Intracellular SERS monitoring of drug release from plasmonic-assisted biosilica nanoparticles

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Abstract. Nanoscale delivery systems have been investigated for therapy due to their advantages, including the sustained delivery of drugs to cells and reduction of systemic toxicity compared to conventional treatments. However, their application is still hampered by experimental challenges, such as the investigation of the drug release in cells rather than *in vitro*. Here, we describe a hybrid nanoplatform for monitoring the drug release in living colorectal cancer (CRC) cells by Surface-Enhanced Raman Scattering (SERS). Specifically, the anticancer drug Galunisertib is encapsulated in diatomite nanoparticles (DNPs) decorated by gold nanoparticles (AuNPs) and capped by gelatin. The combination of DNP loading capacities with the Raman enhancement of Galunisertib provided by AuNPs enables bio-imaging and drug delivery without using fluorophores or markers, avoiding fluorescence-quenching issues. Thanks to the Raman enhancement of Galunisertib, the drug release profile is monitored and quantified in living cells by SERS with a femtogram scale resolution. When the gelatin shell is digested by proteases, Galunisertib is released and its SERS spectrum decreases, allowing real-time quantification in CRC cells. The therapeutic efficiency of the Galunisertib delivery platform offers an alternative route for lowering drug dose and toxicity.

1 Introduction

The research for nanoscale Drug Delivery Systems (DDS) is driven by their potentiality to reduce the systemic toxicity associated with small-molecule administration. Although many advantages have been reported in the medical application of nanoparticles (NPs), their use in cancer treatment is hampered by diverse experimental challenges, such as investigating the drug release behavior in cells [1]. The quantification of the intracellular released drug is crucial to understand the effective amount of the molecule that reaches the site of action for disease treatment. Sophisticated analytical tools (*e.g.*, High-Performance Liquid Chromatography HPLC, fluorescence or absorbance spectroscopy) allows studying the drug release profile of nanoscale DDS *in vitro*, but not in living cells, and the time required for sample preparation and running hinders tracing the intracellular release in real-time modalities. To avoid these limitations, it is necessary to develop sensitive platforms enabling the real-time intracellular monitoring of chemotherapeutics to improve the therapeutic efficacy of DDS and get a deeper understanding of how they react to the cell environment. Recently, Surface-Enhanced Raman Spectroscopy (SERS) has offered advancements in label-free biosensing thanks to the near-field optical amplification of molecules provided by metal nanostructures, such as metal NPs [2]. The plasmonic and electromagnetic properties of gold NPs (AuNPs) offer high-sensitivity and Raman enhancement factors up to 10^{12} for absorbed or attached molecules on metal surfaces or NPs. Therefore, the combination of the strong Raman enhancement of molecules provided by AuNPs with the loading capacity of porous NPs can be used to develop hybrid platforms capable of both delivering and real-time quantifying the released drug in cells [3]. In the last years, inorganic diatomite nanoparticles (DNPs) made of diatom biosilica have been explored as DDS thanks to their intriguing properties for biomedical applications, such as the porous structure, long half-life, chemical stability, and ease-to-tune surface properties [4,5]. The tunability of the DNP surface allows to modify NPs through several chemical

approaches and provides them with novel captivating properties. Among all, the decoration of the DNP surface with AuNPs confers to DNPs optical properties suitable for bioimaging applications via SERS analysis [6]. In this study, the small molecule Galunisertib (LY), currently used for colorectal cancer (CRC) treatment, is encapsulated in a hybrid nanoplatform made of DNPs decorated by AuNPs and capped by gelatin (DNP-AuNPs-LY@Gel). The monitoring of DNP internalization in cells and quantification of the LY release is made possible by the plasmonic resonance of AuNPs providing a highly sensitive SERS readout of LY. Therefore, the intracellular release of LY is measured in CRC cells by SERS and correlatively quantified to provide the LY sensogram evolution with a sub-femtogram scale resolution [7] (Fig.1).

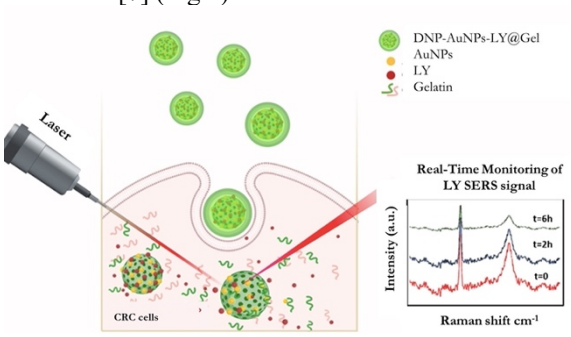

Fig.1. Scheme of the hybrid nanoplatform internalization and real-time monitoring of the released drug. The gelatin shell around the hybrid system acts as a pH-sensitive drug gatekeeper. It is disrupted in the acidic microenvironment of the CRC cells, enabling the controlled release of LY in malignant cells. The monitoring and quantification of the released drug are performed by SERS technique.

2 Results and Discussion

The hybrid nanoplatform was developed through an *in-situ* synthesis of pegylated AuNPs on the amino-modified surface of DNPs. Before this, the diatom powder, mainly constituted by diatom skeletons having different sizes and shapes, was ultrasonicated, purified, and filtered to obtain DNPs with a mean diameter of 400 (50) nm. The hydroxyl groups (-OH) exposed on the surface of the nanostructured silica were then activated by a Piranha treatment and an amino-silanization process was carried out according to methods described earlier [8]. The amino groups ($-NH_3^+$) promoted the nucleation of pegylated AuNPs on the surface of DNPs thanks to the electrostatic attraction to the negatively charged groups of the dicarboxylic polyethylene glycol (PEG) used as a stabilizing agent. The presence of AuNPs was confirmed by Transmission Electron Microscopy (TEM) investigation (Fig. 2A), showing that the surface of DNPs was covered by a homogeneous carpet of AuNPs after the synthesis process. According to the TEM investigation, the mean size of the spherical AuNPs was about 25 nm (calculated by Image J software).

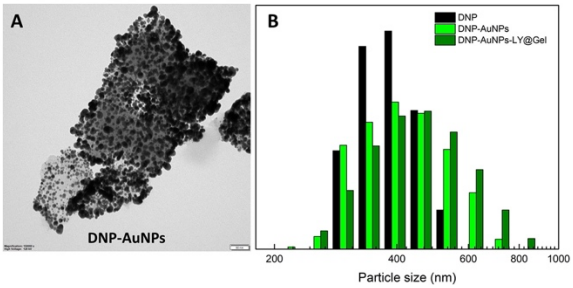

Fig.2. A**)** TEM image of a DNP decorated by 25 nm- in size spherical AuNPs. The scale bar is 50 nm. **B)** Size distribution (nm) of DNP before surface functionalization (black), after the decoration with AuNPs (light green), and gelatin capping (dark green).

Although DNPs show an irregular shape due to the process of ultrasonication performed to reduce the diatom powder at the nanoscale level, their biocompatibility and safety have been extensively demonstrated *in vitro* and animal models [9]. Moreover, the presence of metal NPs on the silica structure conferred to the DNPs a localized surface plasmon resonance (LSPR) at 576 nm wavelength, which enabled monitoring the functionalization *via* absorbance analysis (UV-Visible spectroscopy). The mean diameter of the hybrid system (DNP-AuNPs) calculated by Dynamic Light Scattering (DLS) was equal to the bare DNPs (400 ± 50 nm), revealing that the process did not influence the size and stability of the nanoplatform (Fig. 2B) Then, the hybrid system DNP-AuNPs was loaded with the anticancer small molecule Galunisertib (LY2157299), a Transforming Growth Factor β (TGF β) Receptor Inhibitor able to block the metastatic process in diverse types of cancer, including CRC. The loading of LY in the nanoplatform was performed by both physisorption (through the porous structure) and electrostatic attraction forces between the drug molecules and the surface of DNP-AuNPs. Finally, the loaded nanoplatform was covered by a shell of cross-linked gelatin to avoid burst release phenomena and promote a pH-controlled release of the drug *in vitro*. The encapsulation of LY in the nanocomplex did not cause changes in the LSPR of the hybrid suspension, due to the small size of LY. Rather, the LSPR peak of the gelatin-functionalized hybrid system (DNP-AuNPs-LY@Gel) red-shifted by 10 nm after the gelatin shell formation, due to a change of the local refractive index at AuNPs surroundings. The gelatin shell caused an overall size increment of 50 nm in the final nanoplatform that showed a mean diameter of 450 (50) nm. The generation of a gelatin shell surrounding the NPs was effectively confirmed by the analysis of the LY release from the nanoplatform, which followed a sigmoidal and a pH-responsive behavior (Fig. 3A). The loading capacity (LC) of the DNP-AuNPs-LY@Gel system was investigated by dispersing the hybrid nanoplatform in Phosphate Buffer Saline solution (PBS) for 60 hours and

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quantifying the amount of released drug by HPLC. The LC was estimated to be 20 (4) µg/mg of DNPs. Figure 3a shows the drug release profiles performed both in acidic and physiological conditions. The gelatin shell around the DNP-AuNPs-LY@Gel system was responsible for the controlled and pH-responsive LY release. The acidic microenvironment, as cancer cell microenvironment as well, promoted the relaxation of the gelatin chains and the release of the drug in the medium. The release profile reached a plateau corresponding to 100% of the total encapsulated drug after 60 hours, ensuring that no drug dispersion phenomena occurred in the system. In physiological conditions, instead, the gelatin shell retained the drug and hampered its release from the nanoplatform. Therefore, the pH-responsive profile exhibited by the developed DDS can address the release of LY in malignant cells, in which the acidic microenvironment is caused by high levels of lactate generated by cell activity [10].

The investigation of the drug release *in vitro* is generally considered mandatory to understand the nanoplatform behavior in different pH environments. The *in vitro* release tests offer many advantages in the comprehension of the DDS stability and performance, but they cannot replicate the cell environment at all, especially the malignant one. To overcome this limitation, we developed a SERS hybrid nanoplatform that allowed us to investigate, real-time monitor, and quantify the LY release in living CRC cells without the need of fluorescence payloads or markers, avoiding fluorescence-quenching issues or modification of the nanoprobe surface [11]. To this aim, we studied the Raman fingerprint of LY alone between 1300-1650 cm^{-1} and the SERS fingerprint of the LY from the DNP-AuNPs-LY@Gel nanoplatform dried on a coverslip. The enhancement factor (EF) analysis confirmed that drug molecules strongly interacted with the AuNPs decorating the nanoplatform, providing an EF of the LY SERS signal of 4.5 x 10^5. The most intense SERS vibration, ascribed to a combined action of ring C–N stretching and bending in the drug structure, was found at 1360 cm^{-1} and used for monitoring the LY intracellular release *in vitro* and cell. The release behavior of the DNP-AuNPs-LY@Gel platform was first investigated by SERS in PBS solutions 5.5 and 7.4 and then in the CRC cell. To this aim, 1.5 µg of the nanoplatform (in which 30 ng of LY was loaded according to the reported LC) was deposited on a CaF_2 slide. The release profile investigated by SERS measurements was compared with the HPLC investigations reported in Figure 3a, confirming that the acidic microenvironment triggered the LY release from the nanoplatform in a sigmoidal and pH-responsive manner. The nanoplatform released 50% of the total encapsulated drug after 24 hours in PBS solution 5.5, as already observed by HPLC and reported in Fig 3a. Furthermore, thanks to the strong SERS enhancement of the LY signal provided by the hybrid nanoplatform, we managed to quantify the amount of drug released from a single nanovector unit in the medium at each time interval. Each DNP unit contained 0.25 fg of LY and released 0.125 fg after 24 hours of immersion in the PBS solution pH 5.5, corresponding to 50% of the total loaded mass. Conversely, the gelatin shell was tightly folded in PBS solution 7.4 mimicking the physiological conditions and the LY release was prevented (data shown in [7]).

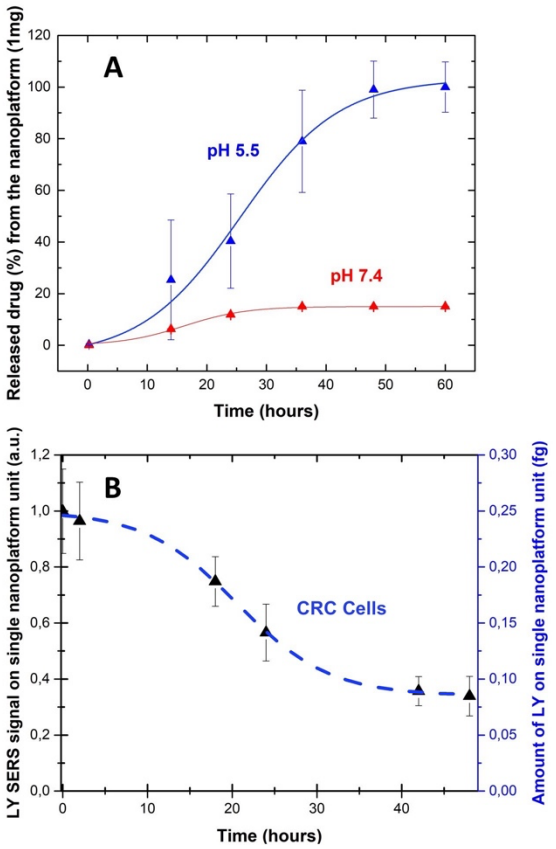

Fig.3. A) Cumulative release (%) of LY from DNP-AuNPs-LY@Gel (1mg) in PBS pH 5.5 (blue line) and 7.4 (red line). B**)** Sensogram of the LY SERS signal evolution after release in CRC cells. The SERS signals are correlated to the HPLC analysis to quantify the amount of drug-loaded in /released from a single nanovector unit. Left Y reports the normalized LY SERS intensity counts monitored on the nanoplatform. Right Y reports the amount of LY measured in a single nanovector unit.

The great advantage offered by this hybrid nanosensor was the possibility to real-time monitor the drug release in the LS-174T cell line and quantify the effective internalized dose. The LS-174T cell line was chosen due to its metastatic potential and mesenchymal phenotype related to an upregulation of the TGF βI receptor pathway [12]. The release of LY from the DNP-AuNPs-LY@Gel nanoplatform was expected to block the TGF β-induced metastatic process, promoting the transformation from epithelial to mesenchymal phenotype.

For the intracellular release tests, the DNP-AuNPs-LY@Gel was incubated with cells at a concentration of 50 µg/mL and the LY SERS signal was monitored in a cluster of cells at different times (Fig 3B). The intracellular release followed a sigmoidal trend

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similar to one investigated by HPLC in PBS 5.5 since the acidic tumor microenvironment favored the LY release from the gelatin shell. The delivered LY in cells was about 30% of the total drug after 18 hours. Considering that each DNP unit contained 0.25 fg of LY, the LY delivered in the cell by a single DNP corresponded to 0.075 fg after 18 hours. The released LY was 50% (0.125 fg for DNP unit) after 24 h and the remaining mass of drug (0.16 fg) was released after 48 h. So, the combination of metal NPs with porous DNPs enabled an efficient SERS intracellular drug tracing, sensing, and quantification in living CRC with a sensing resolution down to 7.5×10^{-18} g. The therapeutic outcomes of the LY DDS were finally tested in the LS-174T cell line to analyze whether the therapeutic efficacy of the drug could be enhanced by the nanoplatform or not. We assessed the biocompatibility of the nanoplatform in the CRC cell line and indicated as safe for the treatment a concentration of the DNP-AuNPs-LY@Gel suspension of 50 μg/mL (in which 2.5 μM of LY was loaded according to the reported LC). Since the chosen drug was capable of binding to the TGF β receptor and affecting the downstream genetic pathway, we evaluated the expression levels of E-Cadherin (involved in the epithelial process) and Snail-1 and Twist-1 (involved in the mesenchymal one) genes upon treatment with 50 μg/mL of the hybrid system. The encapsulation of LY in the nanoplatform enhanced its ability to block metastases by downregulating the pro-metastatic genes and enhancing cell epithelialization. As a consequence, the treatment with the hybrid nanoplatform induced a strong morphological cell transformation from elongated and spindle-shaped (aggressive and metastatic) to rounded (less aggressive and wild-type) cells within 48 hours.
Considering these results, it was clear that the encapsulation of LY in the hybrid nanoplatform made the LY therapeutic effect more than three times stronger than the conventional administration of the drug.

3 Conclusions

In this work, a hybrid nanoplatform is developed to trace and quantify the Galunisertib intracellular release with a sub-femtogram scale resolution. Galunisertib is loaded in the DNP with a loading capacity of 20 $\mu g/mg^{-1}$ of DNPs and its release is label-free monitored with extreme sensitivity in living cells thanks to the enhancement of the Galunisertib SERS signal provided by gold nanoparticles. The gelatin shell prevents burst release effects and addresses the drug release in malignant cells, where the acidic microenvironment promotes gelatin degradation. The modulation of the expression levels of E-Cadherin Snail-1, and Twist-1 genes is significantly higher upon treatment with the loaded nanoplatform than the free Galunisertib. The delivered drug promotes the transformation of the LS-174T cancer cells from mesenchymal to epithelial-like phenotype efficiently. Thereby, it is possible to lower the administered drug dose through the encapsulation of Galunisertib in the developed system to reduce the reported systemic effects.

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