IL NUOVO CIMENTO **46 C** (2023) 132
DOI 10.1393/ncc/i2023-23132-8

COMMUNICATIONS: SIF Congress 2022

Diatom-based SERS substrates for applications in biomedicine

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received 2 March 2023

Summary. — Surface-enhanced Raman scattering (SERS) is a powerful spectroscopic analytical tool for diagnostic, pharmaceutical and biochemical studies. Demand for improved sensitivity, specificity, and cost efficiency constantly drives advances in SERS substrates development. Herein, the recent results in SERS substrate production based on naturally occurring diatom shells and their applications in biomedicine are presented.

1. – Introduction

Surface-enhanced Raman scattering (SERS) is a spectroscopic tool that involves the enhancement of the Raman signal of adsorbed analyte molecules on plasmonic nanostructures. Therefore, it combines the intrinsic advantages of Raman spectroscopy with high sensitivity, facilitating the trace detection of analytes [1]. A SERS platform can be fabricated using several bottom-up chemical synthesis procedures or advanced top-down nanofabrication technologies [2-4]. These techniques allow controlling the surface features or spacing, sizes, shapes and aggregations of the metallic nanostructures, providing reproducible SERS enhancement factors, EF, (up to 5-7 orders of magnitude). Unfortunately, these production methods are generally prohibitively expensive, complex, and ultimately unsuitable to mass production. In search of less expensive methods for SERS substrate production, in recent years scientists have taken inspiration from naturally occurring nanostructures, as for instance diatom microalgae. Diatoms are characterized by hydrogenated and nanostructured porous silica shells, the frustules, whose optical properties can be exploited in biophotonics and biomedical applications [5]. Metallization of

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diatom biosilica frustules can trigger plasmonic effects that in turn can find application in highly sensitive and low cost SERS detection platforms. SERS substrates were obtained after uniform thermal deposition of Au (20–50 nm layer) onto *Pseudo-nitzchia multistriata* frustule valves [6]. The 3D hierarchical morphology of the frustule, comprising several nanopores and interrelated periodicities, hardly reproducible by lithographic approaches, allows for efficient coupling with external optical radiation, good enhancement of the Raman signal (EF $= 5 \times 10^6$) and good reproducibility. The 3D vertically extruded side edges of the valve favor a high near-field localization, providing strong SERS signals and optimal interaction with cells. The optical and chemical properties of such natural nanostructured materials can be applied to the development of plasmonic-assisted diatomite nanoparticles for local SERS sensing. Diatomite nanoparticles (DNPs) are derived from fossilized diatom frustules and show high surface-to-volume ratio and high chemical stability. They can be easily prepared in the size range of 100–400 nm and can be functionalized. DNPs possess nanopores useful for loading and release of small molecules and, most importantly, they are not toxic. Indeed, metallic nanoparticles located near or inside the periodic nanopores of diatomite can form hybrid photonic-plasmonic modes producing SERS enhancement.

In this contribution, a hybrid nanocomplex constituted of a DNP decorated by gold nanospheres (AuNPs) and capped by a layer of gelatin was developed for monitoring the drug release in living colorectal cancer cells (CRCs) at a femtogram scale by SERS. The combination of the drug-loading capacity of DNPs with the strong Raman enhancement enabled combining therapeutic purposes with label-free intracellular drug monitoring.

2. – Materials and methods

The hybrid nanoplatform was obtained as previously described in [7]. DNPs were amino-modified using APTES and then decorated by in situ synthetized AuNPs. The DNPs-AuNPs were then loaded with the drug Galunisertib (LY) and finally capped with a gelatin layer. SERS microscopy was performedby using an inverted confocal Raman microscope (Xplora Inv, Horiba – Jobin Yvon) using the laser line at 638 nm (50 mW, He-Ne) and a 60× water immersion objective lens [7]. CRCs were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. For Raman imaging, cells were plated on poly-L-lysine-CaF_2-coated coverslips. Raman images were acquired by raster scanning the cells through laser focus (638 nm, laser power 20 mW), with a step size of 0.5 μm, and acquiring a 2D array of Raman spectra on a chosen region. The chemical false color Raman maps were generated through the multivariate curve resolution-alternative least square (MCR-ALS) method [7].

3. – Results and discussion

Diatomite nanoparticles are 3D porous silica nanostructures that have been obtained by cost-effective steps, including crushing, purification, and size-based separation from diatomite natural deposits. Figure 1(a) shows the TEM images of the hybrid diatomite nanocomplex. The DNPs showed a typical size of 350–400 nm, an irregular shape with pores of 20–30 nm useful for loading and release of small drugs (yellow box). The DNPs were uniformly decorated with 10–20 nm AuNPs (red box) providing high performances in terms of Raman enhancement (EF up to 6 orders of magnitude) and reproducibility of the SERS signal (standard deviation within 15%). The nanosystem showed very

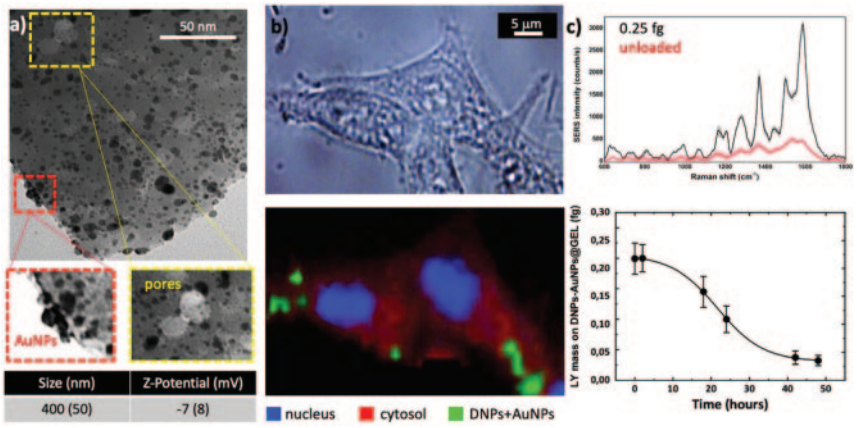

Fig. 1. – (a) TEM image of DNPs-AuNPs complex. The zooms highlight the presence of AuNPs aggregates (red box) and nanopores (yellow box). (b) Bright field and Raman microscopy images of colorectal cancer cells incubated with 50 μg/mL of DNPs-AuNPs for 18 h. (c) SERS signal and its standard deviation of 0.25 fg of LY loaded by the DNPs-AuNPs nanosystem. Time-dependent LY SERS signal from the nanocomplex in living cells.

low *in-vitro* cytotoxicity with concentrations of about 400 μg/mL up to 72 h. By nitrogen absorption/deposition isotherm analysis, it was demonstrated that the presence of AuNPs does not alter the loading capacity of the DNPs as well as pore availability [8]. The DNP uptake kinetics in cells has been preliminary evaluated by Raman imaging and confocal fluorescence microscopy [9]. The results demonstrated that after 6 hours of incubation, both internalized DNPs and DNPs clusters localized on the cell membrane were present. The number of cells showing the DNPs uptake remains almost constant after 18 hours, while their localization at the periphery of the nucleus increases. After 72 hours, the DNPs are still inside the cells in the perinuclear region [9]. The small molecule Galunisertib (LY), used for colorectal cancer treatment, was encapsulated in the hybrid nanoplatform and it was, finally, capped with a gelatin layer showing a pH-dependent degradation. The characterizations of DNPs revealed an increment of the mean size up to about 450 nm and an almost neutral surface ζ-potential (-7 ± 8 mV). The loading capacity of the nanocomplex can be easily tuned by varying the amount of gelatin in the external shell rather than the mass of DNPs. The hybrid nanosystem can integrate multiple functionalities allowing imaging and drug delivery goals simultaneously without using any fluorophore or external marker, avoiding fluorescence-quenching issues or surface alterations of the nanovector. The plasmonic assisted nature of the nanocomplex allowed its direct visualization in living cells by label-free Raman imaging, and was additionally used for studying the drug release in real-time [7]. Figure 1(b) shows the Raman map of the CRCs incubated with 50 μg/mL of DNPs-AuNPs for 18 h. The false colour Raman map assessed that the nanocomplex was internalized and localized in few clusters distributed through the cell cytoplasm. The strong Raman enhancement of LY molecules close to AuNPs allowed for their label-free intracellular monitoring. Figure 1(c) shows the SERS signal of the 0.25 fg of LY loaded by the DNP-AuNPs complex. The SERS spectrum of the complex DNP-AuNPs incubated with LY revealed the enhancement of the LY signal of about 6 orders of magnitude. The SERS spectra were well reproducible and the intensities on a selected nanocomplex and on different clusters could varied up to about 10% showing a really good inter-sample reproducibility.

The loading capacity of the nanocomplex, as well as the calibration of the SERS signal, was determined by analyzing the release solution by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) after 48 h, and it resulted to be $20 \pm 4\,\mu$g per mg of DNP-AuNPs complex [7]. The DNP-AuNPs nanosystem, thanks to the external gelatin layer, exhibited a *p*H-dependent release behavior with a LY release faster in acidic than physiological microenvironment. Indeed, at physiological *p*H (7.4) the gelatin layer was compact and the drug was retained in the system. In the presence of cancer cells, where high levels of lactate generate an acidic microenvironment, the gelatin shell is biodegraded and the LY release promoted. To calibrate the SERS signal of the LY loaded by the nanocomplex, we measured the LY SERS signal from the DNP-AuNPs platform in different *p*H environments (5.5 and 7.4) *in vitro* as a function of time. The results were compared with the HPLC investigations. The amount of LY released from the carrier was quantified via SERS according to the nanosystem loading capacity calculated by HPLC. The local real-time sensing of the LY release in living CRCs is provided by analysing the LY SERS signal as a function of the incubation time up to 48 h with an unprecedented resolution of 7.5×10^{-18} g, as reported in fig. 1(c). The DNP-AuNPs nanocomplex was incubated with living cells and the LY SERS signal was monitored in a cluster of cells at different times. The delivered LY in cells after 18 hours of incubation was about 30% of the total encapsulated drug. The remaining 70% of the drug was released within 48 hours. Crucially, the release of LY from the DNP-AuNPs complex in colorectal cancer cells inhibited their proliferation and induced the reversion to a normal phenotype with greater efficiency compared to the free drug, as demonstrated by analysing the expression level of specific genes (E- CADHERIN, SNAIL-1, and TWIST-1) [7]. The delivery of LY from the hybrid platform enhanced its ability to block the metastatic process by down regulating the genes inducing cancer metastasis and enhancing cell epithelialization, as confirmed by confocal microscopy analyses [7].

These studies showed that the hybrid SERS nanoplatform allowed tracing and quantifying the drug intracellular release with a femtogram resolution. The encapsulation of LY in the hybrid system improved its efficacy and therapeutic outcomes, lowering the amount of drug required to inhibit the metastatic process in cancer cells [7].

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The research was funded by the Italian Association for Cancer Research (AIRC IG Grant No. 21420), Campania Region POR-CIRO and Consiglio Nazionale delle Ricerche (SAC.AD002.173.025).

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