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Controlled release of targeted anti-leukemia drugs azacitidine and decitabine monitored using surface-enhanced Raman scattering (SERS) spectroscopy

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Abstract: A new targeted drug delivery system with controlled release of anti-cancer drugs, azacitidine and decitabine, was investigated to enhance the efficacy of cancer treatment and reduce the effects of high drug toxicity to healthy tissues. The proposed drug nanocarriers are based on gold nanoparticles (AuNPs) modified with mercaptobenzoic acid (MBA) linker to enable the immobilization of azacitidine (AZA) and decitabine (DAC) on AuNPs in the form of AuNP@MBA/AZA,DAC entities. The cancer cell recognition was accomplished by covalently binding folic acid (FA) ligands to para-aminothiophenol (PATP) in the mixed SAM shell on gold nanoparticle nanocarriers, AuNP@MBA,PATP. The FA ligand was used due to the strong expression of folic acid receptors (FR) in the membrane of cancer cells. This enables the functionalized carriers to target only cancer cells owing to the efficient FA-FR binding property. The amide bonds between the linkers and azacitidine/decitabine are pH sensitive and undergo acid hydrolysis in a low pH environment of the cytosol in cancer cells. Using the solutions of different pH, the release of azacitidine/decitabine was monitored by surface-enhanced Raman scattering spectroscopy (SERS) measurements of the MBA Raman modes at 1586 cm^{-1} and 1074 cm^{-1}. At pH 7.4, the release of the drug was found to be negligible, while at pH 4.0 and 5.5 a continuous drug release was observed over 3 hours. The utilization of SERS monitoring for the drug release was based on the strong Raman signals which are generated by the MBA linker when it is bound to a plasmonic AuNP. During the immobilization of azacitidine/decitabine on AuNP carriers, the SERS signals are strongly reduced due to the shielding by drug molecules but they increase sharply upon the drug release confirming the amide bond breakage and successful drug delivery.

Keywords: targeted drug delivery; controlled drug release; anti-cancer drugs; Raman spectroscopy; SERS; gold nanoparticles.

Introduction

Targeted delivery of anti-cancer drugs offers considerable reduction in damage to healthy cells and other side effects while increasing the overall drug efficacy in cancer treatment [1-6]. Unlike the traditional intravenous drug infusion approach, the targeted drug delivery systems are based on carriers seeking out the cancer cells rather than equally affecting all cells, healthy and unhealthy. The drug release is then induced by the physiochemical conditions at the target. For instance, a high glutathione concentration [7-9] or lowered pH in cancer cells [2,4,9] can be utilized to induce the drug release. Herein, we report on our investigations of targeted delivery of a group of anti-cancer drugs which act as inhibitors of DNA methylation, thus hindering the silencing of genes which prevent carcinogenesis and cancer growth [10]. These hypomethylation drugs have increasingly been administered to treat leukemia and other cancers [11]. To reduce the side effects of these drugs, such as myocarditis [12], neutropenia (40%) [13,14], and thrombocytopenia (20%) [14], and to increase the treatment efficacy (one-year survival rate of acute myeloid leukemia for >60 year old is 28% [15]), the development of targeted delivery and controlled drug release has been pursued and holds a promise for hundreds of thousands of patients worldwide.

The drugs used in this research were azacitidine (AZA, also known as Vidaza®) and decitabine (DAC, also known as Dacogen®). They are used to treat myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) [16-19]. MDS is due to a bone marrow failure where not enough healthy red blood cells are being produced and this can ultimately lead to leukemia [17]. As a pyrimidine analog, DAC can incorporate itself into DNA, while AZA can intercalate into both DNA and RNA, thus disrupting the mRNA and protein metabolism [16]. Both drugs under study, AZA and DAC, function as DNA

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methyltransferase inhibitors [20,21] and help protecting anti-tumor genes [22,23].

AZA and DAC, as cytidine derivatives, become triphosphorylated once in the cell and incorporate themselves into DNA or RNA. It has been found that

ca. 10-20% of AZA at the diphosphorylated state can be converted into diphosphorylated DAC via ribonucleotide reductase [20]. Once they are incorporated into a DNA/RNA strand they inhibit DNA methyltransferase-1 (DNMT-1) [13,24]. Often in the case of cancer, tumor suppressors are silenced due to methylation [13,24]. When a DNMT-1 encounters AZA or DAC it becomes trapped due to the extra nitrogen in the aromatic ring of triazine with respect to the cytidine. Since DNMT-1 cannot be released, the methylation and silencing of genes, needed to maintain healthy cell, is stalled. Due to this inhibitor activity, AZA and DAC are often called the hypomethylating agents [13,21]. AZA can also induce lethal mutations in human immunodeficiency virus HIV1 [25] and at high doses are toxic to human cells.

To diminish the anti-cancer drug toxicity to healthy cells, a new approach to the drug administration, utilizing targeted drug delivery with nanocarriers able to recognize cancer cells, has extensively been investigated in recent years. In the case of AML, several receptors overexpressed in cancer cells have been considered for cancer cell recognition, including a family of folate receptors (FR) [26,27], with most common FRα and FRβ, which share most of the homology and are anchored in the cell membrane through glycosyl phosphatidinylinositol linkages. They show similar folate binding ability, as well as the same mechanism of endocytosis-based folate internalization [28]. The convenient binding chemistry and lack of immunogenicity make the folate ligands one of the best choices for targeting cancer cells [19,29-31].

The controlled release can be accomplished in multiple ways. In this work, we have utilized gold nanoparticle carriers (AuNPs) with anti-cancer drugs covalently bound to the SAM of NP shells by amide bonding. Thus, the drug release was accomplished weakening of amid bonds in a low-pH cytosol of cancer cells. The carriers were modified with mercaptobenzoic acid (MBA), 4-Aminothiophenol (PATP), azacitidine (AZA) and/or decitabine (DAC), and folic acid (FA). The drug release was monitored using surface-enhanced resonance Raman scattering (SERS) spectroscopy. This technique offers very high sensitivity and enables detecting processes occurring in a monolayer covering AuNPs. We have recently applied this technique to study DNA damage [2], detection of cancer biomarkers [3], and drug release from nanocarriers [4]. The exceptional sensitivity of SERS technique is due to the high Raman signal enhancement observed when chemical compounds are close to the surface of nanostructured materials emanating surface plasmon fields [32-36]. In the present work, it allowed us to monitor the loading of anti-cancer drugs, azacitidine and decitabine, onto the modified AuNP nanocarriers and their release in simulated conditions of neoplasia-induced acidity change in cancer cells.

Experimental Section

Materials

The anti-cancer drug azacitidine (AZA; IUPAC: 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one, also known as Vidaza®) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and decitabine (DAC; IUPAC: 4-Amino-1-(2-deoxyβ-D-erythro-pentofuranosyl)-1,3,5-triazin-2(1*H*)-one, also known as Dacogen®) was obtained from SelleckChem (Houston, TX , USA). Gold nanoparticles (AuNPs) with a 50 nm diameter coated with a protecting citrate monolayer were purchased from Nanopartz (Loveland CO, USA). A 4-mercaptobenzoic acid (MBA) stock solution of 0.1 M in dimethyl sulfoxide (DMSO), purchased from Alfa Aesar (Tewksbury, MA, USA), was prepared using 0.1 mmol MBA from Acros Organics (Thermo Fisher Company). This stock solution can be reused if refrigerated. The stock solution of N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was also prepared and stored at 4 °C. The NHS was purchased from Sigma-Aldrich and the EDC from ProteoChem. A 0.1 M solution of NHS was prepared by weighing 1 mmol of NHS and adding it to a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer with pH of 5.0. A 0.2 M solution of EDC was prepared by weighing 2 mmol of EDC and adding it to a MES buffer with pH of 5.0. The EDC stock solution must be kept frozen when not in use. Two different anti-cancer drugs were covalently bound to AuNPs, as described in the next section. Folic acid, purchased from Sigma-Aldrich, and 4-Aminothiophenol (PATP), purchased from Sigma-Aldrich, were used in the second part of the experiments. A stock solution of 0.1 M PATP was prepared by adding 0.1 mM of PATP to 1 mL of DMSO. This solution was refrigerated when not in use.

Modification of AuNPs with azacitidine and decitabine

400 µL of AuNP solution were centrifuged for 30 minutes and the supernatant was removed. The AuNPs were then modified with a 1 mM MBA solution which was prepared by mixing 990 µL of DMSO and 10 µL of the 0.1 M stock solution of MBA. 400 µL of 1 mM MBA was added to the AuNPs, mixed thoroughly and left to incubate at room temperature for 3 hours. After three hours, the AuNP/MBA solution was centrifuged until a pellet was formed and the supernatant was then removed. A 2 mM EDC + 5 mM NHS in MES buffer with pH 5.5 was then prepared by mixing 940 µL of MES

buffer, 10 μL of 0.2 M EDC and 50 μL of 0.1 M NHS. 400 μL of the EDC/NHS solution was added to the AuNPs and left at room temperature for an hour

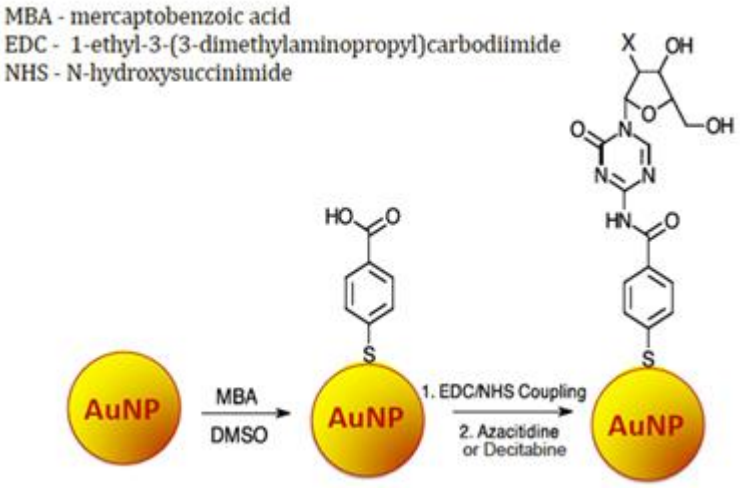

Figure 1. Modification of AuNP with azacitidine or deitabine. The X coming off the ribose ring represents either a hydrogen for decitabine or OH for azacitidine.

The anti-cancer drugs (azacitidine or decitabine) were dissolved in water to prepare a 10 μg/mL solution. After the incubation of MBA-capped AuNPs with EDC/NHS solution, the AuNPs were centrifuged and the supernatant removed. Then, 400 μL of the anti-cancer drug was added to AuNPs (Figure 1) and incubated at room temperature for 18 hours. After that, the AuNPs were pelleted and placed in buffers of different pH: 4.0 (acetate buffer), 5.5 (acetate buffer), 6.0 (MES buffer), or 7.4 (PBS buffer). Raman scans were taken over 3 hours to monitor the release of anti-cancer drug from the modified AuNPs. The Raman peaks at 1586 cm^{-1} and 1074 cm^{-1}, due to the ring vibration modes, were monitored.

Modification of AuNPs with azacitidine/decitabine and folic acid

400 μL of AuNP solution were centrifuged for 30 minutes and the supernatant was removed. The AuNPs were then modified with MBA and PATP solution with 7 μL of 1 mM MBA, 3 μL of 1 mM PATP and 990 μL DMSO. 400 μL of the MBA/PATP solution was added to the AuNPs and left for 3 hours. After pelleting the AuNPs, 400 μL of the EDC/NHS solution was added and left for 1 hour. Afterwards the anti-cancer drug (AZA or DAC) was added and left at room temperature for 18 hours. Another 400 μL of EDC/NHS was added after removing the supernatant from the AuNPs and again incubated for an hour. Finally, 400 μL of 10 μg/mL folic acid was added and left at room temperature for 18 hours (Figure 2).

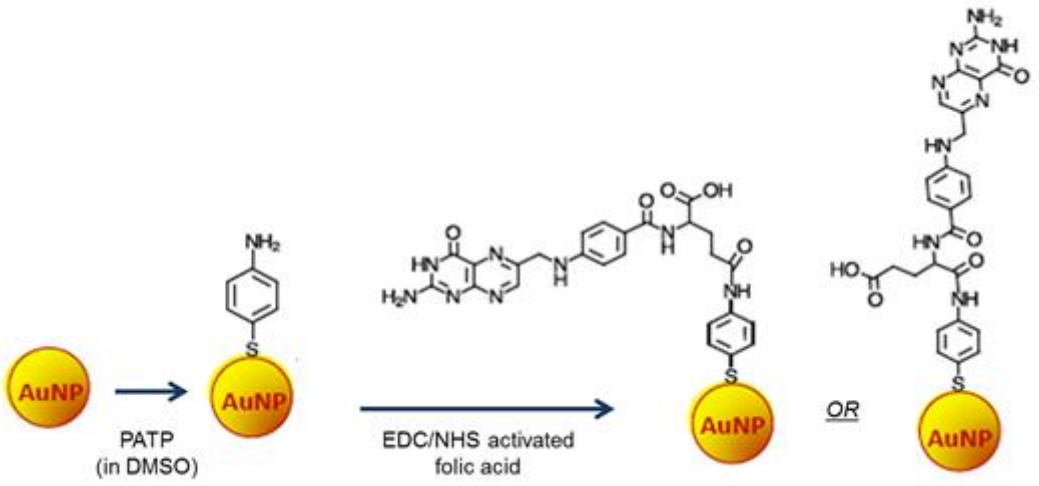

Figure 2. Modification of AuNPs with PATP and folic acid. There are two possible binding sites on folic acid to bind to PATP.

Folic acid activated with EDC/NHS was added after the anti-cancer drug to make sure that FA binds only to PATP and not to MBA (Figure 3). The remaining pellet was placed in buffers of different pH: 5.5 (acetate buffer) or 7.4 (PBS buffer). Raman scans are taken over 3 hours to monitor the release of the anti-cancer drug from the AuNPs. The Raman bands at 1586 cm^{-1} and 1074 cm^{-1} are due to the MBA ring vibration modes.

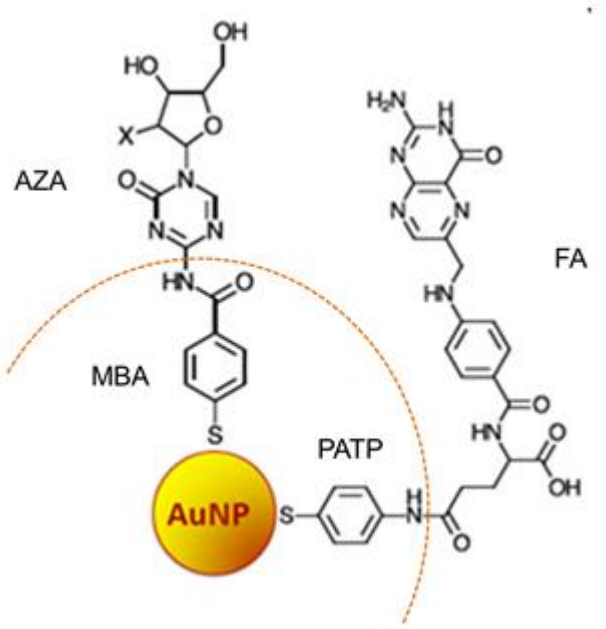

Figure 3. A AuNP@MBA,PTPA nanocarrier uploaded with both the anti-cancer drug (AZA) and targeting ligand, folic acid (FA).

Results and Discussion

Monitoring drug release from AZA/DAC-modified AuNP nanocarriers

The rate of the drug release from AuNP-based nanocarriers was investigated using buffer solutions of different pH. The Raman spectra of clustered AZA/DAC-modified AuNP nanocarriers, shown in Figure 4, were taken over 3 h course during the drug release experiments.

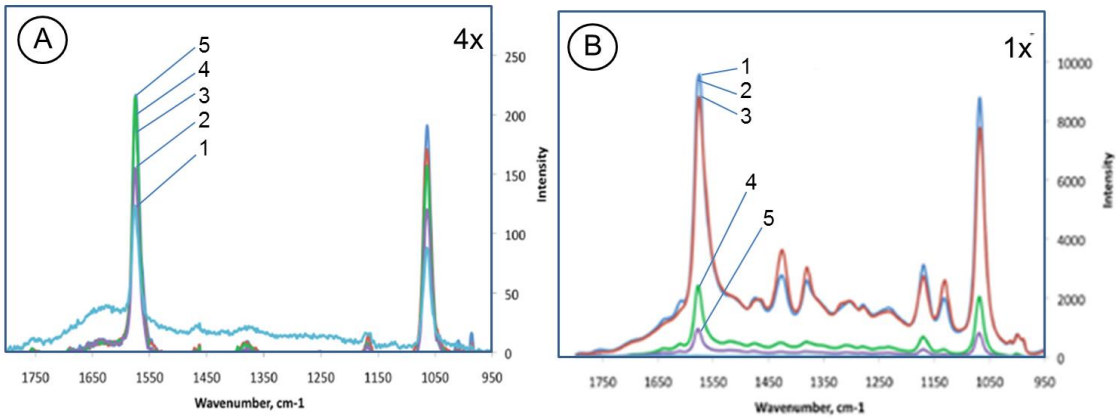

Figure 4. Temporal evolution of SERS spectra for $AuNP_{50nm}$@MBA/AZA in buffer solutions of pH: (A) 7.4 and (B) 5.5, after soaking time *t* [min]: (1) 0, (2) 30, (3) 60, (4) 120, (5) 180. The increase of MBA peak heights at 1586 cm^{-1} and 1074 cm^{-1} indicates on release of AZA from the surface.

The results show clearly a fast release of azacitidine in medium of a low pH 5.5 and no release at a high pH of 7.4. The temporal evolution of the relative Raman peak intensities at 1586 cm^{-1} and 1074 cm^{-1} observed during the soaking of AuNP@MBA/AZA nanocarriers in buffer solutions of pH ranging from 7.4 down to 4.0 are presented in Figure 5. The drug requires an induction time, typically ca. 50-60 min, after which a fast release phase is observed. During this phase, lasting usually ca. 60-70 min, most of the drug is being dissolved from the nanocarriers, uncovering the MBA Raman

marker in the shell SAM of nanocarriers, which results in the sharp increase of the Raman peaks at 1586 cm^{-1} and 1074 cm^{-1}.

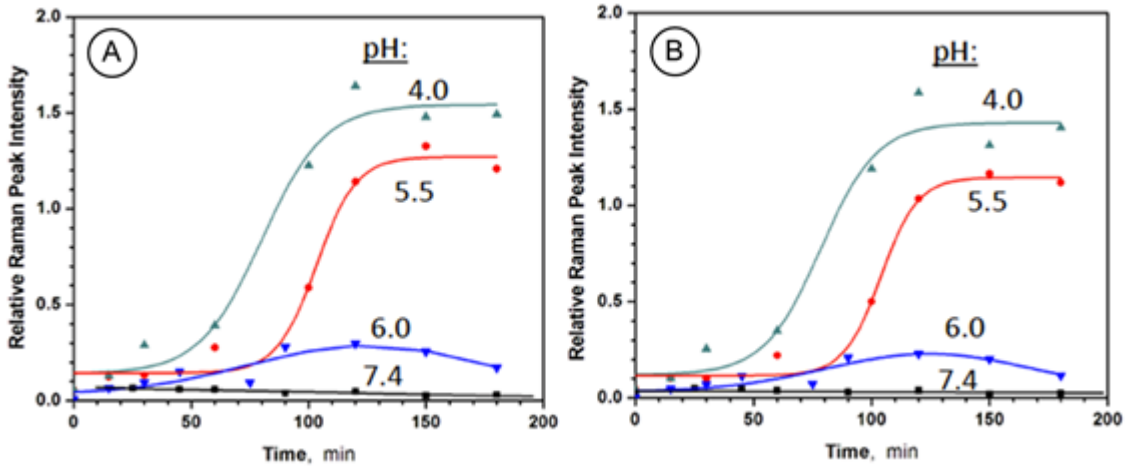

Figure 5. Release of azacitidine (AZA) from $AuNP_{50nm}$@MBA/AZA in buffer solutions of pH: (1) 7.4 and (2) 6.0, (3) 5.5, and (4) 4.0, as measured by the relative Raman peak intensity increase for peak at: (A) 1586 cm^{-1} and (B) 1074 cm^{-1}.

Similar behavior has been observed for $AuNP_{50nm}$@MBA/DAC nanocarriers, as illustrated in Figure 6. The release of decitabine in solution of pH 4.0 has been achieved in 2 h. At pH 5.5, the release of decitabine was ca. 30% slower.

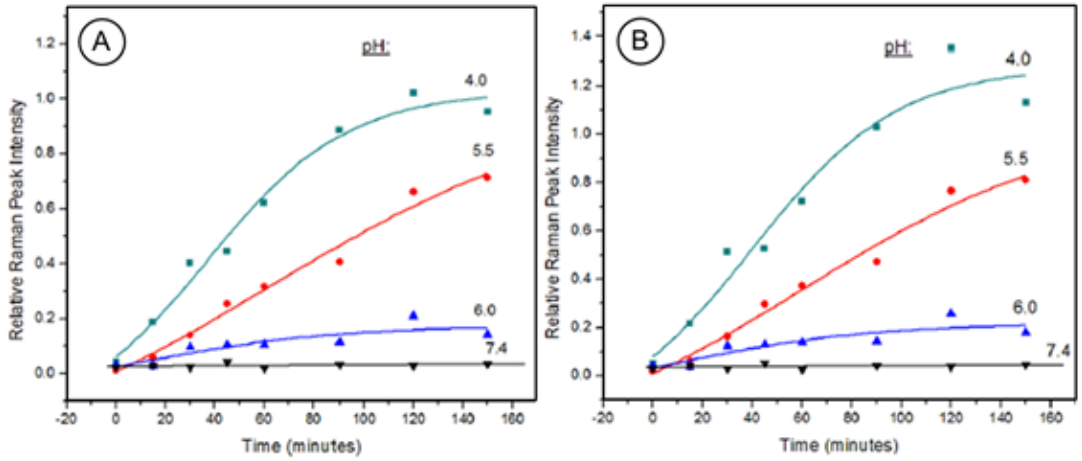

Figure 6. Release of decitabine (DAC) from $AuNP_{50nm}$@MBA/DAC in buffer solutions of pH: (1) 7.4 and (2) 6.0, (3) 5.5, and (4) 4.0, as measured by the relative Raman peak intensity increase for peak at: (A) 1586 cm^{-1} and (B) 1074 cm^{-1}.

Thus, these experiments confirm that the amide-bonded drugs uploaded onto the $AuNP_{50nm}$@MBA nanocarriers can be released in cancer cells characterized by a lowered pH, by acid-induced hydrolysis (acidolysis) of amide bonds. At the same time, the nanocarriers do not release the anti-cancer drugs in healthy tissue, where the physiological pH is ca. 7.4.

Release of AZA/DAC from FA-receptor targeted AuNP nanocarriers

The drug release from AuNP-based nanocarriers targeted for cancer cells was also investigated using buffer solutions of different pH. The Raman spectra of clustered $AuNP_{50nm}$@MBA,PATP/AZA,FA have been obtainedduring the course of 3 h of the drug release experiments (Figure 7). Again, the results show clearly a fast release of azacitidine in solution of low pH of 5.5 and no drug release at a physiological pH of 7.4. The temporal evolution of the relative Raman peak intensities at 1586 cm^{-1} and 1074 cm^{-1} observed during the soaking of AuNP@MBA.PATP/AZA,FA nanocarriers in buffer solutions of pH ranging from 7.4 down to 4.0 are presented in Figure 8. The obtained results confirm that in the presence of FA, the release of the anti-cancer drug, azacitidine, is readily achieved in 2 h when pH is low (pH = 5.5), while no drug release is observed at pH = 7.4.

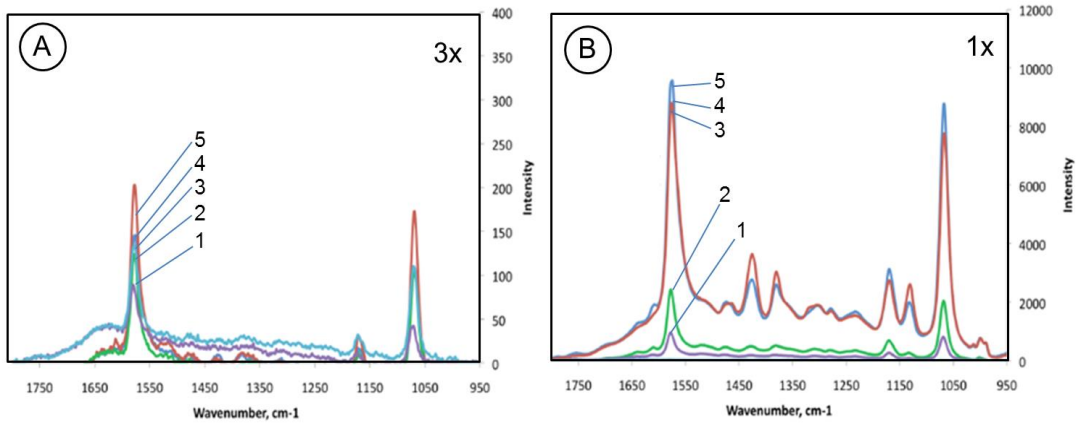

Figure 7. Temporal evolution of SERS spectra for $AuNP_{50nm}$@ MBA,PATP/AZA,FA in buffer solutions of pH: (A) 7.4 and (B) 5.5. The increase of MBA peak heights at 1586 cm^{-1} and 1074 cm^{-1} indicates on release of AZA from the surface.

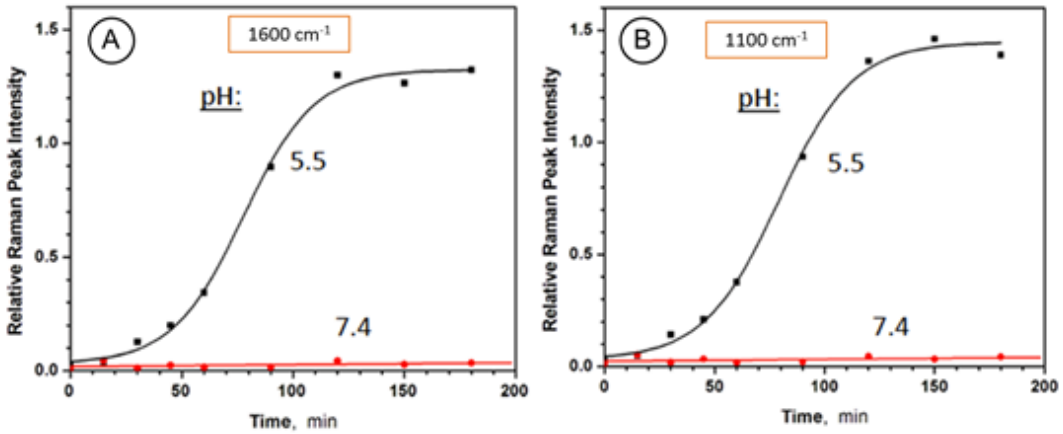

Figure 8. Release of azacitidine (AZA) from targeted nanocarriers $AuNP_{50nm}$@MBA,PATP/AZA,FA in buffer solutions of pH: (1) 7.4 and (2) 6.0, (3) 5.5, and (4) 4.0, as measured by the relative Raman peak intensity increase for peak at: (A) 1586 cm^{-1} and (B) 1074 cm^{-1}.

Similar behavior has been observed for $AuNP_{50nm}$@MBA,PATP/DAC,FA nanocarriers, as illustrated in Figure 9. The release of decitabine in solution of pH 5.5 has been achieved in 2 h. At pH 7.4, the release of decitabine was negligible.

Thus, the addition of PATP-bound FA to the AuNP nanocarriers would not influence much the anti-cancer drug delivery, except that the available space on the nanocarrier surface is somewhat diminished by PATP. In applications, the use of 10-15% of the surface coverage of AuNPs for targeting ligands (here, FA) is a reasonable tradeoff.

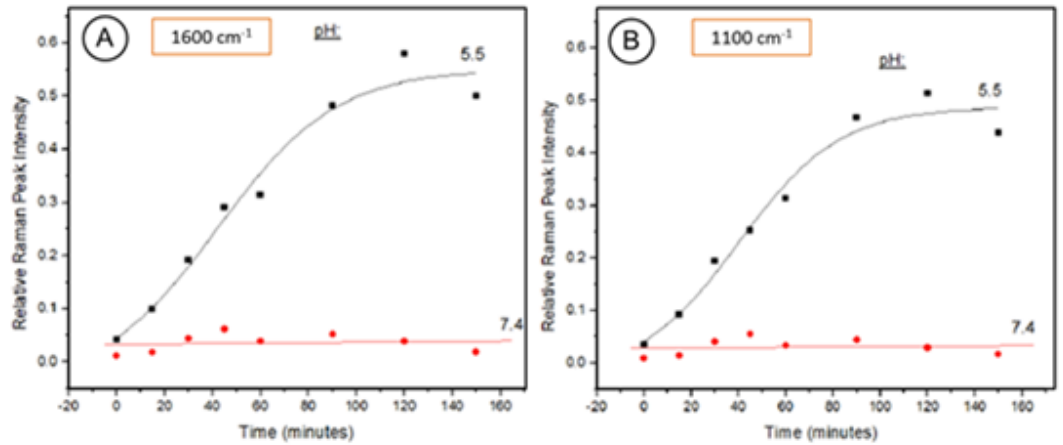

Figure 9. Release of decitabine (DAC) from targeted nanocarriers $AuNP_{50nm}$@MBA,PATP/DAC,FA in buffer solutions of pH: (1) 7.4 and (2) 6.0, (3) 5.5, and (4) 4.0, as measured by the relative Raman peak intensity increase for peak at: (A) 1586 cm^{-1} and (B) 1074 cm^{-1}.

Mechanistic aspects of targeted drug delivery and controlled release using AuNP nanocarriers

The gold nanoparticles, modified with MBA, enable the immobilization of drugs, azacitidine and decitabine, on AuNP in the form of AuNP@MBA/AZA,DAC nanocarriers. The cancer cell recognition was accomplished by covalently binding FA ligands to PATP in the mixed SAM shell on gold nanoparticle nanocarriers, AuNP@MBA,PATP. The FA ligand was used due to the strong expression of folic acid receptors (FR) in the membrane of cancer cells. This enables the functionalized carriers to target only cancer cells since folic acid ligands bind to the FRs. The amide bonds between the linkers and azacitidine/decitabine are pH sensitive and undergo hydrolysis in a low pH environment of the cytosol in cancer cells. Using the solutions of different pH, the release of azacitidine/decitabine was monitored by SERS measurements of the MBA Raman modes at 1586 cm^{-1} and 1074 cm^{-1}. At pH 7.4, the release of the drug was negligible, while at pH 4.0 and 5.5 a continuous drug release was observed over 3 hours.

The mechanism of SERS monitoring of the drug release is based on the strong Raman signals observed from the MBA linker, which is a known Raman marker. After the immobilization of azacitidine/decitabine on a AuNP carrier, the SERS signal is reduced due the shielding by the drug. During the drug release, the SERS signal increases manifesting the amide bond breakage and confirming the successful drug delivery.

Conclusions

The AuNPs can be modified with MBA and PATP as linkers to immobilize azacitidine/decitabine and folic acid. This is possible due to the covalent bonds between the MBA and the anti-cancer drug, and the covalent bonds between the PATP and folic acid. The release of the drugs can be efficiently monitored using the MBA Raman signals, enhanced by the plasmonic field of gold nanoparticle core to attain ultra-high sensitivity. Hence, the SERS measurements provide clear evidence of the drug release when the carrier is placed in a low pH environment.

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