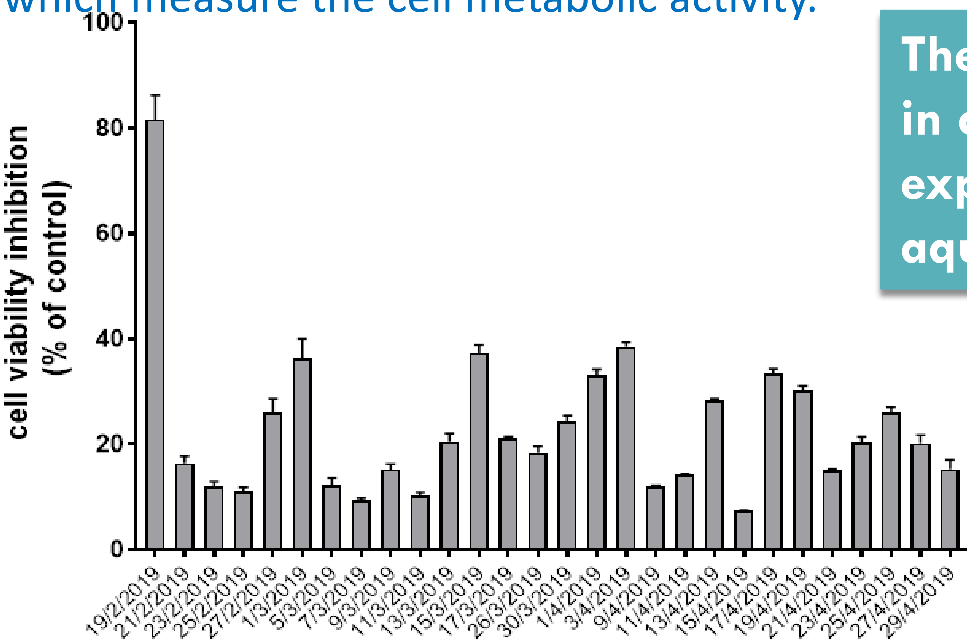


Fig.Cytoxoxicity of PM10, expressed as % cell mortality and assessed by the MTT test on A549 cells exposed for 24h to PM10 aqueous extracts.

The MTT shows an increase in cell mortality after 24h of exposure to the PM10 aqueous extracts.

It measures the activity of the enzymes that catalyze the reduction of MTT bromide of 3- (MT-4,5-dimethylthiazol-2-yl)- diphenyltetrazolium, usually a yellowish salt, in **formazan**, which has a purple colour.

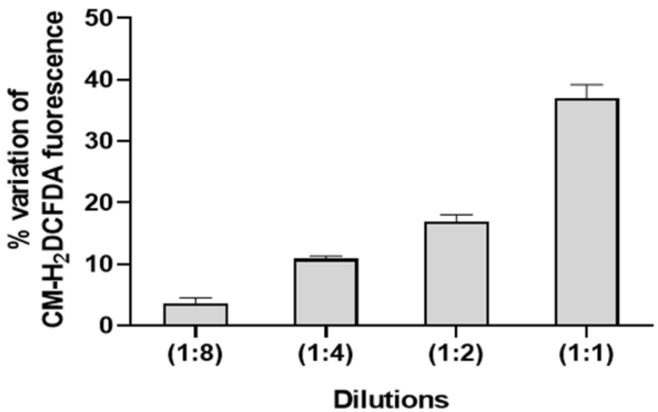
A statistically significant inhibition of the NAD(P)H-dependent cellular oxidoreductase enzymes was observed following the exposure of the cells for 24h to the aqueous extracts of PM_{10} compared to control cells.

The CM-H2DCFDA probe

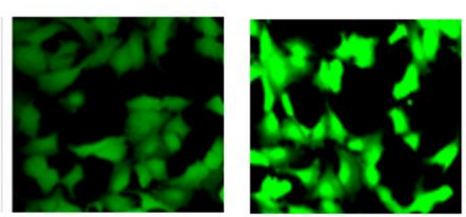
The cell-mediated oxidant generating capacity of PM_{10} was assessed by the measurement of the intracellular oxidative stress using the intracellular ROS-sensitive fluorescent probe CM-H2DCFDA on A549 cells exposed for 24h to the same aqueous PM_{10} extracts.

This result demonstrates that PM_{10} mediates ROS production in the cells in a dose-dependent manner.

(A)



(B)



Control cells

PM_{10} exposed cells

(C)

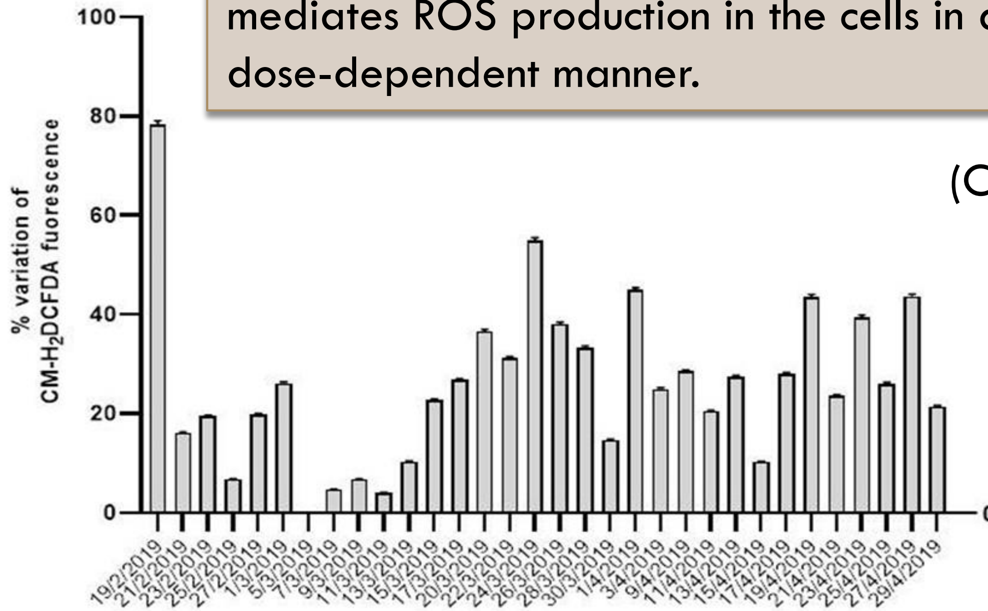
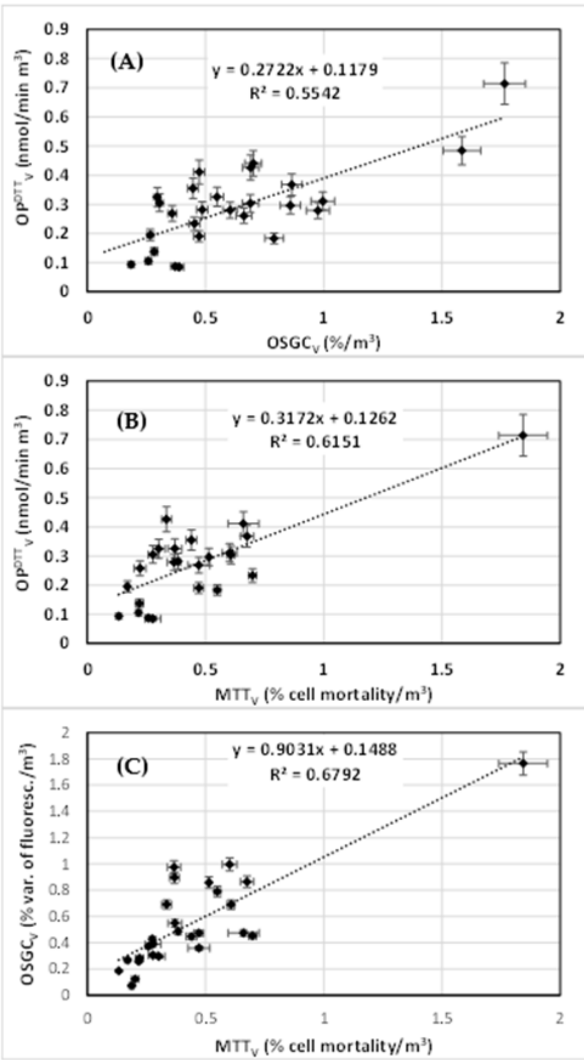


FIG.(A) Percentage variation of fluorescence intensity in **A549** cells exposed for 24h to increasing dilutions of the aqueous extract obtained from PM_{10}. (B) Percentage variation of fluorescence intensity in A549 cells exposed for 24h to aqueous extracts. (C) Representative confocal images of control and PM exposed A549 cells charged with the probe CM-H_2DCFDA.

The *correlation* analysis



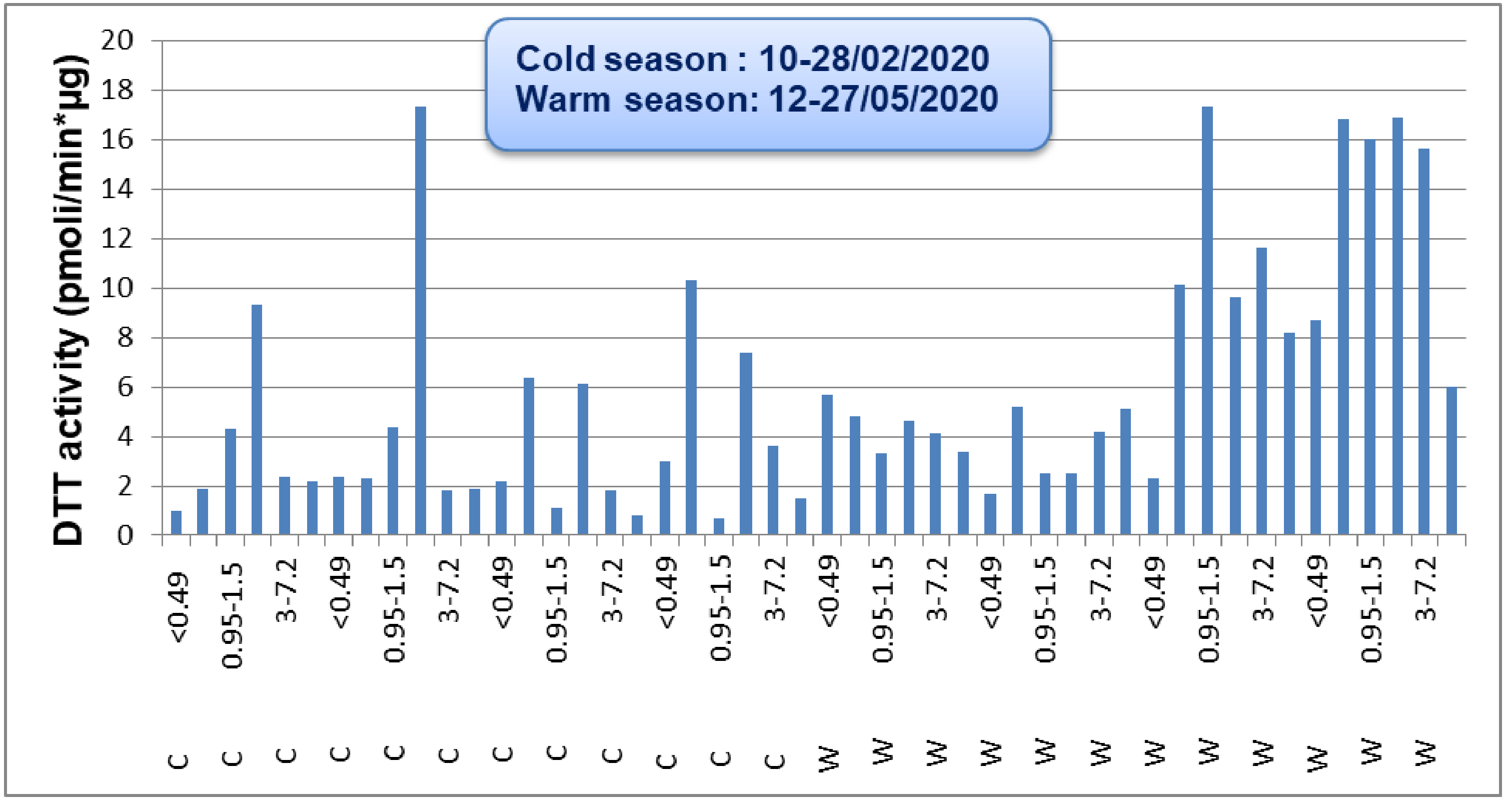
To investigate the **relationship between the outcomes of the cellular and acellular assays** on the PM_{10} samples, a comparison of the results obtained with the different tests was performed.

- ✓ The intracellular oxidative stress induced by PM is statistically correlated (A) to the acellular oxidative potential: **the ability of PM_{10} to generate intracellularly reactive species is related to its oxidative potential.**

- ✓ The **oxidative potential is related to cytotoxicity** (B), which is a **cellular endpoint** with integrates all the potentially toxic effects that PM_{10} can exert in the cell.

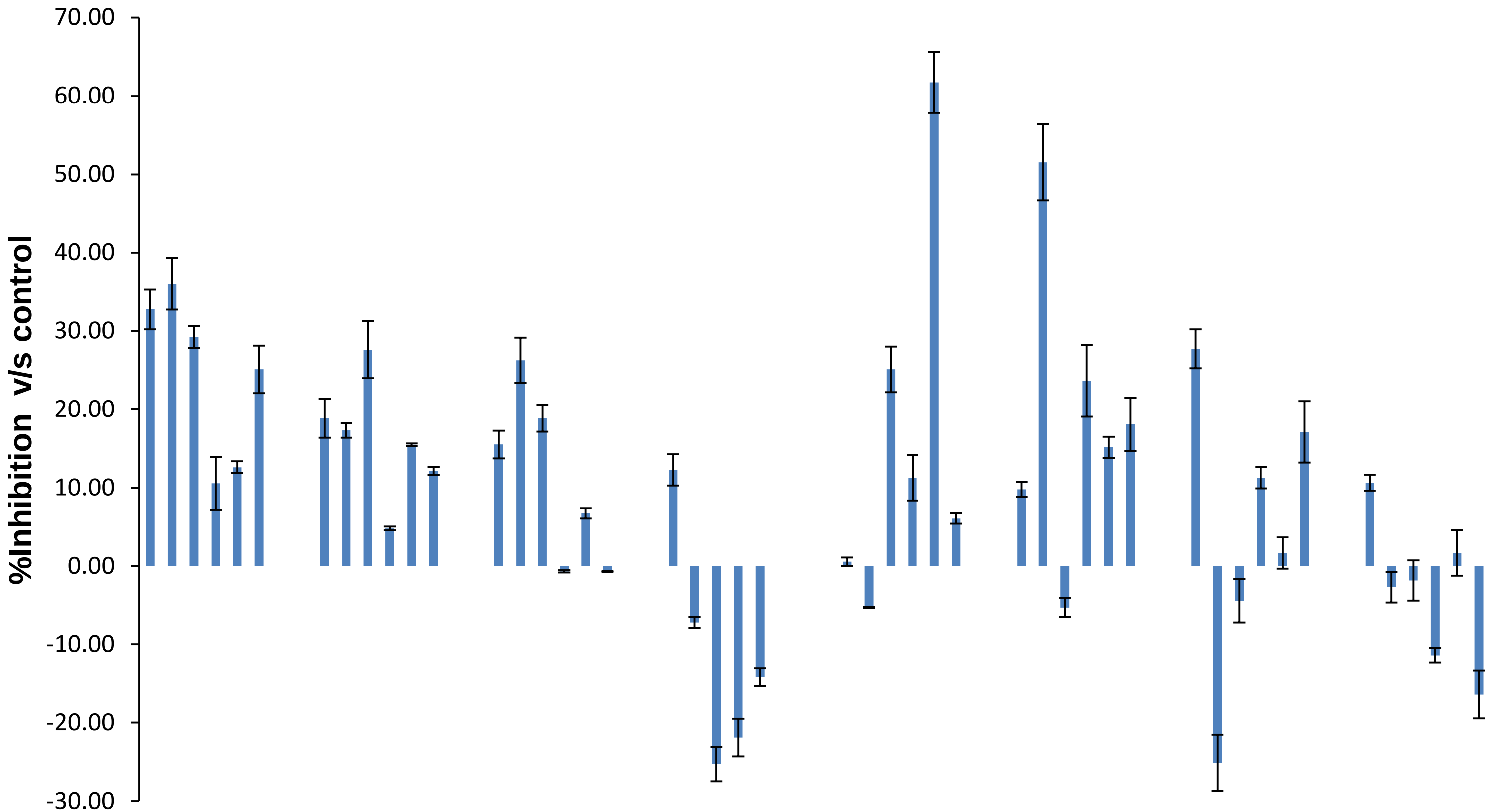
- ✓ **Cytotoxicity is related to the PM oxidative stress-inducing capacity** (C), suggesting that intracellular oxidative stress plays a key role in the multiple mechanisms underlying PM_{10} cytotoxicity.

The analysis on Greek samples



To improve the knowledge of the relationship between the OP assessed by the use of the DDT oxidation principle and the cellular effects of PM_{10}, the study was deepened through the study of the water-soluble fraction of size-segregated urban particulate matter collected in the urban agglomeration of **Thessaloniki** (Greek) (in collaboration with prof. Samara Costantini, University of Aristotele, Thessaloniki).

The analysis on Greek samples



Some samples, in particular during the summer season, showed **hormesis,** represented by negative value of the % inhibition. Hormesis consists in a biphasic dose-response to an environmental agent with a low dose stimulation and a high dose inhibition of a biological function.

Conclusions

- The sensor operates well, both in **BIA** that in **FIA**: it is reactive towards the DTT and shows low LOD, an high sensitivity and selectivity (absence of interference of real PM aqueous extract in the electrochemical response to DTT).
- **SEM-EDS** and **XPS** have been useful for the chemical and morphological characterization. The properties of the sensor are considerably influenced by the Gold Nanoparticles.
- The tests on **real samples** were a proof of concept for the electrochemical sensor: the goal was to establish the suitability of the sensor to measure the OP of PM and it seems successfully reached.
- The **MTT assay** shows an increase in cell mortality after 24h of exposure to the PM aqueous extracts. The **CM-CM-H2DCFDA** shows the intracellular oxidative stress induction by PM which, in turn, results in an increased citotoxicity.
- **Significant correlation between** MTT^V and $OPDDT^V$. It was found a statistically significant correlation between acellular $OPDTT^V$ assay and intracellular oxidative stress $OSGC^V$ results. These results support the idea that **the oxidative potential of PM could be an indicator of cytotoxicity of PM.**

Articles published on peer-reviewed scientific journals

- Lionetto M.G., Guascito M.R., Caricato R., Giordano M.E., De Bartolomeo A.R., Romano M.P., Conte M., Contini D. "Correlation of PM10 oxidative potential with ecotoxicological and cytotoxicological potential measured at an urban background site in Italy", MDPI Atmosphere, 2019, 10, 733; doi:10.3390/atmos10120733

- Lionetto, M.G.; Guascito, M.R.; Giordano, M.E.; Caricato, R.; De Bartolomeo, A.R.; Romano, M.P.; Conte, M.; Dinoi, A.; Contini, D., Oxidative Potential, Cytotoxicity, and Intracellular Oxidative Stress Generating Capacity of PM10: A Case Study in South of Italy, MDPI Atmosphere 2021, 12, 464. https://doi.org/10.3390/atmos12040464

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Development and characterization of a gold nanoparticles glassy carbon modified electrode for dithiotreitol (DTT) detection suitable to be applied for determination of atmospheric particulate oxidative potential

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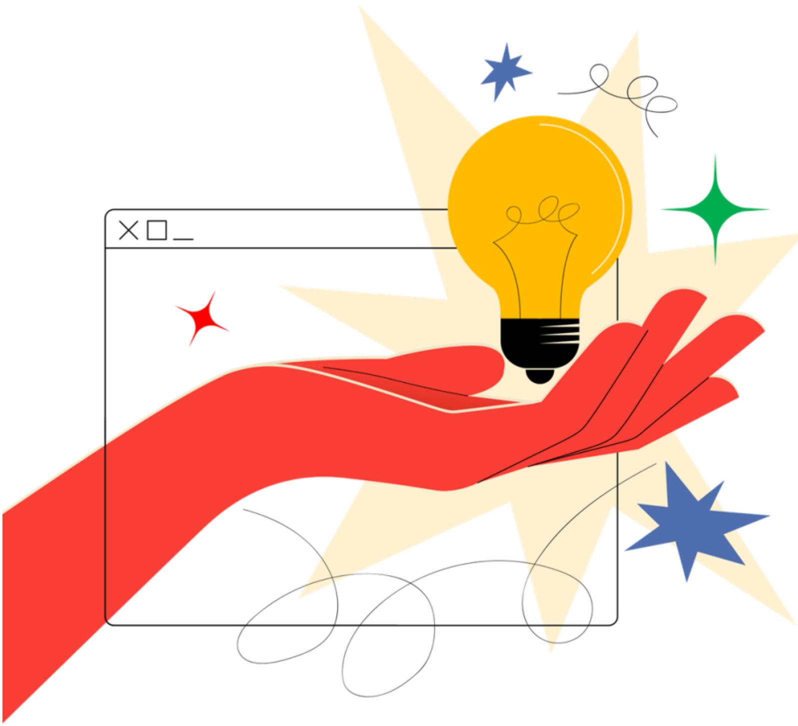

Thanks for the attention!

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